Insulin Stimulates a Novel Mn\textsuperscript{2+}-dependent Cytosolic Serine Kinase in Rat Adipocytes*  

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The cytosolic fraction of insulin-treated adipocytes exhibits a 2-fold increase in protein kinase activity when Kemptide is used as a substrate. The detection of insulin-stimulated kinase activity is critically dependent on the presence of phosphatase inhibitors such as fluoride and vanadate in the cell homogenization buffer. The cytosolic protein kinase activity exhibits high sensitivity (ED\textsubscript{50} = 2 × 10\textsuperscript{-10} M) and a rapid response (maximal after 2 min) to insulin. Kinetic analyses of the cytosolic kinase indicate that insulin increases the V\textsubscript{max} of Kemptide phosphorylation and ATP utilization without affecting the affinities of this enzyme toward the substrate or nucleotide.

Upon chromatography on anion-exchange and gel filtration columns, the insulin-stimulated cytosolic kinase activity is resolved from the cAMP-dependent protein kinase and migrates as a single peak with an apparent M\textsubscript{r} = 50,000–60,000. The partially purified kinase preferentially utilizes histones, Kemptide, multifunctional calmodulin-dependent protein kinase substrate peptide, ATP citrate-lyase, and acetyl-coenzyme A carboxylase as substrates but does not catalyze phosphorylation of ribosomal protein S6, casein, phosvitin, phosphorylase b, glycogen synthase, inhibitor II, and substrate peptides for casein kinase II, protein kinase C, and cGMP-dependent protein kinase. Phosphoamino acid analyses of the \textsuperscript{32}P-labeled substrates reveal that the insulin-stimulated cytosolic kinase is primarily serine-specific. The insulin-activated cytosolic kinase prefers Mn\textsuperscript{2+} to Mg\textsuperscript{2+} and is independent of Ca\textsuperscript{2+}. Unlike ribosomal protein S6 kinase and protease-activated kinase II, the insulin-sensitive cytosolic kinase is fluoride-insensitive. Taken together, these results indicate that a novel cytosolic protein kinase activity is activated by insulin.

Recent studies have provided direct evidence that the insulin receptor, like a number of other growth factor receptors (1–8), is a tyrosine kinase which is activated upon binding of the hormone (9–14). The insulin receptor is capable of catalyzing the phosphorylation of tyrosine residues on its \beta subunit as well as a number of artificial exogenous substrates. Studies in this (15, 16) and other laboratories (17–20) have indicated that the autophosphorylation reaction of the insulin receptor plays a crucial role in regulating the tyrosine kinase activity. Thus, a direct correlation has been established between the tyrosine phosphate content of the insulin receptor \beta subunit and the receptor tyrosine kinase activity. More recently, using site-specific mutagenesis (21) and phosphopeptide mapping techniques (22, 23), two laboratories have provided evidence that the phosphorylation of a specific tyrosine residue on the insulin receptor \beta subunit is essential for receptor kinase activation.

It has been postulated that the insulin receptor may transmit all or part of the hormone signal through its intrinsic kinase activity. Evidence in support of this hypothesis has recently been reported by several laboratories (24–27). In these studies, it has been shown by microinjection of monoclonal antibodies inhibitory to the receptor kinase (24, 25) or by genetic engineering (26, 27) that the ability of the insulin receptor to elicit a number of typical insulin responses is markedly attenuated when its receptor tyrosine kinase activity is inactivated.

The potential role of the insulin receptor kinase in mediating insulin action raises the possibility that the hormone signal may be transmitted through a cascade of phosphorylation reactions. For example, the serine/threonine phosphorylation of the insulin receptor (10, 16, 28, 29), acetyl-coenzyme A carboxylase (30–32), ATP citrate-lyase (33–35), and a number of proteins of unknown identity (36–45) is activated by insulin in intact cells. It has also been demonstrated that extracts derived from insulin-treated 3T3-L1 adipocytes and Swiss 3T3 fibroblasts exhibit a marked increase in kinase activity toward exogenous ribosomal protein S6, indicating that a serine kinase is indeed activated by insulin (46–52). Recently, it has been reported that microinjection of virus-encoded src and abl tyrosine kinases (53, 54) and the insulin receptor kinase (55) into Xenopus oocytes leads to an increase in ribosomal protein S6 phosphorylation on serine residues. These results suggest that a possible linkage may exist between the insulin receptor tyrosine kinase activity and the regulation of ribosomal protein S6 serine kinase.

In this study, we identify a novel Mn\textsuperscript{2+}-dependent insulin-stimulated serine kinase activity in rat adipocyte cytosolic extracts. Our success in detecting this kinase activity is based on a unique extraction procedure which requires the presence of phosphatase inhibitors such as sodium fluoride and vanadate in the homogenization buffer. In the absence of these inhibitors, little or no insulin-stimulated kinase activity can be detected. The insulin-sensitive kinase is highly sensitive to insulin (ED\textsubscript{50} = 2 × 10\textsuperscript{-10} M). Most important, based on its substrate specificity and insensitivity to sodium fluoride, this insulin-activated serine kinase appears to be distinct from the hormone-sensitive ribosomal protein S6 and protease-activated kinases reported earlier by several laboratories.
EXPERIMENTAL PROCEDURES

Materials

PMSF, histones H1, 2B, VS, and V11S, Kemptide, casein, phosphorvit, heparin, dibutyryl adenosine 3'5'-monophosphate, CaMDependent protein kinase inhibitor, phosphoserine, phosphothreonine, and phosphophorysine were purchased from Sigma. [γ-32P]ATP was obtained from Amersham Corp. DEAE-Sephalose and Sephadex G-200 were supplied by Pharmacia Biotechnology, Inc. Precalcelulose plastic sheets were purchased from Brinkmann Instruments. Porcine insulin and desoxaptapetide insulin were gifts from Dr. Ronald Chance (Lilly). Guinea pig anti-insulin serum was kindly provided by Dr. W. Chick (University of Massachusetts Medical School). Molecular weight standards were from Bio-Rad. Rat liver ribosomal 40 S subunit was prepared according to the procedure described by Thomas et al. (56). Substrate peptides were synthesized using an Applied Biosystems peptide synthesizer.

Methods

Rat Adipocyte Isolation and Incubation—Male rats weighing 100-125 g were used (Sprague-Dawley strain, Taconic Farms, Inc., Germantown, NY). Adipocytes were prepared from the epididymal fat pads as described by Rodbell (57) in Krebs-Ringer-Hepes buffer, pH 7.4, containing 30 mg/ml BSA and 2 mM sodium pyruvate. Fat cells were equilibrated in the same buffer without collagenase at 37 °C for 10 min. For further incubation, adipocytes were incubated in the absence and presence of different concentrations of insulin (10-10 to 10-7 M) at 37 °C for various periods of time and then homogenized according to the procedure described below.

Preparation of Adipocyte Cytosols—Fat cells were homogenized in 7 volumes of 20 mM Tris-HCl, pH 7.4, containing 256 mM sucrose, 5 mM EDTA, 10 mM NaF, 200 μM sodium vanadate, 10 mM sodium pyrophosphate, and 1 mM PMSF. Membrane fractions were sediBemented by differential centrifugation as described by McKeel and Jarett (58) with some modifications (59). Following the final centrifugat ion at 150,000 × g, the supernatant was mixed with 0.14 volume of 300 mM Hepes, pH 7.4, in order to increase the buffering capacity of the cytosolic extracts. The excess EDTA in the cell extracts was neutralized by 2.4-fold molar concentrations of MgCl2. Under these conditions, no change in pH was observed when the cell homogenate was exchanged.

Assay of Protein Kinase Activity in Adipocyte Cytosols—Cytosolic fractions (20 μl) were assayed for protein kinase activity using Kemptide as substrate and [γ-32P]ATP as phosphate donor. The phosphorylation reaction was initiated by the addition of 12.5 μl of a mixture containing 61 μM [γ-32P]ATP (specific activity, 25-100 μCi/mmol), 31 mM MgCl2, 9.2 mM MnCl2, 0.1 mM DTT, and 1.9 mg/ml Kemptide and was continued for 30 min at 22 °C. The reaction was terminated by the addition of 34 μl of 10% trichloroacetic acid. The sample was chilled at 4 °C for 30 min and then microcentrifuged for 5 min. 32P-Labeled Kemptide remaining in the supernatant was isolated by adsorption to Whatman P-81 phosphocellulose paper (1 × 1-cm square) using 10-μl samples according to the method of Glass et al. (60). The paper squares were then washed three times in 0.5% phosphoric acid (10 ml/paper square). The Kemptide-associated 32P radioactivity in the paper squares was quantitated by Cerenkov counting. Background 32P radioactivity was determined in each sample phosphorylated in the absence of Kemptide and is usually 10-20% of that in the sample containing the substrate. When other substrate peptides were used, the phosphocellulose paper method was also employed to monitor their extents of phosphorylation. For protein substrates such as histone, ATP citrate lyase, acetyl-coenzyme A carboxylase, ribosomal protein S6, casein, phosphovit, phosphorylase b, glycogen synthase, and inhibitor II, the magnitudes of phosphorylation were quantitated by Cerenkov counting of the 32P-labeled bands following SDS-polyacrylamide gel electrophoresis of the samples.

Phosphoamino Acid Analysis—The 32P-labeled protein bands in the dry gel were located by autoradiography and excised. The phosphorylated proteins were obtained from the gel pieces by incubation in 50 mM NH4HCO3 containing 0.1% SDS. The extracted materials were precipitated in 20% trichloroacetic acid at 4 °C using 50 μg of bovine serum albumin as carrier. The precipitated proteins were washed once with ethanol/ether (1:1, v/v) and then dissolved in 20 μl of H2O containing 1 mg/ml each phosphoserine, phosphothreonine, and phosphotyrosine. The samples were spotted onto a cellulose-precocated plastic sheet and electrophoresed at 1000 V for 2 h in pyridine/acetate/water (1:10:189), pH 3.5. The locations of the different phosphosino acid acids were visualized by ninhydrin staining, and the 32P radioactivity associated with each phosphosino acid spot was monitored by autoradiography.

RESULTS

Effect of Insulin on Protein Kinase Activity in Rat Adipocyte Cytosols—To investigate whether insulin modulates protein kinase activity in rat adipocytes, the phosphorylation of Kemptide by the high speed supernatant of homogenized fat cells was examined following incubation of cells with or without insulin at 37 °C for 30 min. As shown in Fig. 1 (bars E and F), cytosolic extracts prepared in the presence of phosphatase inhibitors such as fluoride, vanadate, and pyrophosphate from insulin-treated cells exhibit a 2-fold higher kinase activity toward Kemptide when compared to controls. The insulin-dependent increase in 32P incorporation is not due to phosphorylation of endogenous proteins in the cell extracts because in the absence of Kemptide, the levels of phosphorylation are the same between the control and insulin-treated groups (data not shown). The detection of the stimulatory effects of insulin on Kemptide phosphorylation appears to be critically dependent on the presence of phosphatase inhibitors in the homogenization buffer. When these phosphatase inhibitors were omitted during disruption of cells, the insulin-stimulated Kemptide phosphorylation by cell extracts tended to be variable and was rarely detectable in some experiments (Fig. 1, bars A and B). In order to obtain a consistent insulin stimulation of the cytosolic kinase activity, phosphatase inhibitors were included in the homogenization buffer in all subsequent experiments.

FIG 1. Effects of phosphatase inhibitors on the preservation of the insulin-stimulated Kemptide kinase activity when added before and after cell fractionation. Following incubation in the presence and absence of insulin (10-7 M) at 37 °C for 30 min, rat adipocytes were homogenized in Tris/sucrose/EDTA buffer, pH 7.4, with or without NaF, sodium vanadate, and sodium pyrophosphate. The cytosolic extracts were prepared by differential centrifugation as described under "Methods." Prior to kinase assays, 1-μl aliquots of cytosolic extracts prepared in the presence of phosphatase inhibitors were mixed with 20 μl of 0.5 mM NaF and 10 μl of 20 mM sodium vanadate with or without 30 μl of water. For extracts prepared in the presence of phosphatase inhibitors, water was also added in identical proportion. Then, 20-μl aliquots of the final cell extracts were assayed for Kemptide phosphorylating activity as described under "Methods." Each value represents the average of three determinations. Bars A and B, minus phosphatase inhibitors; bars C and D, phosphatase inhibitors added after preparation of cytosolic extracts; bars E and F, phosphatase inhibitors present during preparation of cytosolic extracts.
The preservation of the insulin-stimulated Kemptide kinase activity in the presence of phosphatase inhibitors is not due to the difference in the rates of phosphate turnover during kinase assay between the minus and plus inhibitor groups. This is because subsequent addition of fluoride and vanadate to the cytosolic extracts prepared in the absence of these two phosphatase inhibitors fails to maintain the full stimulatory effect of insulin on Kemptide phosphorylation (Fig. 1, bars C and D). These results suggest that the function of the phosphatase inhibitors is to prevent the deactivation of insulin-stimulated kinase activity during cell fractionation, perhaps by inhibiting its dephosphorylation. Furthermore, in view of the fact that the serine residue is the only phosphate acceptor site in Kemptide, these results suggest that a serine kinase activity may be activated in adipocyte cytosols in response to insulin.

The Kemptide kinase activity in the cytosol appears to be highly sensitive to insulin. Thus, incubation of adipocytes with $2 \times 10^{-10}$ and $10^{-8}$ m insulin results in half-maximal and maximal activations of this enzyme activity, respectively (Fig. 2). Furthermore, the action of the hormone on the cytosolic protein kinase appears to be extremely rapid. Maximal stimulation of kinase activity is achieved when cells are exposed to insulin for 2 min at 37°C (Fig. 3). Significantly, the stimulatory effect of insulin is fully reversible upon the removal of the hormone from the incubation buffer. As depicted in Fig. 3, the addition of guinea pig anti-insulin serum to the cell suspensions causes a time-dependent decrease in the insulin-stimulated Kemptide phosphorylation activity in the cell extracts. After an incubation period of 15 min at 37°C with the hormone antisera, the magnitude of insulin stimulation of this enzyme was lowered by 50%. By 30 min at 37°C, the stimulatory effect of insulin was almost abolished.

The activation of the cytosolic kinase by insulin is highly specific. This is because neither desoctapeptide insulin nor epidermal growth factor can mimic the full stimulatory action of insulin on the cytosolic Kemptide kinase. The magnitude of kinase stimulation elicited by desoctapeptide insulin and epidermal growth factor represents 16 and 8% of the maximal insulin stimulation, respectively (data not shown). The diminished ability of desoctapeptide insulin corresponds to its lower affinity to the insulin receptor than insulin.

Chromatographic Resolution of the Insulin-stimulated Kemptide Kinase Activity—The preceding section demonstrates the presence of an insulin-sensitive protein kinase activity in adipocyte cytosols. It is possible that this kinase activity may consist of more than one enzyme. To address this issue, the cytosolic fractions from control and insulin-treated adipocytes were analyzed by ion-exchange and size sieving chromatography. The insulin-stimulated Kemptide kinase activity exhibits high affinity for the DEAE anion exchanger. After one passage through the column, the entire portion of insulin-stimulated kinase activity was adsorbed (data not shown). During subsequent washing with the elution buffer, no detectable hormone-sensitive kinase activity was eluted. When the DEAE column was developed with a linear gradient of 0-0.5 M NaCl, a single insulin-stimulated Kemptide kinase activity peak was eluted between 0.15 and 0.2 M NaCl (Fig. 4). Most important, the stimulatory effect of insulin on the cytosolic kinase is preserved during this chromatography step, and its magnitude is comparable to that in the original crude extracts. Control experiments indicate that this peak activity represents the total kinase activity adsorbed by the column. This is because no residual insulin-stimulated kinase activity was detected in the column resin (data not shown). Furthermore, the sum of hormone-sensitive kinase activity recovered in the eluted fractions accounts for close to 100% of the kinase activity originally applied to the DEAE column. Together, these results suggest that the insulin-stimulated Kemptide kinase activity in adipocyte cytosols may be composed of one enzyme species or multiple kinases of similar charge characteristics.
used contained a high concentration of NaF (100 mM). It has peak kinase activity from the S-200 column was also heavily contaminated by this serum protein. Thus, the specific activity of the insulin-stimulated kinase activity in the S-200 column fractions cannot be calculated.

An important point to note in the two chromatography steps utilized in this study is that the equilibration buffer used contained a high concentration of NaF (100 mM). It has been reported by several laboratories that the insulin-stimulated ribosomal protein S6 kinase (48, 50, 61, 62) and protease-activated kinase II (63) are totally inhibited by such high concentrations of fluoride ion. In contrast, the magnitude of insulin stimulation of the adipocyte cytosolic kinase remains unabated under these conditions, indicating that the Kemptide kinase activity reported in this study is insensitive to NaF and may be distinct from the S6 and protease-activated kinases.

Nature of Stimulatory Action of Insulin on Cytosolic Kinase—In order to define the nature of insulin stimulation on the adipocyte cytosolic kinase, the kinetics of Kemptide phosphorylation by the DEAE- and S-200-purified cytosolic kinase preparations from control and insulin-treated fat cells were examined. To ensure that the assay conditions measured the initial linear rate of Kemptide phosphorylation, 32P labeling of Kemptide by control and insulin-treated cell extracts was monitored over different periods of time at 22 °C. The results of these experiments indicate that the phosphorylation of Kemptide by cytosolic fractions of control and insulin-stimulated adipocytes remains linear up to 60 min under these conditions (data not shown). Therefore, in subsequent experiments designed to assess the kinetic properties of the insulin-sensitive cytosolic kinase, an assay period of 30 min at 22 °C was used.

As depicted in Fig. 6, the elevation in Kemptide phosphorylation by the partially purified cytosolic kinase preparation from insulin-treated adipocytes is due to a marked increase (6-fold) in the V_{max} value of the kinase, whereas its substrate affinity (K_m) is unaffected. This finding suggests that the stimulatory action of insulin on the cytosolic kinase may be mediated through an increase in the amount of activated enzyme present in the cell extracts. The K_m value (100 μM) of Kemptide for the insulin-sensitive kinase appears to be severalfold higher than that reported for cAMP-dependent protein kinase (K_m ≈ 16 μM). These results suggest that these two kinases may be different from each other.
It can be argued that insulin stimulation of this Kemptide kinase may be explained by an increase in affinity of the enzyme toward ATP. Hence, at a given submaximal concentration of this nucleotide, the rate of phosphate transfer to the substrate is increased. In order to address this issue, the kinetic parameters of the cytosolic kinase with respect to ATP were determined. The Kemptide kinase from insulin-treated adipocytes exhibits a 3-fold higher rate of ATP utilization (17.5 fmol/min) when compared to that (5.9 fmol/min) from control. No changes in the ATP Km value (2.5 μM) were observed between the control and insulin-treated groups (data not shown). Together, these results indicate that insulin stimulates the cytosolic kinase by increasing the amount of activated enzyme rather than altering its affinity toward Kemptide and ATP.

Substrate Specificity—The ability of the partially purified insulin-sensitive cytosolic kinase to catalyze the phosphorylation of different substrates is examined in Table I. Among the various substrate peptides and proteins tested, the hormone-stimulated cytosolic kinase exhibits preferential phosphorylation of Kemptide (64), multifunctional calmodulin-dependent protein kinase (66), Ca2+- and phospholipid-dependent protein kinase (67), cGMP-dependent protein kinase (RKPRQGRATSVNFS) (68), and different preparations of histone and ATP citrate-lyase. The magnitudes of insulin-stimulated phosphorylation of these substrate polypeptides range from 70 to 260% above basal.

In terms of the stoichiometry of phosphorylation, ATP citrate-lyase appears to be the best substrate tested so far. The levels of phosphate transfer catalyzed by the hormone-stimulated kinase into ATP citrate-lyase, histone VIIS, and Kemptide after 30 min of incubation at 22 °C were 372, 220, and 2 nmol/mmol, respectively. In contrast, substrate peptides for casein kinase II (66), Ca2+- and phospholipid-dependent protein kinase (67), and cGMP-dependent protein kinase (68) and protein substrates such as casein, phosphitin, phosphorylase b, glycogen synthase, ribosomal protein S6, and inhibitor II were poorly phosphorylated by the partially purified cytosolic kinase preparations derived from control and insulin-treated adipocytes. Furthermore, no consistent insulin-mediated increases in phosphorylation of these substrate peptides and proteins were observed. Although the increase (25%) in acetate-coenzyme A carboxylase phosphorylation due to insulin is rather modest compared to those of Kemptide, histone, and ATP citrate-lyase, the stoichiometry (135 nmol/mmol) of carboxylase phosphorylation by the hormone-activated cytosolic kinase is quite comparable to that of ATP citrate-lyase or histone. It should be pointed out that the increase in ATP citrate-lyase phosphorylation by the insulin-activated kinase is not due to an elevation in phosphohistidine content. This is because the 32P-labeled lyase preparations were electrophoresed on SDS-polyacrylamide gels which were subsequently stained and destained in 1% trichloroacetic acid and 0.3 M HCl, and the liberated phosphoamino acids were resolved by electrophoresis on cellulose-precoated plates. These analyses reveal that the activity of the cytosolic kinase is directed primarily on serine residues of histone and ATP citrate-lyase although relatively minor phosphorylation is also detected on threonine residues (Fig. 7). These results confirm the finding with Kemptide as phosphorylation substrate that a cytosolic kinase specific for serine/threonine residues is indeed activated in adipocytes by insulin.

Identification of Phosphorylated Sites—Fig. 8 shows the behavior of the insulin-stimulated cytosolic kinase in the presence of different concentrations of Mn2+ and Mg2+. Interestingly, this cytosolic kinase exhibits a preferential dependence on Mn2+ ion. Insulin stimulation of Kemptide phosphorylation is readily detectable at Mn2+ ion concentrations.
their phosphoamino acid contents were determined according to the
then excised from the gel. The phosphoproteins were extracted, and
cytosolic kinases, the histone and ATP citrate-lyase preparations
were electrophoresed on a 10% SDS-polyacrylamide gel. The 32P-
activation by the partially purified control and insulin (INS.)-stimulated
cytosolic kinases, the histone and ATP citrate-lyase preparations
were assayed in the presence of both Mg+ and MnZ+. The activity
of the insulin-activated cytosolic kinase appears to be inde-
pendent of Ca++ (data not shown). In fact, at high concentra-
tions of Ca++ (>100 μM), the hormone-sensitive kinase is
inhibited.

pH and Temperature Dependence of the Insulin-stimulated
Cytosolic Kinase—The dependences of the insulin-activated
cytosolic kinase on pH and temperature are shown in Fig. 9.
Although insulin stimulation of this kinase is sustained over
a broad range of pH (Fig. 9A), this enzyme appears to function
optimally at pH 8. As shown in Fig. 9B, the dependence of
the insulin-stimulated kinase on temperature follows a bi-
phasic profile. Peak activity is achieved at 30 °C. Further
increases in incubation temperature result in a progressive
decline in enzyme activity.

Effects of Dithiothreitol and Heparin on the Insulin-stimu-
lated Cytosolic Kinase—In light of the fact that most protein
kinases require the presence of reductant for optimal activity,
the dependence of the insulin-activated cytosolic kinase on
dithiothreitol was examined. As depicted in Fig. 10A, the
erexpression of the insulin-stimulated Kemptide phosphoryl-
ating activity is totally dependent on the presence of the
reductant. In the absence of dithiothreitol, no insulin-stimu-
lated kinase activity can be detected. The permissive effect of
the reductant is progressively increased when its concentra-
tion is raised from 2 to 10 mM. Beyond the optimal dithio-
threitol concentration of 10 mM, the hormone-stimulated
kinase activity tends to diminish.

Earlier studies have demonstrated that heparin is a pow-
erful inhibitor of certain protein kinases including ribosomal
protein S6 kinase (62) and casein kinase II (69). These two
kinases are usually completely inhibited at heparin concentra-
tions below 100 μg/ml. As shown in Fig. 10B, the insulinstimulated cytosolic kinase is totally insensitive to heparin.

![Fig. 7. Phosphoamino acid analyses of 32P-labeled histones and ATP citrate-lyase following phosphorylation by control and insulin-stimulated cytosolic kinases.](image)

![Fig. 8. Differential dependences of the insulin-sensitive cytosolic kinase on Mn++ and Mg++. The Kemptide phosphorylating activities of partially purified cytosolic kinase preparations from control and insulin-treated adipocytes were measured in the absence and presence of the indicated concentrations of Mn++ or Mg ++ ions. The phosphorylation reactions were performed with 25 μM [γ-32P]ATP and 0.75 mg/ml Kemptide at 22 °C for 30 min. Each point represents the mean of two determinations.](image)

concentrations as low as 50 μM. Both the basal and insulinstimulated kinase activities are progressively elevated when the concentrations of Mn++ ions are raised. Thus, the magni-
tudes of the stimulatory effects of insulin on the cytosolic kinase are 76, 200, 290, 350, 470, and 0.75, 0.2, 0.8, 1.6, 6.4, and 25 mM Mn+++, respectively. The maximal enhancing effect of Mn++ on the insulin-stimulated Kemptide phosphorylating activity appears to peak at 6.4 mM and declines thereafter. In contrast, when Mg++ is used, the stimulatory effects of insulin can only be detected within a narrow concentration range of this divalent cation (Fig. 8). Furthermore, the magnitude of insulin stimulation in the presence of Mg++ is much lower than that achieved with Mn++. At the maximally effective concentration of 1.6 mM Mg++, the cytosolic kinase is stimulated 2-fold by the hormone; whereas at an equal molar concentration of Mn++, the insulin-enhanced kinase activity is about 4.5-fold.

The diminution in insulin-sensitive Kemptide kinase activity at concentrations of Mg++ higher than 1.6 mM suggests that this divalent cation may be inhibitory to the kinase at high concentrations. Interestingly, such an inhibitory effect of Mg++ can be reversed when Mn++ is included in the phosphorylation buffer (data not shown). These results explain the detection of insulin stimulation in some of the experi-
ments presented above even when the cytosolic kinase is assay-
ed in the presence of both Mg++ and Mn++. The activity of the insulin-activated cytosolic kinase appears to be inde-

![Fig. 9. pH and temperature dependences of the insulin-stimulated cytosolic kinase. The Kemptide phosphorylating activities of partially purified cytosolic kinase preparations from control and insulin-treated adipocytes were assayed at the indicated pH and temperatures for 30 min. The results for the temperature experiments are expressed as the difference between insulin-stimulated and basal kinase activities. Each value shown represents the mean of two determinations. O, control; ●, plus 10-7 M insulin.](image)

![Fig. 10. Effects of DTT and heparin on the insulin-stimulated cytosolic kinase. The activities of partially purified prepara-
tions of control and insulin-stimulated cytosolic kinases were meas-
ured at the indicated concentrations of DTT and heparin using
Kemptide as substrate. The assay period was 30 min at 22 °C. O, control; ●, plus 10-7 M insulin. Each point represents the average of duplicate determinations.](image)
On the contrary, the magnitudes of insulin stimulation of the cytosolic kinase tend to increase slightly when the concentrations of heparin are raised.

_Distinction between the Insulin-stimulated Cytosolic Kinase and cAMP-dependent Protein Kinase_—In view of the fact that the insulin-stimulated kinase specifically catalyzes phosphorylation of a number of typical CAMP-dependent protein kinase substrates such as histone and Kemptide, attempts were made to determine whether these two kinases are related. One approach was to investigate whether the cAMP-dependent kinase will behave chromatographically in a manner similar to the insulin-sensitive cytosolic kinase. In these experiments, adipocytes were exposed to dcAMP to generate the active catalytic subunit of CAMP-dependent protein kinase in the cytosol. The cytosolic fraction was prepared in the same fashion as that treated with insulin. The results of these experiments indicate that the Kemptide phosphorylating activity is elevated by 10-fold in extracts of dcAMP-treated fat cells when compared to control. However, unlike the insulin-stimulated cytosolic kinase which adsorbs to DEAE-Sephacel (Fig. 4), the catalytic subunit of cAMP-dependent protein kinase fails to bind to the anion-exchange resin (data not shown). Furthermore, the activity of the insulin-stimulated cytosolic kinase is not affected by the cAMP-dependent protein kinase inhibitor (data not shown). Taken together, these results strongly indicate that the insulin-stimulated cytosolic kinase is different from the cAMP-dependent protein kinase.

**DISCUSSION**

This study provides direct evidence that insulin stimulates a novel $M_\text{r} = 50,000–60,000$ serine kinase in the cytosol of rat adipocytes. The key requisite for the detection of this hormone-sensitive kinase is the inclusion of phosphatase inhibitors in the homogenization buffer. As evidenced in Fig. 1, the marked stimulatory effect of insulin on this cytosolic kinase is preserved when fluoride, vanadate, and pyrophosphate are present during cell fractionation. The omission of these phosphatase inhibitors results in little or no detectable insulin-stimulated kinase activity in the cytosolic fraction. The high sensitivity and rapid response of this kinase to insulin are comparable to a number of classic insulin responses such as stimulation of hexose and amino acid transport and glycogen synthase activation. Collectively, these evidences strongly suggest that the stimulation of this cytosolic kinase by insulin may represent an important physiological process in rat adipocytes which are exposed to the hormone.

Two insulin-stimulated protein kinases have been well-documented prior to this study. They are the ribosomal protein S6 and protease-activated kinases (46–52, 70). However, the hormone-activated cytosolic kinase reported in this study appears to be unrelated to these two kinases. This is because there are several major differences in enzymatic properties between the adipocyte cytosolic kinase described here and the S6 and protease-activated kinases. With respect to substrate specificity, the insulin-stimulated adipocyte cytosolic kinase exhibits preference to histone, Kemptide, and ATP citrate-lyase and does not phosphorylate ribosomal protein S6 and casein, which are known to be substrates for the S6 and protease-activated kinases (46–52, 62, 70). Conversely, S6 and protease-activated kinases do not phosphorylate histone, Kemptide, and ATP citrate-lyase (48, 50–52). Furthermore, the hormone-sensitive kinase reported here is insensitive to NaF because under our assay conditions, 60 mM NaF was included in the phosphorylation buffer. In contrast, the S6 and protease-activated kinases are reported to be completely inhibited by such a high concentration of fluoride ion (48, 49, 50, 62, 63). Another striking difference between the cytosolic kinase and S6 and protease-activated kinases is that the insulin-stimulated cytosolic kinase prefers Mn$^{2+}$ to Mg$^{2+}$, whereas the reverse is true for the S6 and protease-activated kinases (62, 62, 63). In fact, it has been reported that the S6 kinase from 3T3-L1 adipocytes is strongly inhibited by Mn$^{2+}$ (50). In view of these apparent differences in substrate and divalent cation specificities and NaF sensitivity, it is highly unlikely that the insulin-stimulated adipocyte cytosolic kinase that we have discovered is related to the S6 and protease-activated kinases.

In light of the inability of the insulin-sensitive cytosolic kinase to phosphorylate substrate peptides for casein kinase II, Ca$^{2+}$- and phospholipid-dependent and cGMP-dependent protein kinases, plosvin, phosphorylase b, glycogen synthase, and inhibitor II, the hormone-activated kinase is unlikely to be related to the different kinases responsible for phosphorylating these substrate peptides and proteins. Furthermore, as evidenced by the differential interaction with the DEAE anion exchanger between cAMP-dependent protein kinase and the insulin-stimulated cytosolic kinase and the lack of effect of CAMP-dependent protein kinase inhibitor on the insulin-sensitive kinase, these two protein kinases are clearly dissimilar. Interestingly, the insulin-activated cytosolic kinase is capable of phosphorylating ATP citrate-lyase and acetyl-CoA carboxylase, suggesting that this adipocyte kinase may be responsible for the increased phosphorylation of these two enzymes in intact cells treated with insulin (30–35). An early report (35) indicated that soluble extracts from insulin-treated adipocytes exhibits a 50% higher phosphorylating activity toward exogenous ATP citrate-lyase and acetyl-CoA carboxylase when compared to control. It is possible that these reported kinase activities may be related to the hormone-sensitive kinase described in this study. However, further investigation will be required to determine whether this is the case.

It was recently reported (71) that the addition of insulin to wheat germ agglutinin-agarose detergent extracts of IM-9 cells leads to an increase in histone serine kinase activity. Our observation indicates that the insulin-sensitive adipocyte cytosolic kinase does not adsorb to the lectin column (data not shown). Thus, it is unlikely that the adipocyte enzyme is related to the IM-9 lymphocyte kinase. We have recently reported (61) that in adipocyte microsomes, a serine protein kinase activity is also stimulated following insulin treatment of intact fat cells. This hormone-sensitive kinase preferentially phosphorylates histone and Kemptide. However, it does not utilize multifunctional calmodulin-dependent protein kinase substrate peptide, ATP citrate-lyase, and acetyl-CoA carboxylase as phosphorylation substrates in contrast to the insulin-stimulated cytosolic kinase described in this report. Furthermore, a portion of the insulin-stimulated microsomal kinase activity is specifically recognized by antiphosphotyrosine antibodies, suggesting that phosphotyrosine residues may be present in this particulate kinase (61). On the other hand, the hormone-activated cytosolic kinase exhibits no immunoreactivity with the antiphosphotyrosine antibody preparation (data not shown). In view of the differences in cellular location, substrate specificity, and interaction with antiphosphotyrosine antibodies between the microsomal and cytosolic kinases, it is unlikely that they represent the same enzyme species.

In view of the finding that the hormone-stimulated kinase activity emerges as one single peak when resolved by two sequential chromatography columns of different discriminating properties (DEAE ion-exchange and S-200 size sieving in
Figs. 4 and 5, respectively), it seems likely that this kinase activity is composed of one enzyme species. However, the possibility remains that the hormone-sensitive kinase activity may consist of multiple kinases of similar size and charge characteristics. Experiments are in progress in this laboratory to examine this issue further.

An important finding of this study is that insulin increases the \( V_{\text{max}} \) of this kinase activity rather than altering its affinities for the Kemptide substrate or ATP. These results imply that the insulin stimulation of this kinase will be sustained in intact cells where substrate concentrations are likely to be variable and the nucleotide concentrations will be in the millimolar range. The physiological significance of the Mn\(^{2+}\) dependence of the insulin-stimulated cytosolic kinase is not clear. In view of its high sensitivity to manganese ion, this kinase may remain functional in vivo where the Mn\(^{2+}\) concentration is normally low.

The persistence of insulin stimulation of the cytosolic kinase following two chromatography steps indicates that the hormone-induced change(s) in this enzyme activity is quite stable. This finding raises the possibility that the activation by insulin may involve some covalent modifications of this kinase. At present, it is not clear what the nature of this putative covalent modification may be. A likely candidate is the possibility that the hormone-sensitive kinase activity rather than altering its affinity for the Kemptide substrate or ATP. These results suggest that a functional role of these phosphatase inhibitors is to prevent the cytosolic kinase from dephosphorylation during fractionation, thus maintaining the enzyme in the activated state. This interpretation implies that the cytosolic kinase is stimulated when it is phosphorylated in response to insulin.

It is conceivable that the insulin-dependent activation of the cytosolic kinase may involve other covalent alterations such as limited proteolysis, methylation, sulfation, acetylation, acylation, ribosylation, oxidation, or reduction. Clearly, such a role of kinase regulation may be insulin-stimulated phosphorylation. This hypothesis is based on the evidence (Fig. 1) that the stimulatory effect of insulin on the cytosolic kinase is preserved when the cytosolic fraction is prepared in the presence of fluoride and vanadate, which are potent phosphatase inhibitors. These results suggest that the insulin-induced phosphorylation of these phosphatase inhibitors is to prevent the cytosolic kinase from dephosphorylation during fractionation and maintaining the enzyme in the activated state. This interpretation implies that the cytosolic kinase is stimulated when it is phosphorylated in response to insulin.

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