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Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*

[serine(threonine) protein kinase/3T3-L1 adipocytes]

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ABSTRACT **Exposure of 3T3-L1 cells to insulin stimulates a soluble, serine(threonine)-specific protein kinase that phosphorylates microtubule-associated protein 2 (MAP-2) *in vitro*. The enzyme, termed MAP kinase, was isolated from insulin-treated or control cells radiolabeled with $^{32}P_i$. A 40-kDa phosphoprotein was found to elute in exact correspondence with enzymatic activity during hydrophobic interaction and gel filtration chromatography of extracts from cells stimulated with insulin. Both MAP kinase activity and the phosphoprotein were absent in fractions prepared from untreated cells. The ^{32}P incorporated into the 40-kDa protein was stable during treatment with alkali. Phospho amino acid analysis confirmed that the radiolabel was primarily incorporated into phosphotyrosine and to a lesser extent phosphothreonine. In addition, MAP kinase was incompletely but specifically adsorbed by antibodies to phosphotyrosine. We conclude, based on these data and additional studies from this laboratory, that MAP kinase is phosphorylated on tyrosine *in vivo*. The data are consistent with the possibility that MAP kinase may be a substrate for the insulin receptor or another insulin-regulated tyrosine kinase.**

Alteration of the phosphorylation state of target enzymes is one of the principal mechanisms by which insulin acts to regulate cellular functions (1). Recently, evidence has begun to accumulate supporting the notion that activation of both the insulin receptor tyrosine kinase and soluble serine(threonine) protein kinases is required to bring about cellular responses to the hormone. Insulin receptors that lack functional tyrosine kinase activity have been engineered by *in vitro* site-directed mutagenesis. Experiments with these altered receptors indicate that tyrosine kinase activity is required for normal signaling by the receptor (2–4). The ribosomal protein S6 becomes phosphorylated in cells treated with insulin, apparently as the result of activation of a specific serine(threonine) kinase (5–8). The activity of the insulin-stimulatable S6 kinase purified from *Xenopus* oocytes is dependent on its phosphorylation on serine or threonine residues (9). Direct regulation of one or more serine(threonine) kinases or phosphatases by tyrosine phosphorylation has been proposed as an attractive hypothesis to explain how activation of the insulin receptor kinase might result in altered phosphorylation of proteins on serine or threonine (e.g., see refs. 5 and 10). Reports suggesting the existence of a serine(threonine) kinase that is phosphorylated on tyrosine and regulated by insulin have recently appeared (11, 12).

We have identified a serine(threonine) kinase that is activated by insulin treatment of 3T3-L1 cells (11). The enzyme, microtubule-associated protein (MAP) kinase, phosphorylates and activates S6 kinase II from *Xenopus* oocytes (8) *in vitro* and may therefore participate in a cascade of insulin-stimulated protein kinases (T.W.S., L.B.R., E. Erikson, and J. L. Maller, unpublished data). Here, we present evidence that MAP kinase is itself a phosphoprotein containing phosphotyrosine. This finding is consistent with the possibility that MAP kinase is phosphorylated and activated by the insulin receptor or another tyrosine kinase.

Tyrosine kinase activity is shared by growth factor receptors and viral transforming proteins, suggesting that such activity may be important in the regulation of cell growth and/or metabolism (reviewed in ref. 13). Although it is expected that physiologically important substrates for the growth factor receptor kinases (other than the receptors themselves) do exist, virtually no direct experimental evidence is available to support this view. Of those proteins known to undergo phosphorylation on tyrosine *in vivo*, only the receptors themselves and calmodulin have clearly defined biochemical functions (13, 14). This report provides direct evidence that MAP kinase, an enzyme subject to insulin-dependent regulation, is phosphorylated on tyrosine *in vivo*.

MATERIALS AND METHODS

Cell Culture and Partial Purification of MAP Kinase. 3T3-L1 cells were grown and differentiated as described by Rubin *et al.* (15) and used on days 5–8. Cells were treated with insulin (80 nM) for 10 min. Harvesting, homogenization, preparation of extract supernatants, and assays for MAP kinase activity were performed as reported (11). We have developed a method (to be described in detail elsewhere) for rapid partial purification of MAP kinase from 3T3-L1 cells that resolves the enzyme from contaminating kinases and phosphatases and results in an enzyme preparation that is stable during storage at −70°C. Briefly, extract supernatants of cells from 10 plates (100 mm) prepared in buffer C (11) without glycerol were applied to DEAE-cellulose and step-eluted with 0.35 M NaCl. The eluate was applied to a phenyl-Superose FPLC (fast-protein liquid chromatography) column (Pharmacia), from which activated MAP kinase was eluted by a simultaneous gradient of decreasing salt concentration and increasing concentration of ethylene glycol. Peak fractions from phenyl-Superose chromatography were concentrated in the presence of bovine serum albumin and stored at −70°C. The stored fractions were either used for experiments or further purified by gel filtration chromatography on a Superose-12 FPLC column (Pharmacia) as described in the text.

***In Vivo* Labeling with ^{32}P.** Cells were incubated with [^{32}P]orthophosphate [1–3 mCi per plate in 8 ml of Krebs–Ringer bicarbonate/Hepes buffer (11); 1 Ci = 37 GBq] for 2 hr and processed as described above. Fractions from phenyl-Superose chromatography were resolved by $NaDodSO_4$ gel electrophoresis on 12% polyacrylamide gels. The gels were fixed and silver stained (Bio-Rad).

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Abbreviation: MAP, microtubule-associated protein.
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Analysis of Phosphorylated Amino Acids. Proteins were recovered from dried gels (16). Phospho amino acid analysis was performed by the method of Cooper *et al.* (17).

Autoradiography and Quantitation by Densitometric Scanning. Dried gels were exposed to Kodak X-Omat AR 5 film and a DuPont Lightning Plus intensifying screen at −70°C. Autoradiograms were scanned with an Eikonix camera and the Gould system.

Immunoadsorption by Anti-Phosphotyrosine Antibodies. Antibodies to phosphotyrosine (18) were generously provided by Morris White (Joslin Diabetes Center). Protein A coupled to Sepharose Cl4-B was purchased from Pharmacia. The antibody was adsorbed to protein A-Sepharose to yield a concentration of 0.12 mg per ml of gel. Fifty microliters of a 1:1 suspension of the gel with or without preadsorbed antibody was added to 75 μl of the pooled peak fractions from Superose 12 chromatography, containing bovine serum albumin (1 mg/ml), leupeptin (1 μg/ml), pepstatin (2 mM), and aprotinin (200 units/ml), and incubated at 4°C for 4 hr with periodic agitation. The antibody–protein A-Sepharose complex was pelleted by centrifugation and the supernatant was assayed for MAP kinase activity. The pelleted beads were washed with buffer containing bovine serum albumin and then extracted with buffer containing 10 mM *p*-nitrophenyl phosphate for 30 min at 4°C. The beads were pelleted once again and the supernatant was assayed for MAP kinase activity released from the complex.

RESULTS

Copurification of MAP Kinase and a 40-kDa Phosphoprotein. Our earlier observations suggested that activation of MAP kinase involves phosphorylation of the enzyme (11). We therefore expected that it should be possible to correlate MAP kinase activity with the presence of a phosphoprotein during purification of the enzyme from cells labeled with [^{32}P]orthophosphate. Extracts of cells that had been labeled *in vivo* with $^{32}PO_4$ and treated with either insulin or diluent for 10 min were subjected to chromatography on DEAE-cellulose and then applied to a phenyl-Superose FPLC column. Fractions eluted from the latter column were assayed for MAP kinase activity with MAP-2 as substrate. MAP kinase eluted in a single peak (Fig. 1*A*). The presence of MAP kinase activity in these fractions was dependent on exposure of the cells to insulin. MAP kinase activity was barely detectable in fractions derived from untreated cells and constituted <5% of that isolated from insulin-treated cells (Fig. 1*A*).

Proteins in fractions from phenyl-Superose chromatography were resolved by $NaDodSO_4$ gel electrophoresis and labeled proteins were visualized by autoradiography (Fig. 1 *B* and *C*). A 38- to 42-kDa phosphoprotein, referred to hereafter as pp40φSup, was found to coelute with MAP kinase activity during phenyl-Superose chromatography of extracts of cells treated with insulin (Fig. 1*B*). Quantitation of the radioactivity present in pp40φSup by densitometric scanning of autoradiograms revealed a precise correlation of the amount of labeled phosphoprotein with MAP kinase activity (Fig. 1*A*). pp40φSup was also visualized by silver staining of $NaDodSO_4$ gels. The density of the stained band correlated exactly with the level of MAP kinase activity in each fraction (Fig. 1*A*). As described above, identical chromatographic separation of extracts from untreated cells yielded fractions with barely detectable kinase activity. After resolution of these fractions on $NaDodSO_4$ gels, pp40φSup was also undetectable by either silver staining or autoradiography (Fig. 1*C*; data not shown). Thus, stimulation of 3T3-L1 cells with insulin results in the appearance, in fractions eluted from phenyl-Superose, of both a ^{32}P-labeled 40-kDa phosphoprotein, a corresponding protein band detected by staining, and MAP kinase activity, all of which are essentially absent in equivalent fractions from untreated cells.

To more critically evaluate the correlation of pp40φSup and MAP kinase, the enzyme recovered from phenyl-Superose chromatography was further purified by gel-filtration chromatography. Extracts of 3T3-L1 cells, prelabeled with [^{32}P]orthophosphate and then exposed to insulin for 10 min, were subjected to DEAE-cellulose and phenyl-Superose chromatography. Peak fractions from the phenyl-Superose column were applied to a Superose 12 FPLC column. MAP kinase activity elutes from this column with an apparent molecular mass of 35 kDa (19). The amount of pp40φSup in each fraction, determined by either autoradiography of the ^{32}P-labeled protein or by the density of the silver-stained band, correlated precisely with the activity of MAP kinase in those fractions (Fig. 2).

Detection of Phosphotyrosine in pp40φSup. Phosphate esters of tyrosine are more stable to alkali hydrolysis than those of serine and threonine (16). Because of this property, alkali treatment of gels has been successfully used to detect proteins containing ^{32}P-labeled phosphotyrosine residues

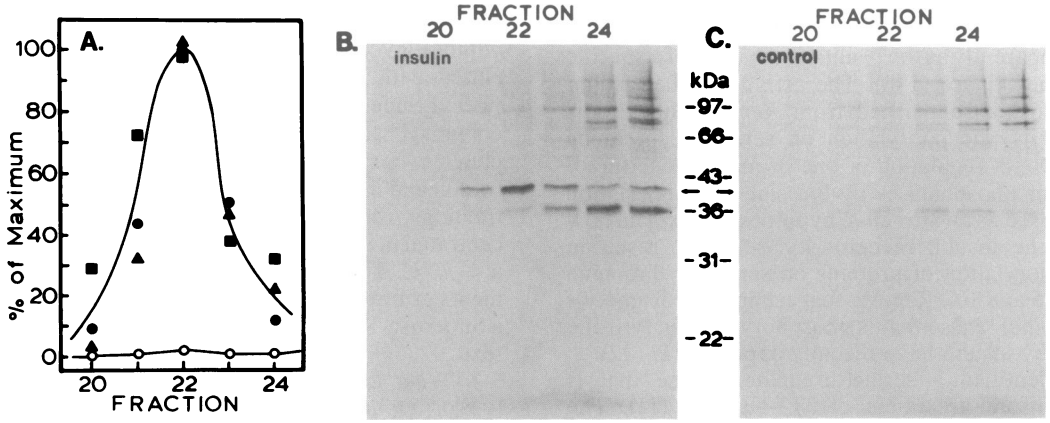

FIG. 1. Coelution of MAP kinase and a 40-kDa phosphoprotein from phenyl-Superose. ^{32}P-labeled 3T3-L1 cells were treated with insulin or diluent. Extracts were fractionated by DEAE-cellulose and phenyl-Superose chromatography. (*A*) Fractions eluted from phenyl-Superose were assayed for MAP kinase activity (●, insulin treated; ○, control). (*B* and *C*) The same fractions were subjected to $NaDodSO_4$/PAGE and analyzed by autoradiography (*B*, insulin treated; *C*, control). Migration of molecular mass standards and pp40φSup (arrow) is indicated. The amount of pp40φSup in each fraction was determined by densitometric scanning of either these autoradiograms (▲) or silver-stained gels (■) (*A*). Maximal activity of MAP kinase was 35,800 cpm of ^{32}P incorporated into MAP-2 in 10 min. The peak fraction derived from untreated cells catalyzed the transfer of only 540 cpm. The results depicted in this and other figures are representative of three or more independent experiments.

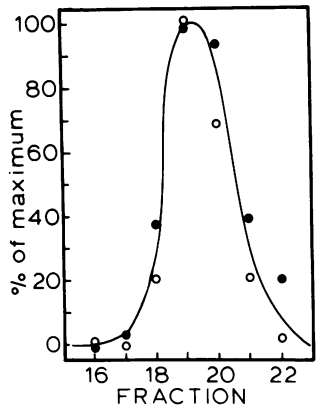

FIG. 2. Coelution of MAP kinase and pp40φSup from gel-filtration chromatography. MAP kinase was purified from insulin-treated cells by DEAE-cellulose and phenyl-Superose chromatography and then subjected to chromatography on Superose 12. An aliquot of each fraction was assayed for MAP kinase activity (●); 100% corresponds to 2800 cpm of ^{32}P incorporated into MAP-2. The remainder of the fraction was concentrated and resolved by NaDodSO$_4$/PAGE. The amount of pp40φSup in each fraction was assessed by densitometric scanning of an autoradiogram (exposed for 3 days) of the gel (○).

(16). ^{32}P-labeled pp40φSup was isolated as described above. Gels containing the protein were incubated in 1 M KOH and compared to equivalent gels not exposed to base. Alkali treatment dramatically reduced the content of ^{32}P in all other proteins detected in the autoradiograms, while labeling of pp40φSup was reduced only slightly (Fig. 3).

To confirm the presence of phosphotyrosine in pp40φSup indicated by the studies of alkali stability of the phosphorylated band, phospho amino acid analysis was performed on that protein excised and eluted from a NaDodSO$_4$ gel. As shown in Fig. 4, pp40φSup contains primarily phosphotyrosine and a lesser amount of phosphothreonine. Interestingly, phosphoserine, the most abundant cellular phosphorylated amino acid, is nearly undetectable.

Immunoadsorption of MAP Kinase with Anti-Phosphotyrosine Antibodies. If MAP kinase is activated by a mechanism involving its phosphorylation on tyrosine, we reasoned that the enzyme might be immunoadsorbed by anti-phosphotyrosine antibodies. Peak fractions from phenyl-Superose chromatography were subjected to gel-filtration chromatography, which affords additional purification and removal of *p*-nitrophenyl phosphate, which would block adsorption by anti-phosphotyrosine antibodies. Peak fractions from the gel filtration column were pooled and aliquots were incubated with anti-phosphotyrosine antibodies, which were preadsorbed to protein A-Sepharose beads. The antibody and bound molecules were then removed by centrifugation. In three independent experiments, 14% ± 2% (SEM) of the total activity present was specifically removed by immunoadsorption with anti-phosphotyrosine antibodies. Incubation of the pelleted beads with buffer containing 10 mM *p*-nitrophenyl phosphate released 70% ± 13% (SEM) of the activity adsorbed by anti-phosphotyrosine antibodies. The specificity of the adsorption of MAP kinase activity by anti-phosphotyrosine antibodies is supported by the observation that inclusion of 10 mM *p*-nitrophenyl phosphate during incubation of the MAP kinase fraction with the antibody blocked the immunoadsorption of the activity; only 1% ± 4% (SEM) of the total activity was immunoadsorbed in the presence of 10 mM *p*-nitrophenyl phosphate.

DISCUSSION

Hydrophobic interaction chromatography of extracts from 3T3-L1 cells treated with insulin resolves a peak of MAP kinase activity that is almost completely absent in comparable fractions from untreated cells (Fig. 1). Furthermore, these fractions contain a phosphoprotein (pp40φSup), the abundance of which, determined by either silver staining or autoradiography, is precisely proportional to the enzymatic activity in each fraction.

The nearly complete dependence of the appearance of MAP kinase activity in phenyl-Superose eluates on prior insulin treatment of the cells is most easily explained by the hypothesis that chromatography on phenyl-Superose separates the activated form of the enzyme from a less active precursor. This would account for the observations that MAP kinase is stimulated 1.5- to 3-fold in crude extract supernatants from these cells, while the peak fractions from phenyl-Superose chromatography routinely show 20- to 60-fold stimulation over equivalent fractions from control cells. The alternative possibility, that the enzyme is inactive in serum-starved cells and undergoes a dramatic 50-fold increase in activity in stimulated cells, is unlikely because the 40-kDa protein detected by silver staining of gels, which correlates with activity, is also absent in fractions from untreated cells. Thus, it appears more likely that the unstimulated form of the enzyme does not elute from phenyl-Superose in the same position as the activated kinase.

The results described here, along with other observations from this laboratory, provide convincing evidence that MAP kinase and pp40φSup are identical. Several independent observations lead to this conclusion. First, MAP kinase

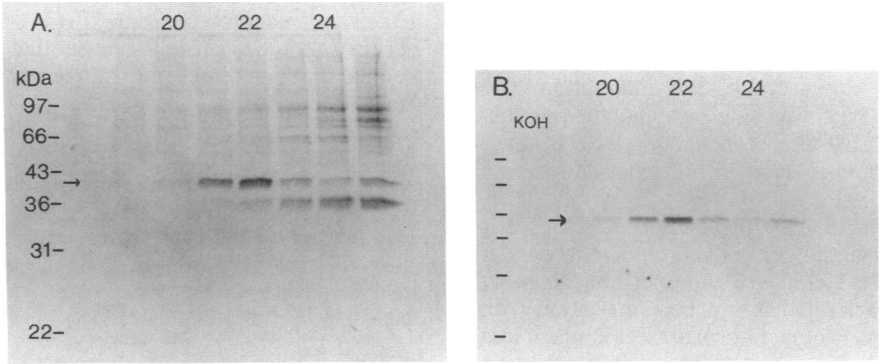

FIG. 3. Alkali stability of pp40φSup. MAP kinase was partially purified from ^{32}P-labeled 3T3-L1 cells treated with insulin through chromatography on phenyl-Superose. Equal portions of fractions eluted from phenyl-Superose proteins were resolved on identical NaDodSO$_4$ gels. Both gels were fixed and silver stained and one (*B*) was subjected to alkali treatment (1 M KOH for 2 hr at 55°C). The alkali-treated gel was then washed with 10% acetic acid/10% ethanol for 2 hr, during which the solution was changed four times. The gels were dried and autoradiograms were prepared by equal exposure (7 days) of both gels to photographic film. The migration of molecular mass standards and pp40φSup (arrow) is indicated. Fraction numbers are noted above the lanes.

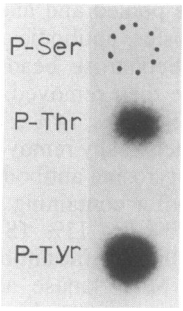

FIG. 4. Analysis of phosphorylated amino acids from pp40φSup. Radiolabeled pp40φSup was prepared as described in Fig. 1. The band was excised from the gel and processed as described. The migration of phosphorylated amino acid standards visualized by reaction with ninhydrin is indicated. ^{32}P-labeled amino acids were visualized by autoradiography for 8 days.

activity and pp40φSup are both detected in eluates from phenyl-Superose only when fractionating extracts of cells that have been exposed to insulin. Both are barely detectable in fractions from unstimulated cells. Second, MAP kinase and pp40φSup eluted from DEAE-cellulose copurify during further chromatographic separations on hydrophobic interaction and gel filtration columns. Each of these procedures provides resolution of proteins based on distinct physical properties. It is unlikely that all of these characteristics would be shared by a protein fortuitously copurifying with the kinase. Finally, we have estimated the molecular mass of MAP kinase by gel filtration chromatography and by glycerol gradient sedimentation (unpublished observations). These methods gave estimates of 35 kDa and 37 kDa, respectively. Since phosphorylation is often observed to retard slightly the migration of proteins in $NaDodSO_4$ gels, the apparent molecular mass of 40 kDa of the protein observed on $NaDodSO_4$ gels is entirely consistent with that estimated by the two other methods. Thus, the phosphoprotein apparently represents the kinase itself rather than a subunit or regulatory factor.

We have previously proposed that MAP kinase might be regulated by a mechanism involving phosphorylation because of the pronounced ability of phosphatase inhibitors to protect MAP kinase activity in crude cell extracts (11). The efficacy of phosphotyrosine and its structural analog *p*-nitrophenyl phosphate in this regard suggested further that phosphorylation on tyrosine might be involved (11). In the present experiments, we have demonstrated directly that MAP kinase isolated from insulin-treated cells is phosphorylated on tyrosine and to a lesser extent on threonine. Since the insulin receptor is a tyrosine kinase, the simplest hypothesis to explain our results is that the receptor phosphorylates MAP kinase and that this or another modification involved in the activation of the enzyme alters its interaction with phenyl-Superose. However, we cannot exclude the possibility that the enzyme is constitutively phosphorylated because we cannot as yet compare its level of phosphorylation with that of a precursor.

Additional support for the hypothesis that MAP kinase is activated by phosphorylation is derived from our experiments, to be described in detail elsewhere, establishing that the enzyme can be inactivated by dephosphorylation with protein phosphatase 2A. Protein phosphatase 1 is much less effective in this regard. Phosphatase 2A acts primarily on phosphoserine/phosphothreonine residues but, unlike phosphatase 1, it also has a significant level of activity toward phosphotyrosine (20). Since we have detected phosphorylation of both threonine and tyrosine in MAP kinase, it is not clear whether phosphatase 2A acts on one or both of these residues. By analogy to other protein kinases, several different phosphorylation sites might be expected to have regulatory significance (21, 22).

MAP kinase is partially, but specifically, immunoadsorbed by anti-phosphotyrosine antibodies, providing additional evidence that the enzyme contains phosphotyrosine. Incomplete immunoprecipitation of proteins containing phosphotyrosine by anti-phosphotyrosine antibodies has been reported in several other instances (12, 23). This may be caused by low affinity of the antibodies for phosphotyrosine residues located within microenvironments of the proteins that inhibit interaction of the antibody and epitope. However, other explanations cannot be excluded. Yu *et al.* (12) have described a membrane-bound serine kinase that is partially immunoadsorbed by anti-phosphotyrosine antibodies. The subcellular localization and substrate specificity of this enzyme differ from those of MAP kinase, but further characterization of the particulate kinase is required to allow definitive comparison of the enzymes.

The fact that several peptide growth factor receptors and viral transforming proteins are tyrosine kinases implies that such activity may be important in conveying intracellular signals that promote growth (13). Efforts to identify substrates for tyrosine kinases have been carried out by one- or two-dimensional gel electrophoretic analysis of phosphoproteins induced by treatment of cells with growth factors (24–26). These studies have identified a protein of ≈42 kDa (pp42) that is apparently a substrate for tyrosine kinases stimulated by a variety of growth-promoting agents including peptide growth factors, phorbol esters, and transforming retroviruses (24–27). Similar analysis of proteins from amphibian oocytes undergoing maturation (a process that can be stimulated by treatment of immature oocytes with insulin) revealed a 42-kDa protein phosphorylated on tyrosine and threonine residues (28). Comparison of these proteins to MAP kinase appears worthwhile.

We have demonstrated that MAP kinase is a phosphoprotein that, when isolated from insulin-treated 3T3-L1 cells, is phosphorylated on tyrosine and threonine residues. MAP kinase is the only insulin-regulated enzyme (other than the insulin receptor itself) that has been shown directly to be phosphorylated on tyrosine. Because the activation of MAP kinase occurs rapidly after exposure of cells to insulin and the phosphorylation state of the enzyme appears to regulate its activity, direct phosphorylation of the kinase by the insulin receptor presents an attractive hypothesis to account for these observations. We have recently demonstrated that MAP kinase phosphorylates and activates ribosomal protein S6 kinase II *in vitro* (T.W.S., L.B.R., E. Erikson, and J. L. Maller, unpublished data). It is quite possible in light of these findings that MAP kinase functions as an intermediate between the receptor tyrosine kinase and at least one other serine(threonine) protein kinase that regulates a specific cellular function. Since the discovery that the insulin receptor is itself a tyrosine kinase that is activated by hormone binding, it has often been proposed that signal transduction from the receptor to intracellular targets could proceed by sequential activation of a series of protein kinases (e.g., see refs. 5 and 10). Analysis of the specific amino acid modifications that regulate MAP kinase activity and evaluation of the ability of MAP kinase to serve as a substrate for the insulin receptor and other tyrosine kinases will allow us to assess the validity of this model.

Note Added in Proof. Additional experiments suggesting regulation by phosphorylation–dephosphorylation of the mitogen-stimulated S6 kinase from Swiss mouse 3T3 cells have been published (29).

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