THE STIMULATION OF pp42^{mapkinase} BY INSULIN DOES NOT CORRELATE WITH ITS METABOLIC ACTIONS IN CELLS OVEREXPRESSING MUTANT INSULIN RECEPTORS

Long Pang§†, Dan F. Lazar†, David E. Moller‡, Jeffrey S. Flier‡, and Alan R. Saltiel§†*

§Department of Physiology, University of Michigan, School of Medicine, Ann Arbor, MI 48109

†Department of Signal Transduction, Parke-Davis Pharmaceutical Division, Ann Arbor, MI 48105

‡The Charles A Dana Research Institute and Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine, Beth Israel Hospital and the Harvard Medical School, Boston, MA 02215

SUMMARY: Naturally occurring human insulin receptor mutants Ser^{1200} and Thr^{1134}, and a site-directed mutant Arg^{1030} overexpressed in Chinese hamster ovary (CHO) cells, bind insulin with affinities identical to wildtype receptors but are apparently kinase deficient. Cells expressing the Ser^{1200} receptor exhibit insulin stimulation of glycogen synthesis similar to these bearing the wildtype receptor, but fail to mediate insulin-responsive DNA synthesis. In contrast, the Thr^{1134} and Arg^{1030} mutants exhibit no response to insulin. The activity of Mitogen Activated Protein (MAP) kinase in cells transfected with wildtype receptor is more responsive to insulin than that detected in untransfected parental cells, while cells bearing any of the mutant receptors are less responsive than the parental cells. These differences in the stimulation of MAP kinase activity are paralleled by differences in insulin-dependent phosphorylation of the enzyme. These results suggest that the p42 MAP kinase is not universally required for the metabolic effects of insulin. © 1993 Academic Press, Inc.

Although the intracellular events that mediate the diverse actions of insulin remain poorly understood, an increasing body of evidence has implicated a role for regulation of protein phosphorylation. The insulin receptor itself is a tyrosine kinase that undergoes autophosphorylation (1) and catalyzes the tyrosine phosphorylation of several intracellular proteins (1-10). Moreover, insulin also acutely regulates serine and threonine phosphorylations, paradoxically stimulating the phosphorylation of some proteins while causing the dephosphorylation of others, presumably through the activation of serine/threonine-specific kinases and phosphatases (11-14). While the precise link between the receptor tyrosine kinase and serine/threonine kinases and phosphatases has not been identified, one of the most promising candidates discovered thus far to mediate some of the phosphorylations produced by insulin is the family of Mitogen Activated Protein (MAP) kinases. The activities of these enzymes are acutely stimulated by growth and differentiative factors (15-25), including insulin (26), via a mechanism that involves both tyrosine and serine or threonine phosphorylation of the enzyme itself (17, 25, 27-29).

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* To whom correspondence should be addressed.   Fax: 313-996-5668.

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Although the cellular substrates for the enzyme have yet to be clearly identified, MAP kinases may be intermediates in the regulation of other serine kinases, such as the ribosomal S6 kinase II (26). The activation of S6 kinase II has been proposed to mediate a cascade resulting in the stimulation of glycogen synthesis and inhibition of glycogenolysis by insulin, via phosphorylation of site-1 on glycogen-associated protein phosphatase I (30, 31). However, the precise role of the MAP kinase family members in the pleiotropic actions of insulin remains unknown.

One of the most useful tools in dissecting the importance of different signaling pathways in insulin action has been the identification of point mutations in the insulin receptor tyrosine kinase domain, some of which have been found in patients with the type A syndrome (32) of insulin resistance (33-35). Two such mutant receptors, $Trp^{1200} \rightarrow Ser^{1200}$ (33), and $Ala^{1134} \rightarrow Thr^{1134}$ (35) were transfected, expressed, and partially characterized in Chinese hamster ovary (CHO) cells (34-36). Although both mutants are markedly kinase-deficient, cells expressing the Ser^{1200} receptor exhibited a level of insulin-stimulated 2-deoxyglucose uptake and glycogen synthesis similar to that observed with the wildtype receptor under conditions of overexpression. In contrast, insulin-dependent DNA synthesis and induction of GLUT-1 mRNA expression was identical to untransfected cells, supporting the hypothesis that divergence of signaling pathways may begin at the insulin receptor.

MATERIALS AND METHODS

Materials: All reagents were purchased from Sigma except for tissue culture reagents (GIBCO), $[\gamma^{-32}P]ATP$ (3000Ci/mmol) (NEN), mouse anti-MAP kinase monoclonal antibody (Zymed), mouse anti-phosphotyrosine monoclonal antibody (UBI), $\alpha IR1$ anti-human insulin receptor monoclonal antibody (a generous gift from Dr. S. Jacobs).

Cell culture: CHO cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum. Stable clonal CHO cell lines expressing wild-type human insulin receptors (CHO-hIRC), Thr^{1134} mutant receptors (CHO-1134), Ser^{1200} mutant receptors (CHO-1200), or Arg^{1030} mutant receptors (CHO-1030) (a generous gift of Dr. W. Rutter (37)), were maintained in the same medium with the addition of 200 µg/ml Geneticin (G418). Prior to insulin treatment, the medium was replaced with serum-free medium and incubated overnight. Insulin was directly added to the medium and the incubation was continued for the indicated time at 37°C. After hormonal treatment, the medium was removed, and the cell layer was quickly washed three times with 10 ml of ice-cold Ca^{++}-free phosphate-buffered saline (PBS). Cells were collected as described previously (18).

MAP kinase assay: This was assayed as described previously (18, 38).

Anti-phosphotyrosine blotting: CHO cells were grown in 150 mm tissue culture dishes as described above. After insulin treatment, cells were lysed and immunoprecipitated with anti-MAP kinase antisera. The resulting immune complexes were subjected to SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibody as described (39).

***In vitro* assay of insulin receptor autophosphorylation**: CHO cells were grown in 150 mm tissue culture dishes as described above. After insulin treatment, cells were lysed and immunoprecipitated with anti-receptor antibody, $\alpha IR1$. The resulting immune complexes were subjected to *in vitro* kinase assay in a 20 µl reaction mixture as described previously (39). After incubation for 5 min at 24°C, reaction were stopped with Laemmli sample buffer (40) and electrophoresed on 7.2% SDS-PAGE followed by autoradiography.

Thymidine uptake: Insulin stimulated thymidine uptake was measured in 24-well plates. Subconfluent non-transfected, wild-type transfected and Ser^{1200} transfected CHO cells were incubated in Ham's F-12 medium with 0.5% fetal bovine serum for 24 hr. Cells were then washed three times with PBS and exposed to various concentrations of insulin for 16 hr. This medium was then removed, and 0.5 ml of F-12, 0.1% BSA containing 1.0 µCi of [³H]thymidine was added to each well. After 90 min at 37°C, cells were washed three times with ice-cold PBS and once with 10% trichloroacetic acid. Trichloroacetic acid-precipitable material was dissolved in 0.5 ml of 1 M NaOH, and 0.4 ml was transferred to 3 ml of scintillant, neutralized with concentrated HCl, and assayed for radioactivity in a liquid scintillation counter.

Glycogen synthesis: Confluent CHO cells in 6-well plates were incubated in DMEM for 3 hr prior to exposure to insulin. After 30-min incubation at 37°C with insulin, 2 μCi of [U-^{14}C]glucose was added to each well for a final 90-min incubation at 37°C. Cells were then solubilized and the radioactivity in glycogen was measured as described previously (41).

RESULTS AND DISCUSSION

Insulin-induced changes in protein phosphorylation were evaluated in CHO cells that were not transfected (CHO) or transfected with wildtype human insulin receptor (CHO-hIRC), mutant Thr^{1134} (CHO-1134), mutant Ser^{1200} (CHO-1200), or with a mutation Lys→ Arg^{1030} in the ATP binding site of the receptor tyrosine kinase domain (CHO-1030). These transfected cell lines have been previously characterized, and have roughly equivalent numbers of insulin receptors with comparable binding affinities (36). To compare the tyrosine phosphorylations induced by these receptors, lysates from each cell type treated with or without insulin were immunoprecipitated with a monoclonal antibody directed toward the extracellular domain of the human insulin receptor (α-IR1) that recognizes both wildtype and mutant receptors (fig. 1). Following immunoprecipitation, the *in vitro* autophosphorylation of the receptors was evaluated by incubation of immunoprecipitates with [γ-^{32}P]ATP. Because this antibody recognizes only human receptor, autophosphorylation of endogenous CHO receptors was not detected in the CHO cells. In contrast, insulin markedly stimulated tyrosine phosphorylation of the wildtype receptor, but had no detectable effect on any of the three mutant receptors, despite overexposure of these gels (data not
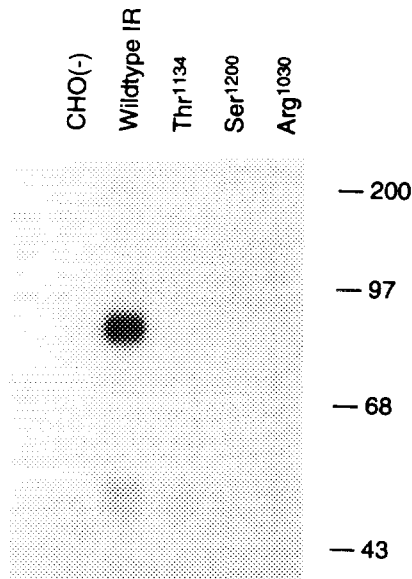

Fig. 1. Insulin-stimulated tyrosine phosphorylation is not detectable in cells with mutant receptors. Untransfected and transfected CHO cells with wildtype and mutant human insulin receptors were grown in 100 mm plates and treated with or without 100 nM insulin for 5 min. Cells were lysed and insulin receptors were immunoprecipitated with α-IR1 followed by an *in vitro* autophosphorylation in the presence of [γ-^{32}P]ATP. ^{32}P-labeled insulin receptors were resolved on 7.2% SDS-PAGE and visualized by autoradiography. Overexposure of the gel for up to 2 weeks revealed no signal in the mutant receptors. The results shown here were repeated twice in separate experiments.

Table 1. MAP kinase activation in the CHO cell lines. The CHO cells transfected with wildtype and mutant receptors were grown in 60 mm dishes till confluent. Prior to the hormonal treatment, cells were serum-starved for 12 hr. Cells were treated with 100 nM insulin for 5 min or 100 nM okadaic acid for 1 hr. Following treatment, cells were lysed and MAP kinase activity was assayed. Results are expressed as the means of triplicate determinations, ±SD. Similar results were reproduced in separate experiments.

Cell Line	MAP kinase (cpm/μg)			Ratio
	No Addition	Insulin	Okadaic Acid	Insulin/OkA
CHO (-)	46.7±1.0	110.9±4.0	153.5±0	0.64
CHO-hIRC	61.2±6.0	117.3±2.0	114.9±9.0	1.06
CHO-1134	35.9±5.0	72.7±3.0	109.0±0.7	0.51
CHO-1200	40.9±2.0	58.7±12	121.5±0.9	0.23
CHO-1030	39.8±1.0	71.1±10	106.1±4.7	0.77

shown). The kinase deficiency of the mutant receptors was confirmed by immunoblotting with anti-phosphotyrosine antibodies (data not shown).

To further evaluate the insulin responsiveness of cells expressing mutant insulin receptors, we compared the activation of MAP kinase in cells treated with insulin and the serine/threonine phosphatase inhibitor okadaic acid (table 1). This agent indirectly activates MAP kinase (42), presumably by inhibiting the dephosphorylation of serine and threonine residues on the enzyme by a receptor-independent mechanism. Basal activity of the enzyme was comparable in untransfected and mutant transfected cells, but was slightly higher in cells transfected with wildtype receptors. Addition to cells of 100 nM okadaic acid produced a 2-3 fold activation of MAP kinase in all cell lines. Insulin stimulated the enzyme in CHO-hIRC cells and was slightly less effective in CHO cells, but caused only modest increases in cells bearing the mutant receptors. Moreover, when
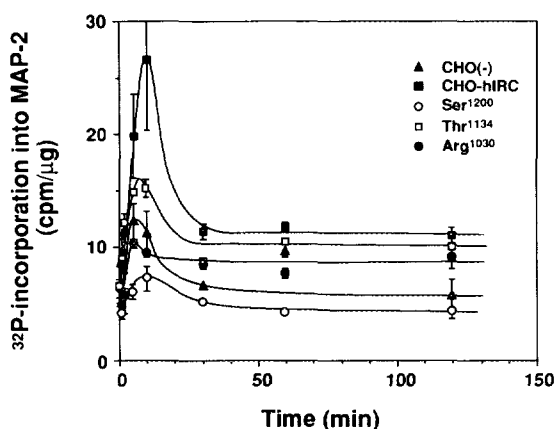

Fig. 2. Time course of the activation of MAP kinase by insulin. The CHO cells transfected with wildtype and mutant receptors were grown in 60 mm dishes till confluent. Prior to hormonal treatment, cells were serum-starved for 12 hours. Each dish of cells was treated with 100 nM insulin for the indicated time. Cells were then lysed and MAP kinase activity was assayed. Lysates were incubated with [γ-^{32}P]ATP in the presence of MAP-2 as described in "Materials and Methods". Reactions were stopped with Laemmli sample buffer and electrophoresed on 7.2% SDS-PAGE, followed by autoradiography. Results are expressed as the means of triplicate determinations, ±SD. Similar results were reproduced in 3 separate experiments.
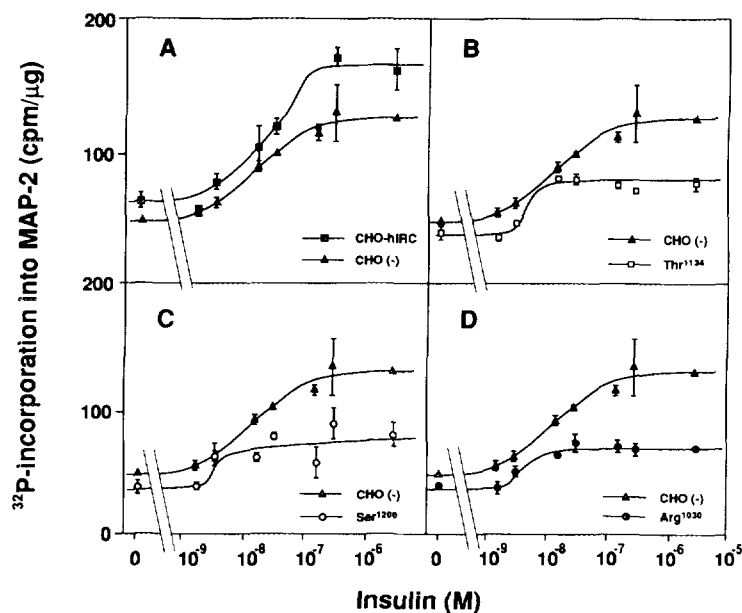
Fig. 3. Dose dependence of MAP kinase activation by insulin. The CHO cells transfected with wildtype and mutant receptors were grown in 60 mm dishes till confluent. Prior to insulin treatment, cells were serum-starved for 12 hours. Each dish of cells was treated with the indicated concentration of insulin for 10 min. Cells were then lysed and MAP kinase activity in cell lysates was assayed as described in figure 2. Results are expressed as the means of triplicate determinations, ±SD. Similar results were reproduced in 3 separate experiments.

expressed as a percentage of activation observed in response to okadaic acid, the effect of insulin was most evident in cells expressing wildtype receptors.

Because the activation of MAP kinase by growth factors is usually transient (38), we examined the time courses of enzyme activation by insulin in CHO cells expressing Thr^{1134}, Ser^{1200}, Arg^{1030} and wildtype human insulin receptor or the parental CHO cells (fig. 2). The time courses of MAP kinase activation by insulin in all 5 cell lines were virtually identical. Maximal activity was achieved by 10 min for all cells, and declined thereafter. Identical results were produced in several repetitions of this experiment, in which 4-5 fold activation of enzyme activity was consistently observed in cells expressing wildtype receptors.

To further explore the sensitivity to insulin of cells expressing the different insulin receptors, the dose dependence of MAP kinase activation was compared (fig. 3). In CHO-hIRC cells, insulin caused a dose dependent increase in enzyme activity, with a half-maximal concentration for activation (EC_{50}) of approximately 30 nM, and a maximal effect occurring at approximately 100 nM (fig. 3A). The parental untransfected CHO cells were equally sensitive to insulin with a EC_{50} for MAP kinase activation of approximately 25 nM, although maximal activity was almost 30% lower than that observed in cells transfected with wildtype receptor. Interestingly, cells expressing all three mutant receptors were significantly less responsive to insulin than wildtype cells or untransfected cells (fig. 3B-D), suggesting that each mutation may produce a dominant negative phenotype for this response of insulin.
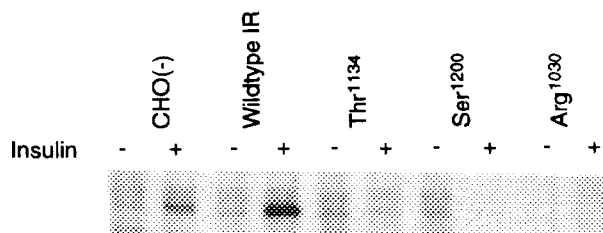
Fig. 4. Insulin-dependent tyrosine phosphorylation of MAP kinase. All 5 cell lines were grown in 150 mm dishes till confluent. Prior to insulin treatment, cells were serum-starved for 12 hr. Each dish of cells was treated with or without 100 nM insulin for 5 min and then harvested in 100 μl 1% SDS. Cell lysates were diluted with 1 ml RIPA buffer and centrifuged. The supernatant was precipitated with 5 μg mouse anti-MAP kinase antibody. Immunoprecipitated MAP kinase was then subjected to SDS-PAGE followed by Western blot with anti-phosphotyrosine antibody. The results shown here were repeated three times in separate experiments.

MAP kinase is activated by mitogens via a mechanism requiring tyrosine and serine or threonine phosphorylation (17, 25, 27, 28, 43). Thus, to further explore differences in signaling from mutant receptors, we compared the effect of insulin on the phosphorylation of immunoprecipitated MAP kinase in these cells. Cells were treated with or without 100 nM insulin for 5 min, and pp42^{mapk} was immunoprecipitated with a monoclonal antibody (fig. 4). Following immunoprecipitation, the enzyme was subjected to SDS-PAGE followed by immunoblotting with an anti-phosphotyrosine antibody. In cells expressing wildtype receptors, insulin dramatically stimulated the tyrosine phosphorylation of the 42 kd MAP kinase (43). In untransfected CHO cells, tyrosine phosphorylation of pp42^{mapk} was also detected, although to a lower extent. In contrast, no insulin-dependent tyrosine phosphorylation of the 42 kd protein could be detected by blotting in any of the cells expressing mutant receptors.

To further compare the insulin responsiveness in cells bearing Ser^{1200} mutant insulin receptors and wild-type insulin receptors, [³H]thymidine incorporation and glycogen synthesis were measured (fig. 5). Cells overexpressing the Ser^{1200} mutation retained insulin responsiveness regarding glycogen synthesis. The insulin dose response was roughly equal to that observed in wildtype receptor-expressing cells (fig. 5A). In contrast, cells expressing the Ser^{1200} receptor were equivalent to untransfected cells regarding insulin-dependent thymidine incorporation, whereas wildtype receptors produced a large response (fig. 5B). Despite the differences in cellular responses to insulin, Ser^{1200} receptors were ineffective in mediating stimulation of the activity of MAP kinase, even producing effects apparent of a dominant negative phenotype (fig. 5C).

The different cellular actions of insulin can be clearly distinguished on the basis of time course, dose response and cell type (13), indicating that more than one signaling pathway may be involved. Indeed, differences in the cellular actions of insulin have been observed in a number of cells expressing truncated (44-47) or mutated (36, 37, 48-57) receptors, suggesting that discrete signals may emerge from the receptor itself. In this study, we have evaluated the effects of point mutations found in the insulin receptor tyrosine kinase domain from patients with insulin resistance. These mutations result in receptors that are markedly kinase deficient, as demonstrated by *in vivo* and *in vitro* kinase assays examining both autophosphorylation (34, 35, 37) and
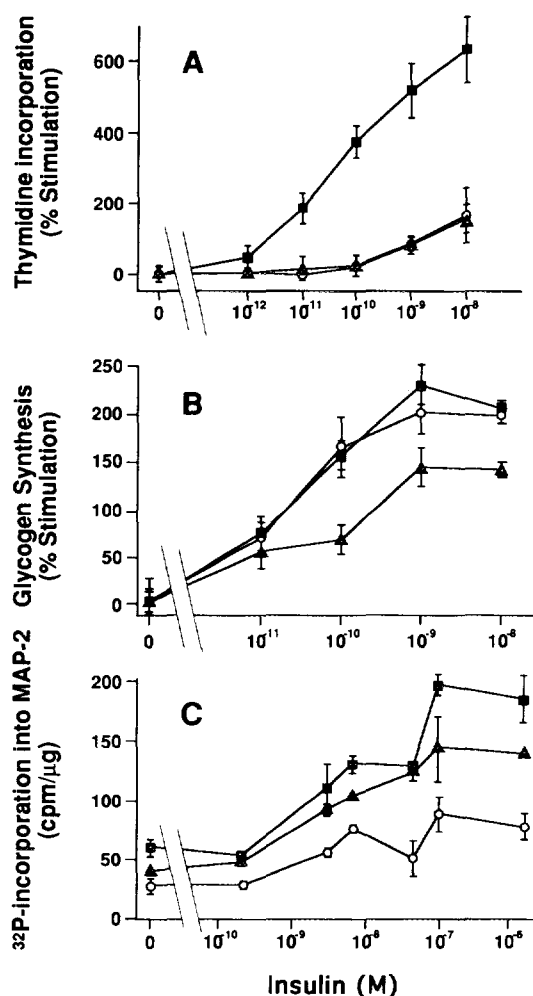
Fig. 5. Comparison of different actions of insulin in CHO cells overexpressing insulin receptors. Three different biological actions of insulin are depicted with untransfected (▲), wildtype-transfected (■) or Ser^{1200} receptor-transfected (O) CHO cells. A) Thymidine incorporation; B) glycogen synthesis, and C) MAP kinase activation were assayed as described in "Materials and Methods". Results are the mean of triplicated determinations ± SD and were reproduced in three separate experiments.

phosphorylation of exogenous substrates (34, 35). However, analysis of four different biologic assays revealed differences in the sensitivity to insulin of cells overexpressing these receptors. Whereas both the Lys→ Arg^{1030} and Ala→ Thr^{1134} mutants were incapable of any measurable response to insulin (36, 37), the Trp→ Ser^{1200} mutation retained responsiveness regarding GLUT 1-dependent glucose transport (37) and glycogen synthesis in overexpressing cells, but was inactive in mediating DNA synthesis. Despite these wide differences in cellular responses to insulin, all of the mutant receptors were similarly ineffective in mediating stimulation of MAP kinase, even producing effects apparent of a dominant negative phenotype, clearly illustrate the differences of these processes in both insulin sensitivity and responsiveness.

It is tempting to conclude from these studies that the stimulation of the p42 MAP kinase, while clearly a result of insulin receptor activation, is correlated with only a subset of the biological actions of insulin. All of these kinase-deficient mutant receptors failed to mediate insulin-stimulated DNA synthesis (36). This apparent connection between MAP kinase and thymidine incorporation may not be surprising, since the activity of this kinase is stimulated by many other growth factors that bind to tyrosine kinase receptors (58). However, the retention of glucose transport and glycogen synthesis stimulation in the face of an absent MAP kinase response in cells overexpressing the Ser^{1200} mutant indicates that this family member is not the major signal for these effects of insulin in transfected CHO cells. The dissociation of MAP kinase activation from these metabolic effects of insulin is further supported by the opposite effects produced by the phosphatase inhibitor okadaic acid, which stimulates MAP kinase activity (42) but attenuates insulin-stimulated glucose transport (59-61) and glycogen synthesis (41, 62). Additionally, an insulin-sensitive PC-12 pheochromocytoma cell line has been described that does not exhibit stimulation of MAP kinase by insulin, despite significant stimulation by NGF and EGF (63). Furthermore, a number of mitogens that activate MAP kinase via Ca^{++} or protein kinase C pathways (25) have no effect or oppose the actions of insulin in these metabolic processes, especially glycogen synthesis (64-67). It must be emphasized, however, that the MAP kinases belong to a family of enzymes, with potential for differential distribution in human tissues as well as different substrate preferences that we may not be able to detect at the present time. Thus, the dissociation of these actions of insulin described here may be related only to the pp42 MAP kinase that is expressed in CHO cells. Furthermore, because of the wide differences in insulin sensitivity of processes such as glucose transport and DNA synthesis in cells overexpressing receptors, mutated partially active receptors may still be capable of producing a full downstream response to insulin, due to the considerable overexpression of receptors in that cell. However, the unambiguous distinction between the dominant negative phenotype regarding MAP kinase stimulation by insulin and the unimpaired stimulation of metabolic activities clearly indicates that MAP kinase is not universally required for these metabolic responses to insulin.

ACKNOWLEDGMENTS

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