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Monoclonal Antibody to the Human Insulin Receptor, but Not Insulin, Stimulates S6 Kinase via Human Insulin Receptors Mutated at Three Major Tyrosine Autophosphorylation Sites

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Abstract Studies were carried out to examine the role of the major insulin receptor tyrosine autophosphorylation sites in stimulation of S6 kinase activity. For these studies, we employed HTC rat hepatoma cells transfected with and expressing human insulin receptors. In cells transfected with and expressing a large number of normal human insulin receptors (HTC-IR cells), the sensitivity of cells to insulin to stimulate S6 kinase was increased tenfold when compared to untransfected wild type HTC cells (HTC-WT cells). However, in cells transfected with and expressing a large number of mutated human insulin receptors where the tyrosines at three major autophosphorylation sites (1158, 1162, and 1163) were mutated to phenylalanines (HTC-F3 cells), there was no change in insulin sensitivity when compared to HTC-WT cells. We next studied the effect of a human-specific monoclonal antibody to the human insulin receptor, MA-5, on S6 kinase activation. In HTC-WT cells, MA-5 did not interact with endogenous rat insulin receptors and thus did not stimulate S6 kinase. In HTC-IR cells expressing normal human insulin receptors, MA-5 stimulated S6 kinase. Interestingly, MA-5, unlike insulin, was also able to stimulate S6 kinase in HTC-F3 cells expressing mutated receptors. In order to further understand the signaling mechanisms by MA-5 and insulin, two potential intermediate protein kinases were investigated. Neither insulin nor MA-5 appears to activate either microtubule-associated protein 2 (MAP-2) kinase or protein kinase C in these cells.

These studies suggest therefore that: 1) insulin and MA-5 may signal S6 kinase activation by independent mechanisms that do not employ either MAP-2 kinase or protein kinase C; and 2) under certain circumstances, S6 kinase appears to be activated by mechanisms that are independent of insulin receptor tyrosine autophosphorylation.

Key words: anti-insulin receptor antibody, mutant receptors, S6 kinase, receptor tyrosine kinase

In most cells insulin is a major anabolic hormone that increases net protein synthesis [1]. The mechanism(s) whereby insulin regulates this function is unknown. The major phosphoprotein in ribosomes undergoing protein synthesis is the 40 S ribosomal protein S6 (M_r = 34,000), whose phosphorylation is enhanced by insulin at multiple serine sites [2–5]. A variety of studies suggest that phosphorylation of protein S6 is involved in the protein synthesis stimulated by insulin and other agents [5–7].

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The serine protein kinases involved in S6 phosphorylation are not completely understood. In vitro, a number of protein kinases phosphorylate protein S6 [8]. However, in vivo, there is limited information available concerning which of these kinases phosphorylates protein S6. A specific soluble S6 kinase activity that is responsible for insulin-induced S6 phosphorylation in vivo has been identified and termed "S6 kinase" [2]. The activation of S6 kinase occurs within minutes at nanomolar concentrations of insulin, paralleling the time course and dose response for insulin stimulated S6 phosphorylation in vivo [2,9,10]. Thus, activation of S6 kinase by insulin appears to be one major mechanism whereby insulin stimulates S6 protein phosphorylation. Furthermore, S6 kinases stimulated by hormones and growth factors have been puri-

fied from mammalian cells and exhibit similar molecular weights of 65 to 70 KDa [11]. These mammalian S6 kinases are immunologically different from Xenopus oocyte S6 kinase II with molecular weight of 90–92 KDa [11,12].

The mechanism whereby insulin activates S6 kinase is unknown. The interaction of insulin with target cells is mediated by a specific tetrameric glycoprotein receptor located in the plasma membrane [13,14]. This receptor consists of two identical extracellular α-subunits (M_r = 130,000 each) that contain insulin binding sites and two identical transmembrane β-subunits (M_r = 95,000 each) that have tyrosine kinase activity in their intracellular domains. When insulin binds to the α-subunit of the receptor, β-subunit tyrosine kinase is activated and various metabolic actions occur [14].

In normal insulin receptors, insulin activation of receptor tyrosine kinase very rapidly leads to the autophosphorylation of a cluster of three tyrosines at residues 1158, 1162, and 1163 [15–17]. There is also phosphorylation of a cluster of tyrosine residues at positions 1328 and 1334, but this phosphorylation is smaller in magnitude and slower in onset than the triple tyrosine cluster [16]. Other tyrosine residues which are potential candidates for autophosphorylation such as 965, 972, and 984 are phosphorylated very poorly, if at all [16]. Concomitant with insulin receptor autophosphorylation, there is a change in receptor β-subunit conformation and further enhancement of tyrosine kinase activity [17–19]. The relationship, however, between these insulin receptor β-subunit changes and activation of S6 kinase is unknown.

Prior studies have shown that either monoclonal or polyclonal antibodies to the insulin receptor can stimulate S6 kinase activity without significant stimulation of receptor tyrosine kinase activity [10,20,21]. Moreover, in an insulin receptor tyrosine autophosphorylation mutant where the tyrosines at 1162 and 1163 [22] or all major three tyrosines at 1158, 1162, and 1163 [10] were mutated to phenylalanines, insulin receptor autophosphorylation was markedly reduced. However, in double tyrosine mutants insulin stimulation of S6 kinase activity remained intact [22]. In a triple tyrosine mutant, insulin effect on S6 kinase activity was not studied in detail. We have previously shown, however, that a monoclonal antibody to the human insulin receptor (MA-5), which had little or no effect on receptor tyrosine kinase activity, stimulated S6 kinase [10]. In order to further investigate the non-tyrosine kinase dependent signaling mechanism induced by this monoclonal antibody to the human insulin receptor, we have utilized HTC rat hepatoma cells expressing either normal human insulin receptors or receptors mutated at all three major tyrosines (1158, 1162, and 1163) or receptors mutated at ATP binding site (1030). These studies demonstrated that overexpression of normal human insulin receptors in HTC cells increased the sensitivity of cells to insulin to stimulate S6 kinase, but not the maximal response; and only MA-5, but not insulin, could interact with receptors mutated at all three major tyrosine residues to stimulate S6 kinase. The results of these studies indicate that these two agents interact with the insulin receptor and stimulate S6 kinase activity via two independent signaling pathways that do not employ activation of either MAP-2 kinase or protein kinase C.

MATERIALS AND METHODS

Transfection and Maintenance of Cultured Cells

HTC rat hepatoma cells were grown in Dulbecco's modified Eagle's H-16 medium supplemented with 10% fetal calf serum and transfected with an expression plasmid containing the human insulin receptor cDNA, either normal or mutated by oligonucleotide-directed mutagenesis as previously described [10,23,24]. The mutant changes tyrosines at all three major autophosphorylation sites (1158, 1162, and 1163) to phenylalanines (HTC-F3). Another mutant changes lysine at an ATP binding site (1030) to methionine (HTC-M1030). Stable transfected cell lines were obtained using the calcium phosphate/glycerol shock method and co-selection for the neomycin resistance gene. Resistant colonies were subcloned and stable lines were tested for insulin receptors by ^{125}I-insulin binding. HTC-IR cells expressing normal human insulin receptors, HTC-F3 cells, and HTC-M1030 cells have approximately 4×10^4, 10^5, and 2×10^5 receptors/cell respectively based on radioimmunoassay [25]. These cells do not contain insulin-like growth factor 1 (IGF-1) receptors [23].

Preparation of Soluble Cellular Extracts of HTC Cells Containing S6 Kinase

Cells were plated in 100 × 20 mm culture dishes and grown to 70%–80% confluency. They

were then incubated for 16 h in serum-free medium. For dose responses experiments, cells were incubated for 30 min in the presence of various concentrations of either insulin or MA-5. For time course experiments, cells were incubated with 10 nM of either insulin or MA-5 for various times (0, 5, 15, 30, 60, 120 min). At the end of incubation, cells were washed three times with ice-cold Tris-Saline, scraped off and spun down at 1,000 rpm for 5 min.

Soluble cellular extracts of cells were prepared by sonication of cells in a buffer (80 mM β-glycerophosphoate, 20 mM EGTA, 15 mM $MgCl_2$, pH 7.3) followed by ultracentrifugation at 200,000*g* for 1 h at 4°C. The supernatants were removed and assayed for S6 kinase activity [2,9,10].

Measurement of S6 Kinase Activity

The S6 kinase activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P] ATP into rat pancreatic ribosomes prepared as described by Sung and Williams [9]. The reaction mixture contained the following: 50 mM Mops (pH 7.0), 1 mM dithiothreitol (DTT), 40 mM β-glycerophosphate, 10 mM EGTA, 7.5 mM $MgCl_2$, 20 μg of soluble cellular extracts, 10 μg of purified rat pancreatic ribosomes, 60 μM [γ-^{32}P] ATP (0.8–1.6 μCi/nmol) as described by Sung et al. [10]. After incubation for 15 min at 30°C, the reaction was terminated by addition of sodium dodecyl sulfate (SDS) stop buffer and boiling. S6 phosphorylation was then analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography [9,10]. For these studies, several separate experiments were carried out and their results are shown either as a representative experiment or as pooled data from several experiments.

Insulin Receptor Tyrosine Kinase Studies

Insulin receptors were prepared as follows. Cells were solubilized in 1% Triton X-100 containing 50 mM Hepes, pH 7.6, 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM sodium orthovanadate, and 0.1 mg/ml aprotinin and centrifuged at 100,000*g* for 1 h followed by wheat germ agglutinin-agarose column chromatography as described previously [26].

Phosphotransferase activity of insulin receptors prepared as above was determined by preincubating receptors (10 ng) with diluent (50 mM Hepes, 150 mM NaCl, pH 7.4), 100 nM insulin or 100 nM MA-5 for 1 h at 24°C in a buffer containing 50 mM Hepes, pH 7.4, 0.1% Triton X-100, 0.025% bovine serum albumin, 1 mM PMSF, 2 mM $MnCl_2$, and 10 mM $MgCl_2$. Next, 10 μM [γ-^{32}P] ATP and 1 mg/ml of poly(Glu, Tyr) were added and incubation was continued for 1 h at 24°C. Reaction was then terminated by addition of 25 mM EDTA, 2.5 mM ATP, and 0.125% bovine serum albumin. Aliquots were spotted on 4-cm^2 discs of Whatman 3 MM paper and dried. Discs were washed in three changes of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, boiled for 2 min in 5% trichloroacetic acid followed by washing in ethyl alcohol and acetone [27]. Radioactivity was determined by liquid scintillation counting.

Measurement of Microtubule-Associated Protein 2 Kinase (MAP-2 Kinase)

Cells grown to 70%–80% confluency were preincubated for 16 h in serum-free medium. They were then treated with insulin or MA-5 for 5 min followed by sonication in a buffer containing 25 mM Tris, pH 7.5, 25 mM NaCl, 2 mM EGTA, 1 mM DTT, 40 mM P-nitrophenylphosphate, 0.2 mM PMSF, and 0.2 mM sodium orthovanadate. The soluble cellular extracts containing MAP-2 kinase were prepared by centrifugation at 10,000*g* for 15 min and assayed for MAP-2 kinase activity in reaction buffer containing 40 mM β-glycerophosphate, pH 7.0, 1 mM DTT, 10 mM $MgCl_2$, 20 mM $MnCl_2$, 5 μg of MAP-2 protein purified from bovine brains [28], and 50 μM [γ-^{32}P] ATP (2.5 μCi) as previously described [29,30]. The reaction was performed at 30°C for 15 min and terminated by SDS-stop buffer. MAP-2 protein phosphorylation was then analyzed by 5% SDS-PAGE and subsequent autoradiography. Controls employing purified rat brain calmodulin-dependent protein kinase indicate that the MAP-2 protein phosphorylation could be increased at least tenfold (data not shown).

The Effects of Prolonged Incubation of Cells With TPA on S6 Kinase Activation

Cells in 100 × 20 mm dishes were grown to 70%–80% confluency and preincubated for 16 h in serum-free medium containing 1 μM of a phorbol ester, 12-0-tetradecanoylphorbol 13-acetate (TPA). After a change of medium with fresh medium, incubation was continued for 30 min in the presence of controls (DMSO or diluent), TPA, insulin or MA-5. Cells were then

processed and their soluble extracts were assayed for their S6 kinase activities as previously described.

RESULTS

S6 Kinase Activation by Insulin in HTC Cells

Stimulation of S6 protein phosphorylation by insulin was observed in all three cell lines studied (Fig. 1): wild type cells (HTC-WT); cells transfected with normal human insulin receptors (HTC-IR); and cells transfected with human insulin receptors mutated at three major tyrosine autophosphorylation sites (HTC-F3). These latter two cell lines were cotransfected with the neomycin resistance gene. HTC-WT cells were transfected only with the neomycin resistance gene. The degree of maximal S6 phosphorylation by 100 nM insulin was similar in all three cell lines (2.6–3.0-fold stimulation).

To study the time course of S6 kinase activation by insulin, HTC cells were incubated with 10 nM insulin for various times and S6 kinase activity measured (Fig. 2). In all three cell lines, S6 kinase activity was significantly increased at 5 min and reached maximal at 30 min. After 120 min of incubation, S6 kinase activity in HTC-WT cells was still increased at 80% of the maximal S6 kinase activity obtained at 30 min. In HTC-IR cells, however, there was a greater decrease in S6 kinase activity subsequent to the maximal stimulation of S6 kinase activity. After 120 min of incubation, only 50% of the maximal S6 kinase activity remained. In HTC-F3 cells, the time course of S6 kinase activation was similar to that in HTC-WT cells. The reason for these biphasic time courses are unknown, but they have been previously observed in other cell lines [31,32].

When HTC-WT cells containing only endogenous rat insulin receptors were incubated for 30 min with increasing concentrations of insulin (10 pM–100 nM), S6 kinase activity was increased in a dose dependent manner. Half-maximal stimulation was observed at 4 nM and maximal stimulation was observed at 10–100 nM (Fig. 3). In HTC-IR cells, the sensitivity to insulin was increased approximately tenfold with half maximal stimulation at 400 pM. In HTC-F3 cells, however, the sensitivity to insulin was not changed when compared to HTC-WT cells. These data suggested that in HTC-F3 cells containing both normal rat insulin receptors and mutated

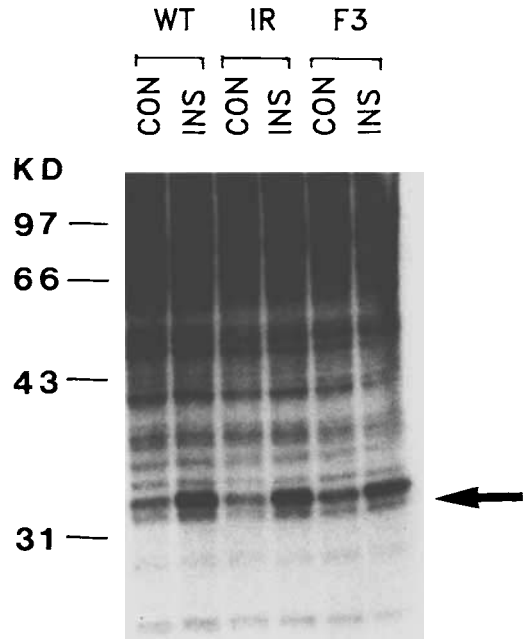

Fig. 1. Autoradiograph of total reaction mixtures containing S6 protein phosphorylated by extracts of HTC cells: untransfected (HTC-WT), transfected with normal (HTC-IR) or mutated (HTC-F3) insulin receptors. Cells in 100 × 20 mm dishes were treated for 30 min with diluent (50 mM Hepes, 150 mM NaCl, pH 7.4; CON) and 100 μM insulin (INS). Following washes in TRIS-saline at 4°C, cells were scraped off the plates, sonicated in buffer, and centrifuged. The resultant supernatants were removed and assayed for 15 min at 30°C as described under Materials and Methods. S6 phosphorylation was analyzed by 10% SDS-PAGE and autoradiography. The arrow indicates S6 protein. A representative of four experiments is shown.

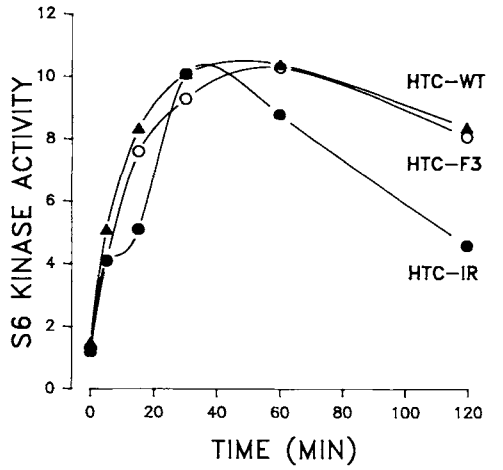

Fig. 2. Time dependent activation of S6 kinase by insulin in HTC-WT (▲), HTC-IR (●), and HTC-F3 (○) cells. Cells were incubated with 10 nM insulin for up to 120 min. Soluble cellular extracts were then prepared and assayed for S6 kinase activities as described in Figure 1. Intensity of S6 phosphorylation was then measured spectrophotometrically. S6 kinase activity is presented as arbitrary units and a representative of three experiments is shown.

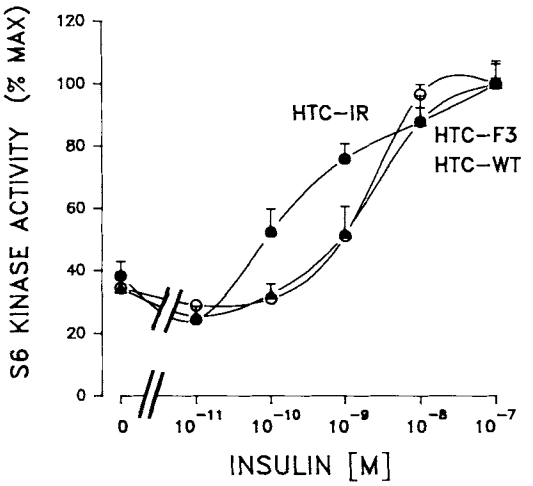

Fig. 3. Dose dependent activation of S6 kinase by insulin in HTC-WT (▲), HTC-IR (●), and HTC-F3 (○) cells. Cells were incubated for 30 min with various concentrations of insulin (100 pM–100 nM). Soluble cellular extracts were then prepared and assayed for S6 kinase activity as described in Figure 1. The data were pooled from several separate experiments. Therefore, the relative intensities of S6 phosphorylation are presented as percent of maximal S6 kinase activity and are the mean ± SEM of four separate experiments. Maximal stimulation was similar in all three cell lines as shown in Figure 1.

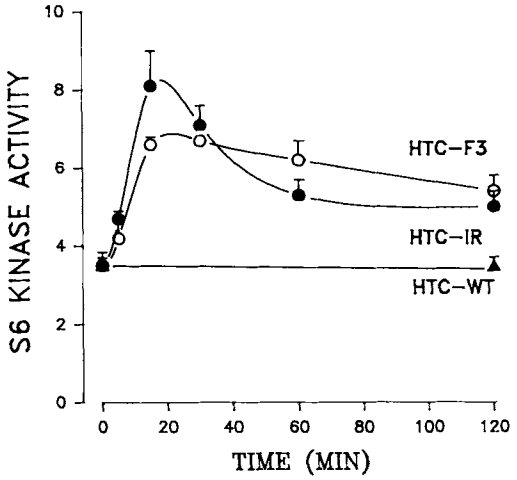

Fig. 4. Time dependent activation of S6 kinase by MA-5 in HTC-WT (▲), HTC-IR (●), and HTC-F3 (○) cells. Cells were incubated with 10 nM MA-5 for up to 120 min. S6 kinase assays for soluble cellular extracts were performed as described in Figure 1. The data are presented as arbitrary units and are the mean ± SEM of three separate experiments.

human insulin receptors, insulin stimulated S6 kinase only via normal endogenous rat insulin receptors.

S6 Kinase Activation by MA-5 in HTC Cells

Next, we studied the effect of a species-specific monoclonal antibody MA-5 on S6 kinase activation in HTC cells (Figs. 4, 5). Cells were incubated with MA-5 for various times and various concentrations and S6 kinase activity measured. HTC-WT cells containing only rat insulin receptors did not respond to human species-specific monoclonal antibody MA-5 (Fig. 5). This result was not unexpected since MA-5 does not cross-react with rodent insulin receptors [25]. In HTC-IR cells, S6 kinase activity was significantly increased at 5 min and reached maximal at 15 min (Fig. 4). After maximal stimulation of S6 kinase by MA-5 in HTC-IR cells, there was a decrease in S6 kinase activity. After 120 min of incubation, S6 kinase activation was increased at only 60% of the maximal S6 kinase activity obtained at 15 min.

In HTC-F3 cells, MA-5, unlike insulin, stimulated S6 kinase activity via mutated insulin receptors. A maximal effect was seen at 15 min.

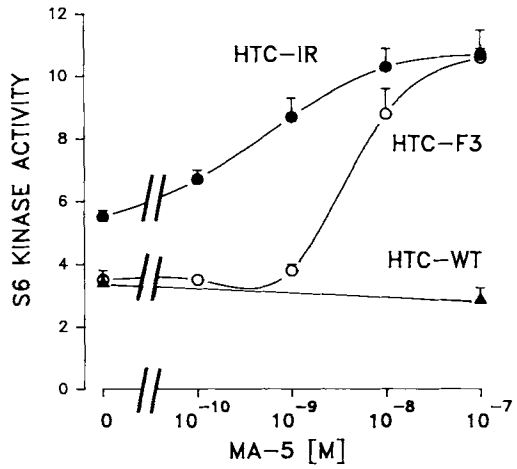

Fig. 5. Dose dependent activation of S6 kinase by MA-5 in HTC-WT (▲), HTC-IR (●), and HTC-F3 (○) cells. Cells were incubated for 30 min with various concentrations of MA-5 (100 pM–100 nM). S6 kinase assays for soluble cellular extracts were performed as described in Figure 1. The data are presented as arbitrary units and are the mean ± SEM of three separate experiments.

After 120 min, 80% of the maximal S6 kinase activity was remained.

To study the dose dependence of S6 kinase activation by MA-5, cells were incubated with increasing concentrations of MA-5 and S6 kinase activity measured (Fig. 5). In HTC-IR cells expressing normal human insulin receptors, MA-5 stimulated S6 kinase in a dose dependent

TABLE I. S6 Kinase Activation by Insulin and MA-5 in Various HTC Cell Lines*

	HTC-WT	HTC-IR	HTC-F3	HTC-M1030
Control	3.4 ± 0.2	5.5 ± 0.2	3.5 ± 0.3	2.7 ± 0.3
100 nM insulin	10.1 ± 0.7	10.1 ± 0.9	9.3 ± 0.2	9.0 ± 1.0
100 nM MA-5	2.9 ± 0.4	10.7 ± 0.2	10.6 ± 0.9	2.5 ± 0.5

*Cells were treated for 30 min with agents. Soluble cellular extracts were then prepared and assayed for S6 kinase activity as described in Materials and Methods. For control, cells were treated with 100 nM normal mouse IgG. These control values were similar to those obtained from cells treated with diluent. Data are expressed as an arbitrary unit of S6 kinase activity and are the mean ± SEM of three separate experiments.

TABLE II. Phosphotransferase Activities of Insulin Receptors Prepared From HTC-WT, HTC-IR, and HTC-F3 Cells Using Poly(Glu,Tyr)*

	HTC-WT	HTC-IR	HTC-F3	HTC-M1030
Diluent	1.6 ± 0.4	0.86 ± 0.03	1.11 ± 0.01	0.16 ± 0.01
100 nM insulin	18.2 ± 0.2	22.04 ± 0.24	2.48 ± 0.04	1.19 ± 0.10
100 nM MA-5	1.3 ± 0.1	0.93 ± 0.00	1.17 ± 0.01	0.19 ± 0.01

*Insulin receptors from HTC cells were prepared by wheat-germ agglutinin column chromatography as described in Materials and Methods. Insulin receptors (10 ng) were then preincubated with diluent, 100 nM insulin, or 100 nM MA-5 and assayed for their phosphotransferase activities toward 1 mg/ml of poly(Glu,Tyr) using 10 μM[γ-^{32}P]-ATP (1.25 μCi/assay). Data are expressed as nmoles of ATP incorporated per mg poly(Glu,Tyr) and are the mean ± SEM of three separate experiments.

manner with half maximal stimulation at 400 pM (Fig. 5). Thus, the sensitivity of cells to MA-5 was very similar to the insulin sensitivity in HTC-IR cells. In HTC-F3 cells, MA-5 stimulated S6 kinase in a dose dependent manner. Half maximal stimulation was observed at 4 nM. These data suggested therefore that MA-5 activated the mutated human insulin receptors and then stimulated S6 kinase. Reasons for the difference in the dose response curves observed in HTC-IR and HTC-F3 cells are not clear. It is possible that there are subtle conformational differences between two receptors, which may account for the difference in sensitivity.

In order to understand if another receptor mutant defective of tyrosine kinase activity could activate S6 kinase in response to MA-5, cells expressing receptors mutated at the ATP binding site (1030) were employed (Table I). In these cells, insulin stimulated S6 kinase to the somewhat similar extent as in other cells via endogenous rat receptors. MA-5, however, failed to stimulate S6 kinase activity.

Insulin Receptor Tyrosine Kinase Studies

Next, we compared the ability of this antibody to stimulate insulin receptor tyrosine kinase activity. Insulin receptors prepared from various HTC cell lines were assayed for phosphotransferase activities toward an exogenous substrate poly(Glu,Tyr) (Table II). When insulin receptors from HTC-IR cells were incubated with 100 nM insulin, poly(Glu,Tyr) activity increased 25-fold from 0.86 to 22.04 nmoles of ATP incorporated per mg poly(Glu,Tyr). 100 nM MA-5 had little or no effect on poly(Glu,Tyr) activity. When the same amount of insulin receptors prepared from HTC-F3 cells were incubated with 100 nM insulin, poly(Glu,Tyr) activity increased only twofold from 1.11 to 2.48 nmoles of ATP incorporated per mg poly(Glu-,Tyr). This small increase is most likely due to the presence of normal endogenous rat insulin receptors [10,23]. Incubation of insulin receptors from HTC-F3 cells with 100 nM MA-5 showed no activation of tyrosine kinase. In M1030 receptors, poly(Glu,Tyr) activity by insulin was similar to the basal activities obtained in other receptors. The basal activity of M1030 receptors was lower than those of other receptors. MA-5 did not activate poly(Glu,Tyr) activity.

Effect of Insulin and MA-5 on MAP-2 Kinase Activity

In order to investigate the signaling pathways linking the activation of the insulin receptor by MA-5 and insulin to activation of S6 kinase, we studied two potential intermediate serine/threonine protein kinases: microtubule-associated pro-

tein 2 kinase (MAP-2 kinase) and protein kinase C.

In 3T3-L1 adipocytes, MAP-2 kinase has been reported to be stimulated by insulin [29]. Moreover, the activated MAP-2 kinase was able to phosphorylate and activate S6 kinase II purified from Xenopus oocytes [30]. To study activation of MAP-2 kinase in HTC cells, cells were treated for 5 min with 100 nM of either insulin or MA-5. Soluble cellular extracts were then prepared and assayed for MAP-2 kinase activities. Both insulin and MA-5 failed to stimulate MAP-2 kinase activity in these cell lines (Fig. 6). An occasional slight increase in MAP-2 phosphorylation in insulin stimulated HTC-IR cells was not statistically significant (107.3% ± 7.3% increase over control presented as mean ± SEM of three experiments). Longer treatment (i.e., 30 min) of cells with both agents also did not increase MAP-2 kinase activity (data not shown). In other experiments, soluble cellular extracts of cells treated with or without insulin were immunoprecipitated with anti-phosphotyrosine antibody and then assayed for MAP-2 kinase activities. However, there was no significant increase in MAP-2 kinase activity in response to insulin (data not shown).

Effects of Prolonged Incubation of Cells With TPA on S6 Kinase Activation

Treatment of cells with TPA has been shown to stimulate S6 kinase, suggesting an intermediate role of protein kinase C [2,33]. In order to determine whether protein kinase C played a role in S6 kinase activation in HTC cells, cells were treated with either vehicle (0.1% DMSO) or 1 μM of TPA for 16 h. The prolonged incubation of cells with high concentration of TPA has been demonstrated to downregulate protein kinase C [34,35].

First, HTC-F3 cells were preincubated for 16 h with vehicle and then incubated for 30 min with 1 μM TPA. TPA stimulated S6 kinase 40% over control. With this protocol, both 10 nM insulin and 10 nM MA-5 also stimulated S6 kinase activity by 110% and 50% over control, respectively. When these cells were preincubated with 1 μM TPA for 16 h to downregulate protein kinase C, the effects of insulin and MA-5 on S6 phosphorylation were still present, whereas the effect of TPA on S6 phosphorylation was completely abolished. Similar experiments performed with HTC-IR cells showed the same results (data not shown). These data suggested therefore that S6 kinase activation by insulin and MA-5 did not require activation of protein kinase C.

DISCUSSION

In the present study, we examined the effects of insulin and human-specific monoclonal antibody MA-5 on S6 kinase activation in HTC rat hepatoma cells transfected with and expressing either normal or mutated human insulin recep-

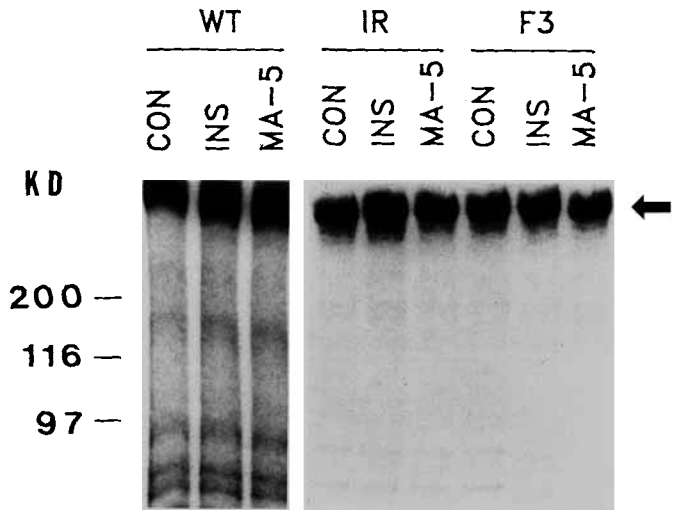

Fig. 6. Effects of insulin or MA-5 on MAP-2 kinase activity in HTC-WT, HTC-IR, and HTC-F3 cells. Cells in 100 × 20 mm dishes were incubated for 5 min with either diluent (CON), 100 nM insulin (INS), or 100 nM MA-5 (MA-5). Soluble cellular extracts containing MAP-2 kinase were then prepared and assayed for MAP-2 kinase activity in reaction mixture containing 5 μm of MAP-2 protein purified from bovine brains and 50 μM [γ-^{32}P] ATP (2.5 μCi) as described in Materials and Methods. A representative of three experiments is shown.

tors. We observed that insulin stimulated this function via normal insulin receptors but not via mutated insulin receptors. In contrast, MA-5 activated S6 kinase via receptors, both normal and mutated at three major tyrosine autophosphorylation sites. These studies suggest therefore that insulin and MA-5 may have employed different signaling pathways to stimulate S6 kinase. These pathways for insulin and MA-5 however do not involve activation of either MAP-2 kinase or protein kinase C.

Insulin receptor is a family of receptors containing intrinsic tyrosine kinase in their intracellular domains, and whose activity is stimulated by ligand occupancy [13,14]. Ligands include naturally occurring hormones, growth factors, and antibodies [13,14]. With these receptors, activation of tyrosine kinase leads to both receptor autophosphorylation on tyrosine residues and tyrosine phosphorylation of cellular substrates [13,14]. It is believed that these processes play a role in mediating many of hormone actions. However, for most receptors, and especially for insulin receptor, the nature of intracellular substrates and the pathways involved subsequent to receptor activation remain unknown [13,14,18,19].

In order to study the potential relationships between insulin receptor tyrosine kinase activation, receptor autophosphorylation, and S6 kinase activation, we and others have employed insulin receptors that have been mutated in the tyrosine kinase domain. One study used Chinese hamster ovary cells transfected with and expressing an insulin receptor mutated at Lysine-1018 in the ATP binding site (equivalent to Lysine-1030 in the exon 11 varient used in our study). In cells containing this mutant, both receptor tyrosine kinase activity and receptor autophosphorylation were abolished [36–39]. In these cells, insulin failed to stimulate S6 kinase activity [36]. We have also studied insulin stimulation of S6 kinase activity in HTC cells transfected with and expressing an insulin receptor mutated at Lysine-1030 and have obtained similar results (data not shown). Another study used Chinese hamster ovary cells transfected with and expressing an insulin receptor mutated at Glycine-996 in the ATP binding site (equivalent to 1008 in the exon 11 varient used in our study). In this mutant, there was also no detectable insulin receptor autophosphorylation. And in these cells, insulin failed to stimulate S6 kinase activity [40]. All of these studies suggest therefore that there is an absolute requirement for an intact ATP binding site in the activation of S6 kinase by the insulin receptor.

Another series of studies have employed receptor autophosphorylation mutants. Two groups have examined cells transfected with and expressing insulin receptors mutated at tyrosine residues 1162 and 1163, and have compared them to cells transfected with and expressing normal receptors [22,41]. In these mutated insulin receptors there was no enhanced receptor autophosphorylation [22,41]. However, in these cells, insulin normally signalled S6 kinase activation [22]. In the present study, using another insulin receptor mutant where three major tyrosine autophosphorylation sites 1158, 1162, and 1163 were changed to phenylalanines, we observed that insulin failed to stimulate S6 kinase via this mutated receptor (Fig. 3). All of these data raise the possibility therefore that Tyrosine-1158 could be important for S6 kinase activation by insulin.

Another series of interesting results have come from studies with polyclonal and monoclonal antibodies to the insulin receptor. While certain antibodies have been shown to stimulate insulin receptor tyrosine kinase activity [42–46], other antibodies appear to have little or no effect on receptor tyrosine kinase activity and receptor autophosphorylation [10,20,21,23,25,47–50]. However, these antibodies have been reported to stimulate a wide spectrum of cellular functions [10,20,21,26,47]. Previously, in mouse fibroblast 3T3 cells transfected with and expressing normal human insulin receptors we demonstrated that three monoclonal antibodies to the insulin receptor stimulated S6 kinase activity under conditions where activation of receptor tyrosine kinase was not observed [10]. Moreover, one antibody, MA-5, stimulated S6 kinase activity with a sensitivity that was greater than insulin. In addition, MA-5 stimulated S6 kinase in HTC-F3 cells when compared with untransfected HTC cells [10]. In these cells the mechanism of S6 kinase activation by MA-5 was not studied in detail. Also, Ponzio et al. [21] have reported that a polyclonal antibody to the insulin receptor stimulated ribosomal S6 phosphorylation without stimulation of receptor tyrosine kinase activity and receptor autophosphorylation. These studies with antibodies, like the studies with receptor autophosphorylation mutants, suggest dissociation between receptor autophosphorylation and certain biological func-

tions of insulin. It should be pointed out, however, that others have suggested that these agonist receptor antibodies may induce low levels of receptor tyrosine kinase activity and receptor autophosphorylation [27,45].

More studies were carried out with both antibodies to the insulin receptor and insulin receptor tyrosine autophosphorylation mutants and compared with insulin-stimulated S6 kinase activation. The data clearly demonstrated that insulin failed to stimulate S6 kinase via these mutated insulin receptors, which argues against the possible role of low, but sufficient enough, level of receptor autophosphorylation (Fig. 3). In contrast, MA-5 stimulated S6 kinase via mutated receptors (Fig. 5). Thus, these studies strongly support the notion that insulin receptor can indeed signal certain biological functions without activation of receptor autophosphorylation when interacted with an appropriate agent.

In contrast to cells transfected with receptor autophosphorylation mutants, in cells transfected with insulin receptors mutated at Lysine-1030 in the ATP binding site, MA-5 did not stimulate S6 kinase (Table I). These results suggest that the intact ATP binding site, but not receptor autophosphorylation sites, may be crucial for MA-5 induced changes in receptor conformation and S6 kinase activation. It is possible that heterodimers are formed between the transfected human insulin receptors and the endogenous rat receptors. In M1030 cells, MA-5, a human-specific monoclonal antibody to the insulin receptor, also failed to activate S6 kinase as in untransfected cells. Whether or not transdominant inhibition within the hybrid receptor occurs in vivo is not clear in the MA-5 induced S6 kinase activation since MA-5 does not interact with normal endogenous rat receptors.

Prior studies have indicated that tyrosine kinase activity of the insulin receptor is regulated by autophosphorylation. In vitro, autophosphorylation of the insulin receptor activates its kinase activity toward exogenous substrates [15]. Moreover, in vivo, phosphorylated and activated receptors can also be isolated from insulin-treated cells [51–53]. In the present studies with HTC-F3 cells and HTC-IR cells, we failed to observe an increase in tyrosine kinase activity with MA-5 (Table II). Moreover, in intact cells treated with MA-5, there was no enhanced β-subunit autophosphorylation and phosphorylation of pp185 [10]. In HTC-F3 and HTC-IR cells, however, MA-5 increased S6 kinase activity. Thus, in these studies it appears that under certain circumstances activation of tyrosine kinase is not a prerequisite for signaling this function. It is probable, however, that insulin receptor tyrosine kinase activation is important for regulating other cellular functions.

In order to further understand the signaling pathways linking stimulation of the insulin receptor to S6 kinase activation, we studied two potential intermediate serine/threonine protein kinases. S6 kinase activation by insulin has been reported to be mediated by serine phosphorylation of the enzyme [2,3,29,30]. Since the insulin receptor tyrosine kinase does not directly phosphorylate and activate S6 kinase, presence of intermediate serine/threonine protein kinases has been proposed in some insulin responsive cells [29,30,54]. In 3T3-L1 adipocytes, insulin has been reported to stimulate MAP-2 kinase activity in a dose dependent manner; this MAP-2 kinase activation precedes the activation of S6 kinase. Furthermore, MAP-2 kinase has been reported to phosphorylate and activate S6 kinase [46]. Accordingly, we studied the activation of MAP-2 kinase by insulin and MA-5 in our HTC cell lines. In transfected HTC cells, however, both insulin and MA-5 failed to stimulate MAP-2 kinase. These results are consistent with the recent findings that mammalian S6 kinase, in contrast to Xenopus S6 kinase, is not activated by MAP-2 kinase [55].

Protein kinase C, another potential intermediate serine/threonine protein kinase, was also studied. In several cells, insulin has been reported to stimulate protein kinase C [56,57]. Furthermore, in insulin responsive cells, protein kinase C has been implicated to stimulate S6 kinase [31,33]. TPA, which is a direct activator of protein kinase C, was utilized to study the role of protein kinase C in S6 kinase activation by insulin and MA-5 in HTC cells. In HTC cells, TPA, like MA-5 and insulin, rapidly stimulated S6 kinase. HTC cells were then preincubated with TPA to downregulate protein kinase C. In HTC-F3 cells, where protein kinase C had been downregulated by TPA, the subsequent effect of TPA on S6 kinase activation was completely abolished. However, the effects of insulin and MA-5 were still present (Fig. 7). These data suggested therefore that activation of protein kinase C may not be crucial for S6 kinase activation by these agents. It should be pointed out, however, that there are many protein kinase C isozymes and thus the possibility exists that some

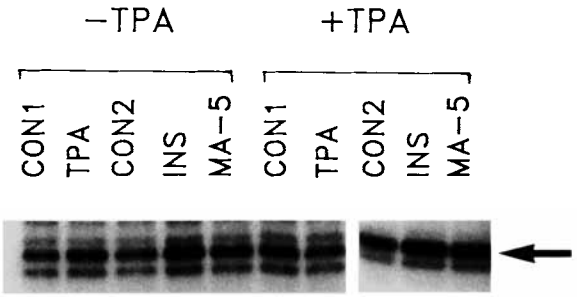

Fig. 7. Effects of prolonged incubation of HTC-F3 cells with TPA on subsequent S6 kinase activation by TPA, insulin, and MA-5. HTC-IR cells in 100 × 20 mm dishes were preincubated with either 0.1% DMSO (−TPA) or 1 μM TPA (+TPA) for 16 h at 37°C. Cells were then subsequently incubated for 30 min with 0.1% DMSO (CON1), 1 μM TPA (TPA), diluent (CON2), 10 nM insulin (INS), and 10 nM MA-5 (MA-5). Soluble cellular extracts were then prepared and assayed for S6 kinase activities as described in Figure 1. The arrow indicates S6 protein. A representative of three experiments is shown.

minor isozymes may not be subject to TPA-induced downregulation and may still play a role in some insulin action [58].

In conclusion, the present data demonstrate that a monoclonal antibody to the insulin receptor, but not insulin, stimulates S6 kinase activity via insulin receptors mutated at three major tyrosine autophosphorylation sites. These studies thus demonstrate a dissociation between 1) mechanisms of insulin receptor activation by MA-5 and insulin; and 2) insulin receptor autophosphorylation and insulin receptor signaling by MA-5. How these agents signal S6 kinase activation remains to be elucidated. Alternative mechanisms for the receptor signaling could include receptor aggregation [21,42,43,46], receptor conformational changes [17–19,59], and generation of a lipid-containing second messenger molecules [60,61]. Further studies will be needed therefore to understand the mechanisms whereby insulin and MA-5 regulate S6 kinase activation.

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