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Regulation of Biological Functions by an Insulin Receptor Monoclonal Antibody in Insulin Receptor β-Subunit Mutants†

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ABSTRACT: We investigated the effects of MA-5, a human-specific monoclonal antibody to the insulin receptor α-subunit, on transmembrane signaling in cell lines transfected with and expressing both normal human insulin receptors and receptors mutated in their β-subunit tyrosine kinase domains. In cell lines expressing normal human insulin receptors, MA-5 stimulated three biological functions: aminoisobutyric acid (AIB) uptake, thymidine incorporation, and S6 kinase activation. Under conditions where these biological functions were stimulated, there was no detectable stimulation of receptor tyrosine kinase. We then combined the use of this monoclonal antibody with cells expressing insulin receptors with mutations in the β-subunit tyrosine kinase domain; two of ATP binding site mutants V1008 (Gly→Val) and M1030 (Lys→Met) and one triple-tyrosine autophosphorylation site mutant F3 (Tyr→Phe at 1158, 1162, and 1163). In cells expressing V1008 receptors, none of the three biological functions of insulin was stimulated. In cells expressing M1030 receptors, AIB uptake was stimulated to a small, but significant, extent whereas the other two functions were not. In cells expressing F3 receptors, AIB uptake and S6 kinase activation, but not thymidine incorporation, were fully stimulated. The data suggest, therefore, that (1) activation of insulin receptor tyrosine kinase may not be a prerequisite for signaling of all the actions of insulin and (2) there may be multiple signal transduction pathways to account for the biological actions of insulin.

Insulin acts upon its receptor to produce a wide range of effects in many cell types, but the mechanism (or mechanisms) whereby the receptor generates transmembrane signals is unknown. The insulin receptor is a tetrameric disulfide-linked glycoprotein consisting of two identical extracellular α-subunits that bind the hormone and two identical transmembrane β-subunits that contain a typical tyrosine kinase motif in their cytoplasmic domains (Goldfine, 1987; Ullrich et al., 1985). The receptor is synthesized as a precursor polypeptide, and is subsequently cleaved into one α- and one β-subunit. When insulin binds to the α-subunit of the receptor, β-subunit tyrosine kinase is activated, tyrosine autophosphorylation of the β-subunit is increased, and various biological functions of

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insulin occur (Czech, 1985; Goldfine, 1987; Jacobs & Cuatrecasas, 1981; Kahn, 1985; Ullrich et al., 1985).

Several independent lines of evidence suggest that insulin receptor tyrosine kinase activity may be involved in certain actions of the hormone. First, insulin receptors mutated at the ATP binding site lose their activity to signal a wide variety of biological functions (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987; Yamamoto-Honda et al., 1990). Second, insulin receptors mutated at one or more of the key tyrosine autophosphorylation sites lose some biological functions (Debant et al., 1988; Ellis et al., 1986; Rafaeloff et al., 1990; Wilden et al., 1990). Third, when antibodies which react with the insulin receptor β-subunit are introduced into the cytoplasm of target cells, certain actions of insulin are blunted (Morgan & Roth, 1987).

A series of interesting observations have come from studies with polyclonal and monoclonal antibodies to the insulin receptor. Certain antibodies have been shown to stimulate insulin receptor tyrosine kinase activity and mimic insulin action (Brindle et al., 1990; Gherzi et al., 1987; Heffetz & Zick, 1986; O'Brien et al., 1987; Roth et al., 1983). Other antibodies, however, appear to have little or no effect on receptor tyrosine kinase activity and receptor autophosphorylation (Brunetti et al., 1989; Forsayeth et al., 1987a,b; Hawley et al., 1989; Ponzio et al., 1988; Sbraccia et al., 1990; Simpson & Hedo, 1984; Soos et al., 1989; Sung et al., 1989; Zick et al., 1984). However, these antibodies stimulate a wide spectrum of cellular functions (Brunetti et al., 1989; Forsayeth et al., 1987a; Ponzio et al., 1988; Soos et al., 1989; Sung et al., 1989). On one hand, studies with these latter antibodies suggest that activation of receptor tyrosine kinase activity may not be necessary to signal all the biological functions of insulin (Brunetti et al., 1989; Forsayeth et al., 1987; Ponzio et al., 1988; Soos et al., 1989; Sung et al., 1989). On the other hand, other studies suggest that these agonist receptor antibodies may induce low levels of receptor tyrosine kinase activity and receptor autophosphorylation and this low level of activation could account for the biological functions of antibodies (Gherzi et al., 1987; Steel-Perkins & Rogh, 1990).

In order to attempt to understand whether antibodies to the insulin receptor can in fact signal biological effects without activation of insulin receptor tyrosine kinase and receptor tyrosine autophosphorylation, we have now carried out studies with an agonist monoclonal antibody to the insulin receptor in cell lines expressing insulin receptor β-subunit mutants. Two cell lines have mutants with alterations in the ATP binding site, and one cell line has a mutant with changes in the three major tyrosine autophosphorylation sites. These studies suggest that there may be multiple signal transduction pathways to account for various biological functions of insulin and certain of these pathways may not require the activation of receptor tyrosine kinase.

Materials and Methods

HEPES, TRIS, MOPS, β-glycerophosphate, 2-mercaptoethanol (2-ME), trypsin, bacitracin, dithiothreitol (DTT), ATP, Triton X-100, sodium vanadate, aprotinin, phenylmethanesulfonyl fluoride (PMSF), and poly(Glu,Tyr 4:1) were from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) of low insulin-like activity was from Reheis Chemical Co. (Phoenix, AZ); porcine insulin was from Elanco Products (Indianapolis, IN); [γ-^{32}P]ATP, α-[^{3}H]aminoisobutyric acid, and [^{3}H]thymidine were from New England Nuclear (Boston, MA); reagents for gel electrophoresis were from Bio-Rad Laboratories (Richmond CA).

Cells Transfected with Mutant Human Insulin Receptors. HTC cells transfected with normal human insulin receptors (HTC-IR), receptors mutated in the major tyrosine autophosphorylation sites at 1158, 1162, and 1163 (HTC-F3), and receptors mutated at lysine-1030 in the ATP binding site (HTC-M1030) were prepared as previously described (Hawley et al., 1989; Sbraccia et al., 1990; Sung et al., 1989). These HTC cells were maintained in Dulbecco's modified Eagle's H-16 medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 incubator.

Chinese hamster ovary (CHO) cells transfected with and expressing normal human insulin receptors (CHO-IR) and receptors mutated at glycine-1008 to valine (CHO-V1008) were prepared as previously described (Yamamoto-Honda et al., 1990). CHO cells were maintained in Ham's F-12 medium containing 10% fetal calf serum.

[^{3}H]Aminoisobutyric Acid (AIB) Uptake. To measure stimulation of [^{3}H]AIB uptake, HTC cells and CHO cells were plated at 5×10^4 and 2×10^4 cells/well in 16-mm multiwell plates and grwon to 80–90% confluence. The media were changed to media containing 0.5% BSA/15 mM HEPES, pH 7.4, and cells were incubated in these media for 16 h. At the time of assay, the media were changed to fresh media, and the cells were incubated with various concentrations of a species-specific monoclonal antibody to the human insulin receptor (MA-5) (Brunetti et al., 1989; Forsayeth et al., 1987a,b; Hawley et al., 1989; Sung et al., 1989) for 2 h at 37 °C. Next, [^{3}H]AIB was added to yield a final concentration of 0.5 μCi (500 μL)$^{-1}$ well^{-1} followed by continuous incubation for 20 min at 37 °C. Cells were then solubilized in 0.03% sodium dodecyl sulfate (Hawley et al., 1989). The data were normalized to the cellular protein concentrations and are mean ± SEM of several separate experiments.

[^{3}H]Thymidine Incorporation. Cells were prepared in multiwell plates and preincubated for 16 h in media without serum as described for [^{3}H]AIB uptake. After the change of media, cells were incubated with various concentrations of MA-5 for 16 h at 37 °C. Next, [^{3}H]thymidine was added to yield a final concentration of 0.5 μCi (500 μL)$^{-1}$ well^{-1} followed by continuous incubation for 2 h. Cells were then processed as previously described (Brunetti et al., 1989).

Measurement of S6 Kinase Activity. Cells in 100 × 20 mm dishes were grown to 80–90% confluences and incubated 16 h in media without serum. Cells were then treated with various concentrations of MA-5 and their soluble cellular extracts prepared (Sung et al., 1989; Tabarini et al., 1985). 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 (1989). 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, and 60 μM [γ-^{32}P]ATP (0.8–1.6 μCi/nmol) as described by Sung et al. (1989). 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 (Sung & Williams, 1989; Sung et al., 1989).

Insulin Receptor Tyrosine Kinase Studies in Vitro. Insulin receptors were prepared as follows. Cells were solubilized in 1% Triton X-100 containing 50 mM HEPES, pH 7.6, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM sodium orthovanadate, and 0.1 mg/mL aprotonin and centrifuged at 100000g for 1 h followed by wheat germ agglutinin–agarose

column chromatography as described previously (Forsayeth et al., 1987a).

Phosphotransferase activity of insulin receptors prepared as above was determined by preincubating receptors (5 ng) with diluent (50 mM HEPES/150 mM NaCl, pH 7.4), insulin, or 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 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 disks of Whatman 3MM paper and dried. Disks were washed in three changes of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate and boiled for 2 min in 5% trichloroacetic acid followed by washing in ethyl alcohol and acetone (Steele-Perkins & Roth, 1990). Radioactivity was determined by liquid scintillation counting.

"Western Blot" Analysis of Tyrosine Phosphorylated Proteins in Vivo. Various HTC cell lines were incubated for 2 min at 37 °C with 100 nM insulin or MA-5. Next, 30 μM phenylarsine oxide was added for 10 s, and the reaction was stopped by rapid aspiration of the medium and freezing the cells in liquid nitrogen (Steele-Perkins et al., 1988; White et al., 1987). The cells were thawed in a buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/mL aprotinin, 2 mM PMSF, and 30 μM phenylarsine oxide. Cell lysates were resolved by 7.5% SDS–PAGE and transferred to nitrocellulose paper (Momomura et al., 1988). The nitrocellulose paper was soaked overnight in BSA buffer (3% bovine serum albumin, 10 mM TRIS, pH 7.5, 50 mM NaCl, and 0.01% sodium azide) and incubated with 2 μg/mL phosphotyrosine antibody for 30 min at 20 °C. After being washed with 10 mM TRIS, pH 7.5, and 50 mM NaCl, the nitrocellulose paper was incubated with ^{125}I-labeled protein A (0.5 μCi/mL) for 30 min. Unbound ^{125}I-labeled protein A was removed by washing twice in 10 mM TRIS, pH 7.5, and 50 mM NaCl, twice with 10 mM TRIS, pH 7.5, 50 mM NaCl, and 0.05% Nonidet P.40, and then twice with 10 mM TRIS, pH 7.5, and 50 mM NaCl. Proteins containing phosphotyrosine residues were then visualized by autoradiography (Pang et al., 1985).

Results

Four HTC cell lines were studied: (1) HTC-WT cells which are untransfected cells that have a relatively low number of wild-type rodent insulin receptors (less than 5000/cell as measured by insulin receptor radioimmunoassay) (Pezzino et al., 1989); (2) HTC-IR cells transfected with and expressing normal human insulin receptors (10^5/cell); (3) HTC-F3 cells transfected with and expressing human insulin receptors mutated at tyrosines-1158, -1162, and -1163 (2×10^5/cell); and (4) HTC-M1030 cells transfected with and expressing human insulin receptors mutated at lysine-1030 (5×10^5/cell).

Three CHO cell lines were studied: (1) CHO-WT cells which are untransfected cells that have a relatively low number of wild-type insulin receptors (4000/cell); (2) CHO-IR cells transfected with and expressing normal human insulin receptors (3.4×10^4/cell); and (3) CHO-V1008 cells transfected with and expressing human insulin receptors mutated at glycine-1008 (2.4×10^4/cell).

Studies of Biological Functions

Stimulation of [^{3}H]AIB Uptake. To test the ability of a human-specific monoclonal antibody to the human insulin receptor, MA-5, to signal metabolic effects via mutant receptors, we studied the uptake of the model amino acid α-aminoisobutyric acid (AIB). HTC-WT cells containing only rat insulin receptors did not respond to human-specific monoclonal antibody MA-5 (Figure 1A). This result was not unexpected since MA-5 does not cross-react with rodent insulin receptors (Forsayeth et al., 1987a). In HTC-IR cells, MA-5 stimulated AIB uptake in a dose-dependent manner with half-maximal stimulation at 670 pM. Interestingly, HTC-F3 cells were more sensitive to MA-5 than HTC-IR cells, whose half-maximal stimulation occurred at 300 pM (Figure 1A). When HTC-M1030 cells were studied, they also increased AIB uptake by 19 ± 4% ($p < 0.01$) in response to MA-5 although they were less responsive to MA-5 than HTC-IR and HTC-F3 cells.

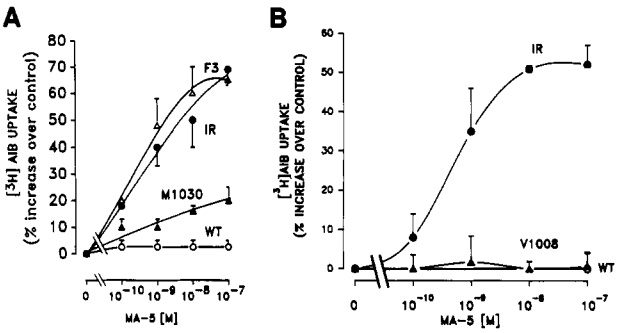

FIGURE 1: Stimulation of [^{3}H]AIB uptake by a human-specific monoclonal antibody, MA-5, in various HTC cell lines (A) and in various CHO cell lines (B). Cells in 16-mm multiwell plates were incubated for 2 h at 37 °C with various concentrations of MA-5. [^{3}H]AIB (0.5 μCi/well) was then added to the cells followed by continuous incubation for 20 min. Data are presented as the percent increase over control and are the mean ± SEM of three of more separate experiments. 100 nM normal mouse IgG was without effect. Basal AIB uptake (picomoles per milligram of protein) in HTC cells was 1.0 (WT), 1.22 (IR), 0.87 (F3), and 1.3 (M1030), respectively. Basal AIB uptake (picomoles per milligram of protein) in CHO cells was 3.87 (WT), 4.58 (IR), and 4.00 (V1008), respectively.

CHO-WT cells, containing only hamster insulin receptors, did not respond to human-specific MA-5 as expected (Figure 1B). In CHO-IR cells, MA-5 stimulated AIB uptake in a dose-dependent manner with half-maximal stimulation at 500 pM. When CHO-V1008 cells were studied, unlike HTC-M1030 cells, they did not respond to MA-5 (Figure 1B).

Stimulation of [^{3}H]Thymidine Incorporation. To test the ability of MA-5 to signal the mitogenic effects of insulin via mutant receptors, we measured stimulation of [^{3}H]thymidine incorporation in serum-deprived cells (Figure 2). In HTC-IR cells, MA-5 stimulated thymidine incorporation in a dose-dependent manner with half-maximal stimulation at 3 nM (Figure 2A). In three other HTC cell lines including HTC-WT, HTC-F3, and HTC-M1030 cells, MA-5 at 100 nM did not significantly stimulate this function. Higher concentrations of MA-5 were not tested due to nonspecific IgG effects.

In CHO-WT cells, MA-5 was without effect. In CHO-IR cells, MA-5 stimulated this function with half-maximal stimulation at 300 pM (Figure 2B). CHO-V1008 cells did not respond to MA-5.

Stimulation of S6 Kinase Activity. Phosphorylation of ribosomal protein S6 by S6 kinase has been proposed to play an important role in protein synthesis in response to hormones including insulin (Hansson & Ingelman-Sundberg, 1985; Nielsen et al., 1982; Thomas et al., 1982). In HTC-IR cells (Figure 3A), 100 nM MA-5 increased S6 kinase activity by (1.95 ± 0.04)-fold (mean ± SEM, $n = 3$). In HTC-F3 cells, 100 nM MA-5 increased S6 kinase activity by (3.03 ±

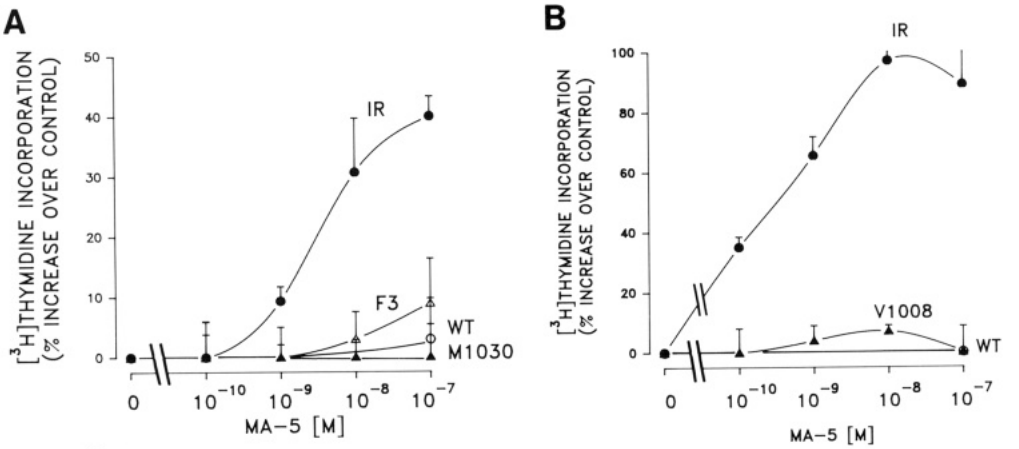

FIGURE 2: Stimulation of [^{3}H]thymidine incorporation by MA-5 in various HTC cell lines (A) and in various CHO cell lines (B). Cells in 16-mm multiwell plates were incubated for 16 h at 37 °C with various concentrations of MA-5. [^{3}H]Thymidine (0.5 μCi/well) was then added to the cells followed by continuous incubation for 2 h. Data are presented as the percent increase over control and are the mean ± SEM of three or more separate experiments. Basal thymidine incorporation was similar in cell HTC cell lines (20.9–29.4 pmol/mg of protein). In all CHO cell lines, basal thymidine incorporation was also similar (4.5–7.4 pmol/mg of protein).

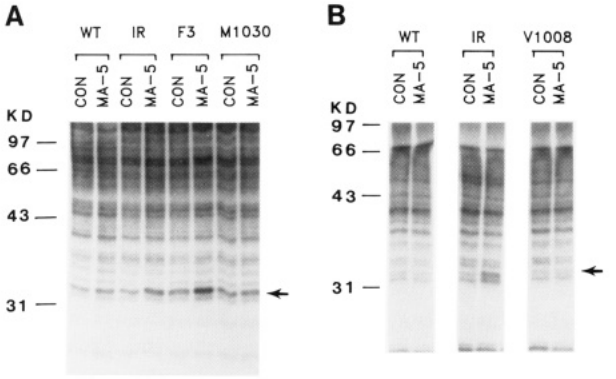

FIGURE 3: Autoradiograph of total reaction mixtures containing S6 protein phosphorylated by extracts of either various HTC cell lines (A) or various CHO cell lines (B). Cells in 100-mm dishes were incubated for 30 min with diluent (50 mM HEPES/150 mM NaCl, pH 7.4) or 100 nM MA-5. Following washes in ice-cold 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.

0.26)-fold (mean ± SEM, n = 3). In HTC-M1030 cells, like in HTC-WT cells, there was no significant increase in this activity by MA-5 (Figure 3A). In CHO-IR cells (Figure 3B), 100 nM MA-5 increased S6 kinase activity by (2.66 ± 0.09)-fold (mean ± SEM, n = 3). In CHO-V1008 cells as in CHO-WT cells, MA-5 was without effect.

Receptor Tyrosine Kinase and Receptor Autophosphorylation Studies

Tyrosine Kinase Studies. The ability of the insulin receptor mutants to phosphorylate an exogenous substrate was further quantitated with isolated, semipurified insulin receptors. The insulin receptors were partially purified by wheat germ agglutinin-agarose chromatography, and tyrosine kinase activities of the insulin receptors were measured using poly(Glu,Tyr) as a substrate (Figure 4). Equivalent amounts of insulin receptors (5 ng) based on radioimmunoassay (Pezzino et al., 1989) were assayed.

In receptors from HTC-IR cells, 100 nM insulin stimulated tyrosine kinase activity approximately 18-fold (Figure 4A). MA-5 had little or no effect on tyrosine kinase activity. With receptors from HTC-F3 cells, stimulation of tyrosine kinase by insulin 3-fold due to the presence of a small amount of normal rat insulin receptors (Hawley et al., 1989; Sung et al., 1989). There was no stimulation of F3 receptor tyrosine kinase by MA-5. In receptors from HTC-M1030 cells, insulin stimulated tyrosine kinase to a similar extent as in receptors

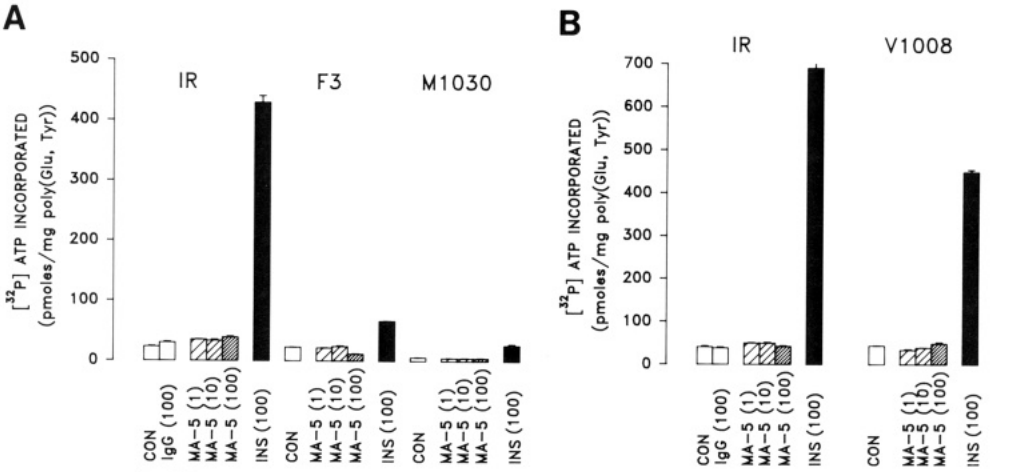

FIGURE 4: Insulin receptor tyrosine kinase activities against an exogenous substrate, poly(Glu,Tyr). Receptors were prepared as described under Materials and Methods. Isolated insulin receptor (5 ng each) from HTC cells (A) and CHO cells (B) was preincubated for 1 h at 24 °C and incubated for 1 h in a reaction mixture containing 10 μM [γ-^{32}P]ATP and 1 mg/mL poly(Glu,Tyr). Data are expressed as picomoles of ATP incorporated per milligram of poly(Glu,Tyr) and are the mean ± SEM of three separate experiments. Numbers in parentheses are concentrations of agents used and expressed as nanomolar (nM). CON = buffer control, IgG = normal mouse IgG, INS = insulin.

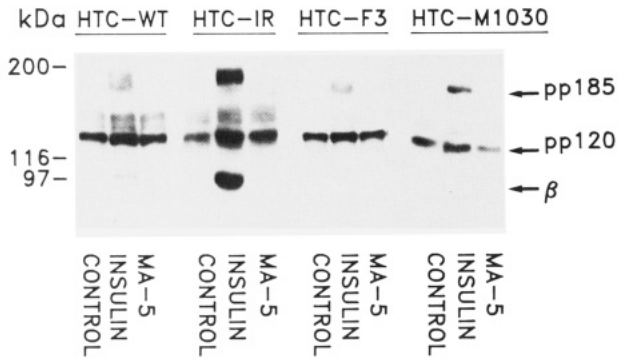

FIGURE 5: Autophosphorylation of insulin receptors and tyrosine phosphorylation of cellular proteins by a 100 nM aliquot of either insulin or MA-5 in intact HTC cell lines using Western blotting. Cells were incubated for 2 min at 37 °C with 100 nM either insulin or MA-5. The cellular lysates were then analyzed by 7.5% SDS–PAGE and Western blotting. Proteins containing phosphotyrosine residues were visualized by autoradiography. β is for the β-subunit of the insulin receptor.

from HTC-F3 cells. No stimulation by MA-5 was observed (Figure 4A). It is unlikely that a small number of insulin-like growth factor 1 (IGF-1) receptors present in insulin receptor preparations made a significant contribution to an insulin-induced increase in the tyrosine kinase activity since insulin does not act via IGF-1 receptors in HTC cells (Heaton et al., 1980).

In insulin receptors from CHO-IR cells, 100 nM insulin stimulated tyrosine kinase activity approximately 17-fold (Figure 4B). MA-5 had little effect on tyrosine kinase activity. In insulin receptors from CHO-V1008 cells, insulin stimulated tyrosine kinase activity by 10-fold. Stimulation of insulin receptors from CH-V1008 cells by insulin may have been due to both normal endogenous rodent insulin receptors and endogenous IGF-1 receptors. There was no stimulation of V1008 receptor tyrosine kinase by MA-5 (Figure 4B).

Intact Cells. In intact cells, the effects of insulin and MA-5 were further studied on insulin receptor β-subunit tyrosine autophosphorylation, and tyrosine phosphorylation of pp185, an endogenous cytoplasmic protein (Figure 5). In all the HTC cell lines unstimulated with agents, there was very little or no phosphorylation of receptor β-subunit. In HTC-WT cells, insulin, but not MA-5, at 100 nM induced a very small increase in tyrosine phosphorylation of both pp185 and receptor β-subunit (Figure 5). When HTC-IR cells were incubated with 100 nM insulin, there was a greater increase in tyrosine phosphorylation of both pp185 and receptor β-subunit than HTC-WT cells. MA-5 in these cells, however, did not increase tyrosine phosphorylation of these proteins. Similar results were obtained in either the absence or the presence of phenylarsine oxide. In HTC-F3 and HTC-M1030 cells, the increase in receptor autophosphorylation and pp185 phosphorylation by either insulin or MA-5 was similar (or somewhat smaller) to that in HTC-WT cells (Figure 5).

White and colleagues have reported that pp185 is a cytoplasmic protein or a group of related cytoplasmic proteins that are the major endogenous cellular substrates for insulin receptor kinase activity (White et al., 1985, 1987; White & Kahn, 1989). These proteins are rapidly phosphorylated, and then their levels of phosphorylation markedly decline. Although the function of pp185 is unknown, the phosphorylation of pp185 is a very sensitive readout of insulin-induced insulin receptor tyrosine kinase activity in intact cells (White et al., 1988). In all the HTC cells lines unstimulated with agents, there was little or no phosphorylation of this substrate. When these cells were incubated with 100 nM MA-5, there was no increase in phosphorylation of pp185 (Figure 5).

In all cell lines, another unidentified protein, pp120, was tyrosine-phosphorylated. This protein was previously observed by our group and others. There was no consistent increase in the phosphorylation of this protein by either MA-5 or insulin (Figure 5).

Previous studies with the CHO cell lines demonstrated that phosphorylation of pp185 was detected at 100 nM insulin in CHO-WT cells (Yamamoto-Honda et al., 1990). We found that in CHO-IR cells, phosphorylation of both the insulin receptor β-subunit and pp185 was apparent at 10 nM insulin (data not shown). The CHO-V1008 cells exhibited the same insulin sensitivity as the CHO-WT cells (Yamamoto-Honda et al., 1990). However, there was no effect of MA-5 on these functions in all CHO cell lines (data not shown).

Discussion

In the present study, we have investigated the effect of an insulin receptor α-subunit monoclonal antibody, MA-5, on transmembrane signaling in HTC and CHO cells transfected with and expressing both normal insulin receptors and insulin receptors mutated in their β-subunit tyrosine kinase domains. In both HTC and CHO cells expressing normal human insulin receptors, MA-5 stimulated three biological functions: AIB uptake, thymidine incorporation, and S6 kinase activation. These effects were not seen in untransfected rat HTC and hamster CHO cells whose receptors do not interact with human-specific monoclonal antibody, MA-5. Under conditions where these biological functions were stimulated, there was no detectable stimulation of tyrosine kinase activity. These studies, therefore, are in agreement with earlier studies from this and other laboratories and suggest that activation of receptor tyrosine kinase may not be a prerequisite for signaling of all biological functions (Brunetti et al., 1989; Forsayeth et al., 1987a; Hawley et al., 1989; Ponzio et al., 1988; Soos et al., 1989; Sung et al., 1989).

It has been suggested, however, that under certain conditions, various antibodies may activate a low level of insulin receptor tyrosine kinase activity and this small activation of tyrosine kinase may be sufficient to account for biological functions observed (Brindle et al., 1990; Gherzi et al., 1987; Steele-Perkins & Roth, 1990). In order to further test the hypothesis that antibodies can activate the insulin receptor to signal biological functions without activation of tyrosine kinase, we combined the use of monoclonal antibody MA-5 with cells expressing insulin receptors having mutations in the β-subunit tyrosine kinase domain. These studies indicated that some functions, including AIB uptake and S6 kinase activation but not thymidine incorporation, were activated by MA-5 in cells with mutant receptors. In these mutated receptors, as with normal insulin receptors, MA-5 did not activate tyrosine kinase. The present studies with mutant receptors are therefore in concert with studies of normal receptors, and provide further evidence that activation of receptor tyrosine kinase may not be essential for insulin receptor signaling of some biological functions.

It was of interest that a monoclonal antibody, MA-5, mimicked some, but not all, of the effects of insulin in cells containing insulin receptors with mutations in the tyrosine kinase domain. We first studied two ATP binding site mutants. The catalytic domain of the insulin receptor β-subunit contains a Gly-X-Gly-X-X-Gly motif (where X represents any amino acid) in the ATP binding site (Hanks et al., 1988; Sternberg & Taylor, 1984; Wierenga & Hol, 1983). This motif is also seen in many other protein kinases and oncogene products. The CHO-V1008 cells used in the present study

had human insulin receptors where the third glycine in the Gly-X-Gly-X-X-Gly motif was changed to valine, and thus these receptors do not bind ATP (Yamamoto-Honda et al., 1990). These mutated receptors failed to signal the three biological functions tested, when stimulated by MA-5. These receptors also failed to respond to insulin, a physiological ligand (Yamamoto-Honda et al., 1990). These studies indicated, therefore, that ATP binding was crucial for signaling by both MA-5 and insulin.

Another ATP binding site mutant we tested had methionine at 1030 changed to lysine. In many tyrosine kinases and oncogene products, there is an invariant lysine 17–23 residues downstream of the third glycine in the Gly-X-Gly-X-X-Gly motif (Hanks et al., 1988; Sternberg & Taylor, 1984; Wierenga & Hol, 1983). This lysine appears to be directly involved in the phosphotransfer reaction, possibly mediating proton transfer (Hanks et al., 1988). Since in certain ATP binding proteins this lysine is not present, it is possible that M1030 receptor lacking this lysine can still bind ATP (Hanks et al., 1988; Sternberg & Taylor, 1984; Wierenga & Hol, 1983). In cells expressing mutated M1030 receptors, there was a small, but significant, increase in AIB uptake by MA-5. Whether heterodimers formed between the transfected human insulin receptors and the endogenous rat receptors is unknown. However, there was no effect on the two other biological functions of insulin including thymidine incorporation and S6 kinase activation. These studies also suggested, therefore, that an intact ATP binding site is important for signaling most functions by MA-5. In M1030 receptors, insulin failed to signal these functions (Rafaeloff et al., 1991).

The third insulin receptor mutant (F3 receptor) used in the present study involved the tyrosine at positions 1158, 1162, and 1163. In normal insulin receptors, insulin activation of tyrosine kinase very rapidly leads to the autophosphorylation of a cluster of three tyrosines at residues 1158, 1162, and 1163 (Herrera & Rosen, 1986; Tornqvist et al., 1988; White & Kahn, 1989). 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 three tyrosine cluster (White & Kahn, 1989). Other tyrosine residues at 965, 972, and 984 are phosphorylated very poorly (White & Kahn, 1989). Autophosphorylation of the insulin receptor has been shown to play a major role in subsequently activating insulin receptor tyrosine kinase activity (Klein et al., 1986; Stadtmauer & Rosen, 1986; Wierenga & Hol, 1983; Yu & Czech, 1986). It is not surprising, therefore, that the tyrosine kinase activity of these receptors stimulated by insulin is far less than seen in normal receptors. However, in these cells, monoclonal antibody MA-5 mimicked stimulation of both AIB uptake and S6 kinase activity. Thymidine incorporation, however, was not stimulated. These studies suggest, therefore, that the presence of the three tyrosines was important for signaling this latter function but not the former ones. It is possible that in this mutant a small amount of receptor autophosphorylation occurred which was involved in signaling. In F3 receptors, insulin normally stimulated AIB uptake and thymidine incorporation (Rafaeloff et al., 1991), but not S6 kinase activation (Sung, 1991).

The insulin receptors prepared from all the cell lines did not respond to MA-5 with enhanced receptor tyrosine kinase activity toward poly(Glu,Tyr) (Figure 4). Insulin, however, stimulated receptor tyrosine kinase activity of normal insulin receptors prepared from HTC-IR and CHO-IR cells (Figure 4). In insulin receptor mutants with changes in the tyrosine kinase domain, including F3, M1030, and V1008 receptors, increases in receptor tyrosine kinase activity by insulin were observed due to the normal endogenous rodent receptors. In the case of the V1008 receptor, stimulation of receptor tyrosine kinase by insulin was higher than what was expected from endogenous hamster receptors only. This result may be due to the exogenous substrate, poly(Glu,Tyr), used in the present studies, since V1008 receptor did not phosphorylate another substrate, histone H2b (Yamamoto-Honda, 1990).

The question arises, therefore, as to how the insulin receptor may signal biological functions without activation of insulin receptor tyrosine kinase. Recently we studied the effects of insulin and ATP on insulin receptor β-subunit conformation (Maddux & Goldfine, 1991). ATP, in a dose-dependent manner, altered the binding of several antibodies directed toward different epitopes in the β-subunit, indicating a major ATP-induced change in receptor conformation. Insulin potentiated the ATP effect; however, the ATP effect was not due to autophosphorylation of the receptor, since both AMP-PNP (a nonhydrolyzable analogue of ATP) and ADP could substitute for ATP. A receptor mutant that had the three major tyrosine autophosphorylation sites mutated to phenylalanines also changed conformation in response to ATP and insulin. In contrast, insulin plus ATP was ineffective in the receptors where the ATP binding site was mutated. These studies suggest, therefore, that insulin and ATP can induce conformational changes in the insulin receptor β-subunit which are independent of either tyrosine kinase and receptor autophosphorylation and raise the possibility, therefore, that conformational changes induced by ATP and insulin may mediate some of the effects of insulin (Maddux & Goldfine, 1991).

These conformational changes chould play a role in insulin receptor signaling by nonconvalently interacting with one or more cellular effector molecules, in order to increase their activities. Monoclonal antibodies such as MA-5 have been demonstrated to induce conformationl changes in the insulin receptor α-subunit (Forsayeth et al., 1987a; Wang et al., 1988). It is also possible that monoclonal antibodies such as MA-5, in the presence of ATP, could induce similar conformational changes in the β-subunit of normal receptors without activation of tyrosine kinase. Further, the conformational change induced by the antibodies could signal biological functions in concert with basal insulin receptor tyrosine kinase activity. Induction of the β-subunit conformational change in the presence of ATP could explain, therefore, how monoclonal antibodies stimulate biological functions in the absence of insulin receptor tyrosine kinase activity.

Registry No. ATP, 56-65-5; S6 kinase, 90698-26-3; insulin, 9004-10-8; insulin receptor tyrosine kinase, 88201-45-0.

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