An Extracellular Domain of the Insulin Receptor β-Subunit with Regulatory Function on Protein-Tyrosine Kinase*

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Anti-insulin receptor monoclonal antibody MA-10 inhibits insulin receptor autophosphorylation of purified rat liver insulin receptors without affecting insulin binding (Cordera, R., Andraghetti, G., Gherzi, R., Adezati, L., Montemurro, A., Lauro, R., Goldfine, I. D., and De Pirro, R. (1987) Endocrinology 121, 2007-2010). The effect of MA-10 on insulin receptor autophosphorylation and on two insulin actions (thymidine incorporation into DNA and receptor down-regulation) was investigated in rat hepatoma Faö cells. MA-10 inhibits insulin-stimulated receptor autophosphorylation, thymidine incorporation into DNA, and insulin-induced receptor down-regulation without affecting insulin receptor binding. We show that MA-10 binds to a site of rat insulin receptors different from the insulin binding site in intact Faö cells. Insulin does not inhibit MA-10 binding, and MA-10 does not inhibit insulin binding to rat Faö cells. Moreover, MA-10 binding to down-regulated cells is reduced to the same extent as insulin binding. In rat insulin receptors the MA-10 binding site has been tentatively localized in the extracellular part of the insulin receptor β-subunit based on the following evidence: (i) MA-10 binds to insulin receptor in intact rat cells; (ii) MA-10 immunoprecipitates isolated insulin receptor β-subunits labeled with both [32S]methionine and [32P]; (iii) MA-10 reacts with rat insulin receptor β-subunits by the method of immunoblotting, similar to an antipeptide antibody directed against the carboxyl terminus of the insulin receptor β-subunit. Moreover, MA-10 inhibits autophosphorylation and protein-tyrosine kinase activity of reduced and purified insulin receptor β-subunits. The finding that MA-10 inhibits insulin-stimulated receptor autophosphorylation and reduces insulin-stimulated thymidine incorporation into DNA and receptor down-regulation suggests that the extracellular part of the insulin receptor β-subunit plays a role in the regulation of insulin receptor protein-tyrosine kinase activity.

The insulin receptor is a transmembrane glycoprotein composed of distinct subunits linked by disulfide bonds: two α-subunits (Mr, 135,000 by SDS-PAGE), comprising the insulin binding site and located entirely at the extracellular face of the plasma membrane, and two β-subunits (Mr, 95,000 by SDS-PAGE) that are protein-tyrosine kinases and span the plasma membrane. Insulin binding to insulin receptor β-subunit causes rapid phosphorylation of tyrosine residues on the receptor β-subunit (for a review, see Ref. 1). Although the structure of insulin receptor is characterized (2, 3) and the relevance of its protein-tyrosine kinase activity is defined (4-9), less information is available on the relative importance of different domains of the molecule in the regulation of its enzymatic activity (10-12). The following data suggest that the insulin receptor is composed of functionally independent domains: first, α-subunits of chimeric receptors both lacking the cytoplasmic portion of the β-subunit and presenting substitutions in this domain bind insulin with normal affinity (13, 14); second, in the absence of the α-subunit, the cytosolic part of insulin receptor β-subunit, expressed in Chinese hamster ovary cells, is a protein-tyrosine kinase more active than the entire receptor (15); third, the protein-tyrosine kinase of the cytoplasmic domain of insulin receptor can be activated by autophosphorylation independently of its organization within the native receptor oligomer (16); fourth, some agents (sodium orthovanadate, hydrogen peroxide, anti-β-subunit monoclonal antibodies) activate insulin receptor protein-tyrosine kinase directly acting on β-subunits (17-19).

Monoclonal antibodies are convenient probes to understand the functional topography of receptor molecules (20-23). In previous reports we demonstrated that protein-tyrosine kinase activity, stimulated in the entire insulin receptor by insulin and insulin mimickers in vitro, can be inhibited by the anti-insulin receptor monoclonal antibody MA-10 (24, 25). Based on this finding we suggested that MA-10 recognizes a district, conserved in human and rat insulin receptor, involved in the regulation of protein-tyrosine kinase activity but different from the insulin binding site (24).

In this paper we investigate: (i) whether MA-10 recognizes insulin receptors in intact rat cells, (ii) whether the portion of insulin receptor molecule, recognized by antibody MA-10, is important to regulate receptor autophosphorylation in intact cells and whether the inhibition of the enzyme by MA-
10 has biological significance, and (iii) whether MA-10 is able to inhibit the protein-tyrosine kinase activity of the isolated β-subunit of insulin receptor.

Data presented herein demonstrate that MA-10 inhibits insulin receptor autophosphorylation and the transduction of two insulin actions in insulin-responsive rat hepatoma cells binding to a region of the receptor molecule which is different from the insulin binding site and which is localized in the protein backbone of the extracellular portion of insulin receptor β-subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin, 125I-β-A-14-monoiodo human insulin (226 mCi/mg), and 125I-β-A-14-monoiodo porcine insulin (230 mCi/mg) were kindly supplied by Novo Industries (Copenhagen, Denmark). γ-[32P]ATP (3000 Ci/mM), [methyl-3H]thymidine (2 Ci/mM), [35S]methionine (1150 Ci/mM), and 125I-protein A (61 mCi/μg) were purchased from Du Pont-New England Nuclear. Hybond-N nylon blotting membranes and Amplify were from Amersham (Buckinghamshire, England). ATP, Triton X-100, phenylmethylsulfonyl fluoride, bacitracin, sodium orthovanadate, N-acetyl-d-glucosamine, diethiothreitol (DTT), N-ethylmaleimide (NEM), L-methionine, normal mouse immunoglobulin G (IgG), and polyethylene glycol 6000 were purchased from Sigma. Tunicamycin was from Boehringer Mannheim. Wheat germ agglutinin-agarose (WGA) was obtained from Vector Laboratories (Burlingame, CA), histone H2F2B was from Cooper Biomedical (Malvern, PA). Protein A-Sepharose CL4B was from Pharmacia (Uppsala, Sweden), G6 desalting columns were from Pierce, SM-2 Bio-Beads, gelatin, and all materials for SDS-PAGE were purchased from Bio-Rad. Pre-stained molecular weight markers for SDS-PAGE were from Bethesda Research Laboratories. Cell culture media, fetal calf serum, and dialyzed fetal calf serum were from Gibco. Tissue culture flasks and dishes were from Falcon. Cell culture media, fetal calf serum, and dialyzed fetal calf serum were from Gibco. Tissue culture flasks and dishes were from Falcon (Oxnard, CA). All other chemicals were from sources cited in Ref. 26.

**Antibodies**—Monoclonal antibody MA-10 is an IgG2b raised in mice against highly purified human placental receptors as previously described (27). MA-10 is devoid of any phosphatase or ATPase activity, it is not able to bind iodinated insulin, although an antibody immunoprecipitates both human and rat insulin receptors (28). Antibody H 65.6 is a monoclonal IgG2b that recognizes a monomorphic epitope present on HLA class I1 DQ molecules.

**Receptor Phosphorylation in Intact Fa0 Cells**—Confluent Fa0 cells (106 cells/plate) were incubated for 1 h at 37 °C in phosphate-free MEM containing 20 mM Hepes buffer, pH 7.4, and 1% dialyzed BSA, followed which [32P]orthophosphate (0.25 mCi/ml) was added. After 60 min at 37 °C, either mAb H 65.6 (105 M) or insulin (10-8 M) in the presence of 10-5 M normal mouse IgG together with 1.5 M MA-10 or with 10-5 M normal mouse IgG. [35S]Methionine Labeling of the Insulin Receptor—Confluent monolayers of Fa0 or Hep G2 cells in 100-mm dishes were incubated in 10% dialyzed fetal calf serum containing 10% [35S]methionine (200 μCi/ml) for 2 h. [35S]Methionine was added, and the incubation was continued further for 30 min. The medium was changed to DME containing 10% fetal calf serum and 10 mM unlabeled L-methionine, and the incubation was continued for up to 5 h.

**M Cells**—M normal mouse IgG together with 1.5 × 105 cpm [125I]-protein A/well were incubated with 4 h at 4 °C. Subsequently cells were washed with ice-cold PBS and incubated with prewarmed binding buffer (50 mM Hepes, pH 8.0, 150 mM NaCl, 10 mM CaCl2, 10 mM MgSO4, 10 mM glucose, 1% BSA) for 15 min at 37 °C. Cells were then moved to 4 °C and either washed twice with prewarmed buffer or four times with fresh binding buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.5 mM sodium acetate buffer, pH 3.5, containing 150 mM NaCl to remove MA-10-[125I]-protein A complexes bound to the cell surface. This procedure, modified from Ref. 29, removes insulin or antibody bound to the cell surface (8). The acid-treated cells were then washed two more times with PBS. Cells were solubilized by the addition of 0.1% SDS, and the radioactivity of the lysates was counted. The radioactivity associated with the cells, washed at pH 7.4 in PBS, represented the total cell-associated ligand, while the radioactivity present in the lysates of acid-washed cells represented the internalized (acid wash-insensitive) MA-10-[125I]-protein A. Nonspecific [125I]-protein A binding was measured incubating cells with 10-5 M normal mouse IgG.

**Insulin Receptor Down-regulation**—Confluent monolayers in six-well dishes were incubated at 37 °C for 20 h in complete medium with mAb H 65.6 (10-7 M) or insulin (10-7 M) in the presence of 10-5 M mAb H 65.6 or 10-7 M MA-10. Following incubation, cells were placed at 4 °C, surface-bound insulin or immunoglobulins were removed by washing twice for a total of 6 min with 500 mM sodium acetate buffer, pH 3.5, containing 150 mM NaCl followed by three additional washes with PBS. The residual insulin or MA-10 binding activity on the cell surface was then determined by incubation with [125I]-insulin or MA-10-[125I]-protein A for 4 h at 4 °C, as indicated above.

**Thymidine Incorporation into DNA—Assays were performed as described in Ref. 4 except that cells were serum-starved in DME containing 0.5% dialyzed BSA for 30 h, and the incubation with insulin and MA-10 prior to the addition of [3H]thymidine was carried out in 0.5% dialyzed BSA instead of 0.5% fetal calf serum.

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supernatant fluid was diluted 20-fold into 50 mM Hepes buffer, pH 7.4 containing 0.1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, and the protease inhibitors (10 μg/ml each) and subjected to chromatography on WGA-agarose as described in Refs. 6 and 24. The insulin receptor was eluted with 0.3 M N-acetylglucosamine. Receptor disulfide bonds were reduced by incubating unlabeled, 125I-

or 35S-labeled WGA-purified receptors at 22 °C with 100 mM DTT and 75 mM Tris (pH 8.5) for 30 min using a modification of the method described by Boni-Schnettler et al. (32). The reaction was stopped by adding NEM (to 100 mM final concentration) and transferring tubes on ice. Then reduced receptors (indicated as "DTT/Tris-reduced") were chromatographed on a 2-ml GF5 desalting column equilibrated with HTG buffer (50 mM Hepes, 0.05% Triton X-100, 0.5% glycerol, 5 mM NEM) and eluted in the same buffer. This procedure results in a near complete removal of DTT and Tris. Insulin receptor β-subunits were immunoprecipitated with the antipeptide polyclonal antibody PC, with MA-10, or with mAb H65.6 as described in other sections.

Reduction of Insulin Receptors into α- and β-Subunits—Insulin receptor disulfide bonds were reduced by incubating unlabeled, 35S- or 125I-labeled WGA-purified receptor preparations at 22 °C with 100 mM DTT and 75 mM Tris (pH 8.5) for 30 min using a modification of the method described by Boni-Schnettler et al. (32). The reaction was stopped by adding NEM (to 100 mM final concentration) and transferring tubes on ice. Then reduced receptors (indicated as "DTT/Tris-reduced") were chromatographed on a 2-ml GF5 desalting column equilibrated with HTG buffer (50 mM Hepes, 0.05% Triton X-100, 0.5% glycerol, 5 mM NEM) and eluted in the same buffer. This procedure results in a near complete removal of DTT and Tris. Insulin receptor β-subunits were immunoprecipitated with the antipeptide polyclonal antibody PC, with MA-10, or with mAb H65.6 as described in other sections.

In vivo cell, autoradiography was performed in duplicate. The average of three independent experiments, performed in duplicate, for each cell line are presented. The values for 100% insulin binding were 8760 and 8200 cpm/106 Hep G2 and Fao cells, respectively; for binding experiments performed at 4 °C and 3870 and 3690 cpm/106 Hep G2 and Fao cells, respectively, for binding experiments performed at 37 °C.

**TABLE I**

| Insulin binding activity | Hep G2 | Fao |
|--------------------------|--------|-----|
| **nM**                   | 4 °C   | 37 °C | 4 °C | 37 °C |
| 0                       | 100    | 100  | 100  | 100  |
| 0.1                     | 81     | 83   | 97   | 93   |
| 1                       | 54     | 49   | 100  | 99   |
| 10                      | 36     | 34   | 98   | 100  |
| 100                     | 28     | 30   | 98   | 98   |

**Fig. 1.** MA-10 binding to human and rat cells. Binding of MA-10, 125I-protein A complexes to the two hepatoma cell lines, rat Fao (○) and human Hep G2 (■), was measured as described under "Experimental Procedures." Cells in six-well dishes were incubated with increasing amounts of MA-10 (from 10−10 to 5 × 10−5 M) and 1.5 × 106 cpm/well 125I-protein A in the absence (——) or presence (——) of insulin (10−6 M) for 4 h at 4 °C. ▲ and △ indicate binding of H65.6-125I-protein A complexes to Fao and Hep G2 cells, respectively. Results are expressed as counts/min (cpm) of 125I-protein A bound to 106 cells and represent the average of three independent experiments performed in duplicate.

**RESULTS**

Antibody MA-10 Binding to Human and Rat Cells—Antibody MA-10 inhibits 125I-insulin binding to human hepatoma Hep G2 cells, 50% inhibition occurring at 10−5 M, but it does not inhibit 125I-insulin binding to rat hepatoma Fao cells even at 10−4 M concentration both at 4 and 37 °C (Table I). MA-10 binds to Hep G2, and insulin (10−6 M) partially inhibits MA-10 binding (Fig. 1); MA-10 also binds to Fao cells but, in this case, insulin (10−4 M) does not affect MA-10 binding (Fig. 1). It is noteworthy that: (i) MA-10 binding to Fao cells is right-shifted about 1 order of magnitude compared to MA-10 binding to Hep G2 cells; and (ii) in the presence of 10−6 M...
insulin, MA-10 binding to Hep G2 cells is similar to MA-10 binding to Fao cells in the absence or in the presence of insulin (Fig. 1). Superimposable results were obtained forming MA-10 binding experiments at 37°C instead of 4°C (data not shown). When Fao and Hep G2 cells are probed with MA-10 in indirect immunofluorescence experiments, in the absence of insulin, both Fao and Hep G2 cells exhibit membrane-associated fluorescence, while coincubation with 10^{-6} M insulin reduces staining of Hep G2 cells but not staining of Fao cells (data not shown). When insulin receptors were down-regulated in both Fao and Hep G2 cells, 125I-insulin and MA-10-125I-protein A binding were reduced to the same extent in both Fao and Hep G2 cells, thus suggesting that MA-10 binds to insulin receptor in these cell lines (Table II).

Effect of Antibody MA-10 on Insulin Receptor Phosphorylation, Thyminide Incorporation into DNA, Insulin Receptor Internalization, and Down-regulation—MA-10 antibody decreases insulin-stimulated protein tyrosine kinase activity of WGA-purified rat insulin receptors without affecting insulin binding (24). To understand whether this phenomenon occurs also when insulin receptors are located in the cell membrane and to define the mechanism by which MA-10 reduces insulin receptor autophosphorylation, the effect of this antibody on insulin receptor phosphorylation in intact cells was studied. In Fao cells, labeled with 32Porthophosphate, insulin (10^{-6} M, for 10 min at 37°C) stimulates 32P incorporation into the insulin receptor -subunit 9-fold compared to controls cells incubated with both normal mouse IgG (Fig. 2, A and B) or with the irrelevant monoclonal IgG2B1 H 65.6 (Fig. 2C). MA-10 reduces insulin-stimulated 32P incorporation into insulin receptor -subunit in a dose-dependent manner, half-maximal effect being at 10^{-8} M (Fig. 2). 32P incorporated into the insulin receptor -subunit was partially resistant to alkaline hydrolisis, and MA-10 reduces the incorporation of both alkalinsensitive and -resistant 32P into the insulin receptor suggesting that the content of phosphotyrosine as well as phosphoserine residues is reduced in cells incubated with MA-10 (Fig. 2, A and B). It is noteworthy that MA-10 (10^{-7} M) does not affect basal (not insulin-activated) insulin receptor autophosphorylation (data not shown) as already demonstrated in partially purified receptors (Ref. 24 and Fig. 8).

In order to verify whether the inhibition of insulin receptor autophosphorylation produced by MA-10 is relevant in terms of insulin action, the effect of MA-10 on insulin-stimulated thymidine incorporation into DNA of Fao cells was studied. In rat hepatoma Fao cells insulin (10^{-6} M) stimulates thymidine incorporation into DNA 2.5-fold and MA-10 (10^{-7} M) inhibits insulin action by 75% (Table III). MA-10 inhibition of insulin-stimulated thymidine incorporation into DNA is dose-dependent with half-maximal effect at 10^{-7} M (data not shown). On the contrary MA-10 does not affect basal thymidine incorporation into DNA of Fao cells (Table III).

The functional relevance of MA-10 inhibition of insulin receptor phosphorylation was investigated also measuring the effect of the antibody on insulin-induced receptor down-regulation. Fao cells were exposed to 10^{-6} M insulin in the presence of 10^{-7} M mAb H 65.6 (control cells) or 10^{-7} MA-10, for 20 h in complete medium, following which the residual binding activity was measured. The insulin binding activity on Fao cells exposed to insulin was reduced by about 40%, while that of cells incubated with insulin plus MA-10 was reduced by 10%, 10^{-7} M MA-10 reduces the insulin-induced receptor down-regulation by 75%. Control experiments showed that MA-10 does not down-regulate insulin receptor in Fao cells per se but, at the same concentration, inhibits insulin effect on receptor down-regulation (Table III). As previously reported (6), MA-10 induces insulin-independent internalization of human insulin receptors. However MA-10 fails to produce the same effect in rat cells: in fact the amount of internalized MA-10-125I-protein A complex is less than 10% of the total antibody bound to Fao cells after 15 min at 37°C, thus suggesting that the epitope of insulin receptor molecule, to which MA-10 binds in rat cells, is not involved in antibody-

### Table II
**Effect of insulin receptor down-regulation on insulin and MA-10 binding to human and rat cells**

| Cultures | Insulin binding activity | MA-10 binding activity |
|----------|--------------------------|------------------------|
|          | Fao | Hep G2 | Fao | Hep G2 |
| Control  | 100 | 100 | 100 | 100 |
| Down-regulated | 67 | 62 | 65 | 64 |

### Table III
**Insulin binding activity**

| Insulin binding activity | Fao | Hep G2 | Fao | Hep G2 |
|--------------------------|-----|--------|-----|--------|
| Control                  | 100 | 100    | 100 | 100    |
| Down-regulated           | 67  | 62     | 65  | 64     |

**Fig. 2.** Insulin receptor phosphorylation in intact cells. Panel A, Fao cells (2 X 10^{6}) incubated with [32P]orthophosphate for 90 min, were treated with either normal IgG, insulin, or MA-10 for 10 min as described under "Experimental Procedures." After cell solubilization insulin receptors were purified, immunoprecipitated with AbPC, and subjected to SDS-PAGE (7.5% polyacrylamide) as described. An autoradiogram of the dry gel, exposed for 16 h at -70°C with an intensifying screen, is shown. The band seen on this autoradiogram is the [32P]-labeled insulin receptor -subunit purified from cells treated with 10^{-7} M normal IgG (lane 1), insulin (10^{-7} M) plus normal IgG (lane 2), and insulin (10^{-7} M) plus 10^{-7} M MA-10 (lane 3). Positions of molecular mass markers are indicated in kDa on the left. Panel B, after the exposure shown in panel A, the gel was rehydrated in 1 M KOH at 55°C for 1 h, then washed in 10% acetic acid, 40% methanol, 1% glycerol for 1 h, neutralized, dried, and subjected to autoradiography (exposure 36 h at -70°C with an intensifying screen). Lanes are as in Panel A. Panel C, Fao cells (2 X 10^{5}), labeled with [32P]orthophosphate for 90 min, were treated with insulin (10^{-7} M) plus 10^{-7} M mAb H 65.6 (lane 1), or with insulin (10^{-7} M) plus MA-10: 10^{-7} M (lane 2), 5 X 10^{-8} M (lane 3), and 10^{-9} M (lane 4) as described under "Experimental Procedures." Insulin receptors were purified, immunoprecipitated with ARA, and analyzed by SDS-PAGE (7.5% polyacrylamide) as described above. An autoradiogram of the dry gel, exposed for 12 h at -70°C with an intensifying screen, is shown. Positions of molecular mass markers (in kDa) are indicated on the left.
mediated receptor internalization.

**Antibody MA-10 Immunoreacts with Human and Rat Insulin Receptors**—As shown in Fig. 3, MA-10 immunoprecipitates both human and rat insulin receptors. At $10^{-8}$ M concentration, MA-10 immunoprecipitates rat insulin receptors 10-15 times less than human insulin receptors. This difference in efficiency is predicted by the difference in MA-10 binding activity to rat and human insulin receptors in intact cells. On the contrary, at saturating antibody concentration ($2 \times 10^{-6}$ M), MA-10 immunoprecipitates both human and rat receptors to the same extent. $[^{35}S]$Methionine pulse-chase experiments also show that MA-10 recognizes both human (not shown) and rat insulin receptor precursor (molecular mass 210 kDa) (Fig. 3C and data not shown).

Next, the possibility that MA-10 immunoprecipitates isolated insulin receptor $\beta$-subunits was investigated. Interchain disulfide bonds of insulin receptors, WGA-purified from human or rat hepatoma cells, were reduced using a modification of the DTT/Tris method reported by Boni-Schnetzler et al. (32). As depicted in Fig. 4, this procedure yields isolated insulin receptor $\beta$-subunits that are immunoprecipitated by an antipeptide antibody directed against the carboxyl-terminal 17 amino acids of the insulin receptor (AbPC). As shown in Fig. 4 antibody MA-10 immunoprecipitates reduced insulin receptor $\beta$-subunits of insulin receptors WGA-purified from $[^{35}S]$methionine-labeled Fao cells. In experiments performed using reduced receptors WGA-purified from human Hep G2 cells, MA-10 immunoprecipitates both $\alpha$- and $\beta$-subunits (Fig. 4). Reduction of class 2 disulfide bonds is not 100% complete: in fact discrete amounts of $\alpha/\beta$ dimers are present and are immunoprecipitated by MA-10. The small amount of rat insulin receptor $\alpha$-subunit evident in the MA-10 immunoprecipitates of reduced rat receptors (Fig. 4) probably depends on the coprecipitation of $\alpha$-subunits linked to $\beta$-subunits through hydrogen bonds or other noncovalent bonds. However the ratio of $\alpha$ to $\beta$ subunits immunoprecipitated from rat DTT-reduced receptors by MA-10 and AbPC (as measured by densitometer quantitation of autoradiograms) was similar (0.21 versus 0.23), while the ratio of $\alpha$- to $\beta$-subunits immunoprecipitated from human DTT-reduced receptors by MA-10 was ~3-fold higher (0.70) (Fig. 4). In order to exclude the possibility that MA-10 immunoprecipitates rat insulin receptor $\beta$-subunits by cross-reacting with the small amount of noncompletely reduced $\alpha$-subunits, also present in our DTT-reduced preparations, we performed immunoblotting experiments probing WGA-purified rat liver receptors with MA-10 or with the anti-insulin receptor $\beta$-subunit antipeptide antibody AbPC. As demonstrated in Fig. 5, both MA-10 and AbPC react with a major band of approximately $M_r$, 95,000 that corresponds to insulin receptor $\beta$-subunits. No immunoreactivity is evident at the molecular weight corresponding to insulin receptor $\alpha$-subunits (Fig. 5).

Finally MA-10 immunoprecipitates in vitro phosphorylated insulin receptor $\beta$-subunits purified from both human placenta and rat liver (data not shown).

**Antibody MA-10 Immunoprecipitates Nonglycosylated Insulin Receptors**—Fao cells were incubated with tunicamycin to inhibit protein glycosylation and labeled with $[^{35}S]$methylamine. Cells were solubilized and the clarified supernatant,
concentrated by polyethylene glycol precipitation, was immuno-
precipitated. MA-10 immunoprecipitated 35S-labeled proteins with M, molecular mass values ranging from 90 to 110 kDa corresponding to nonglycosylated insulin receptor β- and α-subunits (Fig. 6) (34, 35).

An indetical pattern of immunoprecipitation is produced by the antipeptide polyclonal antibody AbPC (Fig. 6). As internal control an aliquot of fully glycosylated insulin recep-
tor, immunoprecipitated by MA-10 from 35S-methionine-
labeled Fao cells, was run in parallel showing the exact position of glycosylated insulin receptor α- and β-subunits.

**Effect of Antibody MA-10 on Autophosphorylation and Kinase Activity of Isolated Insulin Receptor β-Subunits**—WGA-purified human placental insulin receptors were reduced by DTT/Tris treatment and isolated β-subunits purified by im-
nunoprecipitated with AbPC. Autophosphorylation and histone kinase activity of immunoprecipitated material was measured in the presence of normal mouse IgG (10⁻⁸ M) or antibody MA-10 (10⁻⁷ M). As shown in Fig. 7, MA-10 inhibits both insulin receptor beta subunit autophosphorylation and phosphorylation of histone HP2B. Similar results were ob-

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**Fig. 4. Immunoprecipitation of reduced human and rat insulin receptors.** Panel A, Fao cells (2 × 10⁶) were labeled with [35S]methionine for 30 min, washed, and exposed to medium containing 10 mM unlabeled methionine as described under "Experimental Procedures." Insulin receptors were WGA-purified and subjected to the disulfide bond reduction procedure detailed under "Experimental Procedures." The neutralized and alkylated receptors were immunoprecipitated using the polyclonal antibody AbPC (1:100) or a preim-

**Fig. 5. Immunoblotting of rat insulin receptor.** WGA-puri-
ified rat liver receptors were subjected to SDS-PAGE under reducing conditions followed by immunoblotting with 10⁻⁸ MA-10, polyclonal antipeptide antibody AbPC (1:100 final dilution), or nonimmune (NI) rabbit serum as described under "Experimental Procedures." The autoradiogram was exposed for 18 h at -70 °C. The positions of the molecular mass markers are indicated in kDa on the left.

**Fig. 6. Immunoprecipitation of nonglycosylated insulin receptors with MA-10.** Fao cells (2 × 10⁶) were incubated in methionine-free MEM (+10% dialyzed fetal calf serum) in the presence of 20 µg/ml tunicamycin, labeled with [35S]methionine for 30 min, washed, and exposed to DME containing 10 mM methionine for 8 h, as described under "Experimental Procedures," in the presence of tunicamycin (20 µg/ml). Cells were solubilized, Triton X-100 concentration in the clarified supernatant was lowered by chromatography on a SM-2 Bio-Beads column, and the 35S-labeled proteins concentrated by polyethylene glycol precipitation as described under “Experimental Procedures.” Insulin receptors were then immunoprecipitated with MA-10 (2 × 10⁻⁸ M) or with AbPC (10⁻⁷ M), as indicated in the figure, and analyzed by SDS-PAGE (6% polyacryl-
amide) under reducing conditions. In order to localize the exact position of mature (glycosylated) insulin receptor α- and β-subunits, an aliquot of insulin receptors immunoprecipitated by MA-10, as shown in Fig. 4C, was run in parallel (last lane on the right). The autoradiogram was exposed 24 h at -70 °C, molecular mass markers are shown on the left of the figure.
activity of isolated insulin receptor β-subunit. Tris and immunoprecipitated by AbPC preimmune rabbit serum clonal antibody H65.6, used as negative control, did not affect was exposed 16 h at -70°C. Autoradiogram was then conducted in the presence of MnCl₂ as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE (7.5% polyacrylamide), the autoradiogram was exposed for 30 h at -70°C with an intensifying screen. Positions of molecular mass markers (in kDa) are indicated on the left.

Finally it is noteworthy that MA-10 reduces basal (not insulin-stimulated) autophosphorylation of insulin receptor β-subunits while it does not decrease, at any concentration, basal autophosphorylation of the native receptor (Fig. 8).

**DISCUSSION**

We previously reported that anti-insulin receptor monoclonal antibody MA-10 affects both insulin binding and insulin-stimulated receptor protein-tyrosine kinase activity in partially purified human insulin receptors, but that it inhibits this enzymatic activity without inhibiting insulin binding in partially purified rat insulin receptors (24).

The aim of this study was to investigate the mechanism by which MA-10 inhibits insulin receptor protein-tyrosine kinase activity. First the MA-10 binding site to rat insulin receptor was characterized. Data presented herein indicate that antibody MA-10 binds to rat hepatoma Fao cells. By both MA-10-¹²⁵I-protein A complex binding and indirect immunofluorescence MA-10-specific binding to rat as well as to human cells was demonstrated. The fact that MA-10 does not reduce insulin receptor binding and, conversely, insulin does not inhibit MA-10 binding to Fao cells suggests that, in rat cells, MA-10 does not recognize the insulin binding site of insulin receptor molecule. In human hepatoma Hep G2 cells, MA-10 interacts with the insulin binding site since it inhibits insulin binding as insulin (on a molar basis) (27), and furthermore saturating concentrations of insulin reduce MA-10 binding. In the presence of 10⁻⁶ M insulin, MA-10 binding to Hep G2 cells is reduced to the same amount found in Fao cells both in the absence and in the presence of insulin. The demonstration that insulin receptor down-regulation is associated with a reduction of both ¹²⁵I-insulin and MA-10-¹²⁵I-protein A complex binding to Fao cells suggests that MA-10 binds to an extracellular portion of insulin receptor in rat cells.

MA-10 also immunoprecipitates both phosphorylated and native, ³⁵S-labeled rat insulin receptors. This finding is only apparently in contrast with data reported by Forsayeth et al. (27). These authors reported that MA-10 does not immunoprecipitate purified rat insulin receptors. In fact using a relatively low antibody concentration (10⁻⁶ M), the amount of immunoprecipitated rat receptors is 10–15-fold less than the amount of immunoprecipitated human insulin receptors. Optimizing immunoprecipitation conditions and exposing gels for a convenient period of time (up to 3 days at -70°C), it was possible to detect discrete immunoprecipitated material at a molecular weight exactly corresponding to insulin receptor β-subunit using WGA-purified rat liver insulin receptors. It is noteworthy that MA-10 interactions (binding and immunoprecipitation) with the native rat insulin receptor at submaximal antibody concentrations occur with a 10 times lower efficiency compared to MA-10 interactions with native human insulin receptors. Using saturating antibody concentrations (2 × 10⁻⁶ M), MA-10 immunoprecipitates approximately the same amount of insulin receptors from ³⁵S-methionine-labeled rat and human hepatoma cells.

MA-10 immunoprecipitates both α- and β-subunits in DTT-reduced human receptor preparations but reacts only with β-subunits of reduced rat receptors in both immunoprecipitation and immunoblotting experiments. This fact indicates that MA-10 recognizes human and rat insulin receptor β-subunits. While species specificity of the insulin binding site of the receptor is well documented (37–39), data presented here give further evidence to our previous observation (24) that a site involved in insulin receptor protein-tyrosine kinase activity regulation, recognized by MA-10, is conserved through rat and human species.

Data discussed above suggest that MA-10 binds to a region
of insulin receptor molecule common to both human and rat cells and that this region is not the receptor insulin binding site. The insulin receptor region recognized by MA-10 belongs to the protein backbone of the molecule because MA-10 immunoprecipitates not completely glycosylated receptors from tunicamycin-treated rat hepatoma FAO cells.

Experiments were carried out to explore the functional relevance of the insulin receptor region bound by MA-10 in intact rat cells. MA-10 inhibits insulin-stimulated receptor autophosphorylation in intact rat cells without affecting insulin receptor binding. In intact cells a 10-min exposure to insulin leads to the insulin receptor autophosphorylation in both tyrosine and serine residues (40). Recent evidence suggests that after tyrosine autophosphorylation insulin receptors become substrates for a putative serine kinase (1) and that only insulin receptors phosphorylated in tyrosine residues can undergo to serine phosphorylation (6). Our data, demonstrating that MA-10 similarly reduces 32P incorporation into both tyrosine and serine residues of insulin receptor, subunits are concordant with this last finding. The inhibition of insulin-stimulated receptor phosphorylation has biological significance: at the same concentrations, MA-10 reduces to a similar extent insulin-stimulated receptor autophosphorylation, thymidine incorporation into DNA, and insulin-induced receptor down-regulation in rat hepatoma FAO cells. Recent data, obtained in human insulin receptor partially (13) or completely (4-9) devoid of insulin-induced receptor down-regulation in rat hepatoma FAO cells, agree with this hypothesis (42, 43). Data presented in this report give more support to this hypothesis: MA-10 does not inhibit enzyme activity of the isolated insulin receptor β-subunit. This finding indicates that the protein-tyrosine kinase activity of isolated insulin receptor β-subunit can be modulated independently of α-subunit, as suggested by Herrera et al. (16) and that MA-10 binding to isolated β-subunit reduces the basal activity of the enzyme. Recently it has been suggested that the α-subunit of the insulin receptor inhibits protein-tyrosine kinase activity, and, consequently, the physiological responses mediated by the enzyme and the insulin receptor signaling (15). The demonstration that the digestion of insulin receptor α-subunit by trypsin leads to the activation of insulin receptor autophosphorylation in intact cells agrees with this hypothesis (42, 43). Data presented in this report give more support to this hypothesis: MA-10 does not inhibit kinase activity of the β-subunit in the native receptor, that is under the inhibitory effect of α-subunit, but has an inhibitory effect when the β-subunit (protein-tyrosine kinase) is isolated. These findings suggest that MA-10 can substitute the α-subunit for the inhibition of insulin receptor β-subunit protein-tyrosine kinase activity.

In conclusion, MA-10 antibody recognizes the extracellular portion of the insulin receptor β-subunit in rat cells or tissues. Data presented above indicate that (i) the extracellular part of the insulin receptor β-subunit recognized by MA-10 is more conserved, through human and rat species, than the insulin binding domain, and (ii) the extracellular part of the insulin receptor β-subunit is involved in the regulation of insulin receptor phosphorylation and in the transduction of insulin biological actions.

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