Direct Demonstration of Separate Receptors for Growth and Metabolic Activities of Insulin and Multiplication-stimulating Activity (an Insulinlike Growth Factor) Using Antibodies to the Insulin Receptor

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ABSTRACT Insulin and such insulinlike growth factors as multiplication stimulating activity (MSA) are related polypeptides that have common biological activities. Both insulin and MSA produce acute metabolic responses (stimulation of glucose oxidation in isolated fat cells) as well as growth effects (stimulation of [^{3}H]thymidine incorporation into DNA in cultured fibroblasts). In addition, most cells have separate receptors for insulin and insulinlike growth factors, and both peptides have weaker affinity for each other's specific receptors than for their own. To determine, therefore, whether these effects are mediated by receptors for insulin, insulinlike growth factors, or both, we have selectively blocked insulin receptors with a specific antagonist, namely Fab fragments derived from naturally occurring antibodies to the insulin receptor.

In rat adipocytes, 10 μg/ml of antireceptor Fab inhibited insulin binding by 90%, whereas it inhibited MSA binding < 5%. The anti-insulin receptor Fab is without intrinsic biological activity, but acts as a competitive inhibitor of insulin receptors. Blockade of insulin receptors with Fab fragments produced a 30-fold rightward shift in the dose response for stimulation of glucose oxidation by both insulin and MSA. The dose-response curves for stimulation of oxidation by vitamin K_5 and spermine, agents that stimulate glucose oxidation through noninsulin receptor pathways, were not affected by the blockade of insulin receptors with Fab antibody fragments. These data suggest that this acute metabolic effect of both insulin and MSA is mediated via the insulin receptor.

In cultured human fibroblasts, 10 μg/ml of Fab inhibited insulin binding by 90% and MSA binding by 15%. In fibroblasts, however, blockade of the insulin receptor did not alter the dose response for stimulation of thymidine incorporation into DNA by either insulin or MSA. Furthermore, intact antireceptor antibody immunoglobulin (Ig)G, which produces multiple other insulinlike effects, and Fab fragments of antireceptor antibody did not stimulate thymidine incorporation. These data demonstrate directly that the insulin receptor mediates the metabolic effects of insulin and MSA, whereas the growth-promoting action of both peptides is mediated by the MSA receptor or other growth factors.

INTRODUCTION

Varieties of polypeptides have been identified that have growth-promoting and insulinlike activities, but are immunologically distinct from insulin (1–4). These include the insulinlike growth factors[1] IGF-I and IGF-II (1), somatomedin A (2), somatomedin C (3), and multiplication-stimulating activity (MSA) (4). The common biological activities of insulin and insulinlike

A preliminary account of this work has been presented at the National Meeting of American Society for Clinical Investigation, May 1979, in Washington, D. C.
Received for publication 21 November 1980 and in revised form 10 March 1980.

[1] *Abbreviations used in this paper*: IGF, insulinlike growth factors; MSA, multiplication-stimulating activity.

growth factors[2] comprise two categories: metabolic effects, which include the stimulation of glucose transport, glucose metabolism, and antilipolysis; and growth effects, including the stimulation of DNA and RNA synthesis, protein synthesis, cell multiplication, and sulfate incorporation into cartilage (1, 5–10). For most of the acute metabolic effects, insulin is more potent than the insulinlike growth factors, whereas the insulinlike growth factors are generally more potent than insulin with regard to the chronic growth effects (1, 9).

The similar spectrum of action of insulin and insulinlike growth factors can probably be attributed to two factors. First, two of these polypeptides, IGF-I and IGF-II, have many homologous amino acid sequences with insulin (11, 12). Second, although most cells examined have separate, high-affinity receptors for insulin and the insulinlike growth factors (3, 13, 14), each peptide usually shows some affinity for the other's receptors (1, 9, 13, 15). It has been postulated that the common metabolic effects of insulin and the insulinlike growth factors are mediated by the insulin receptor (1, 3, 13, 16), whereas the common growth effects are mediated by the receptors for the insulinlike growth factors (1, 7, 15).

Antibodies that block insulin binding to the insulin receptor were recently described in some patients with insulin-resistant diabetes (17). The intact bivalent antibody mimicked insulin action, whereas monovalent antibody fragments were without biological effects (18), thus providing an inhibitor for insulin at the receptor level. In the present study we have selectively blocked insulin receptors with the specific insulin receptor antagonist Fab fragment to determine directly whether insulin and MSA stimulation of glucose oxidation in rat adipocytes (a metabolic effect) and thymidine incorporation in human fibroblast (a growth effect) are mediated by the insulin receptor, MSA receptor, or both.

[2] We have used the term insulinlike growth factors to refer to a family of closely related polypeptides that includes IGF-I, IGF-II, somatomedin A, somatomedin C, and MSA. These polypeptides share (*a*) common biological activities in adipose tissue, cartilage, and fibroblasts; (*b*) chemical properties (mol wt ≃ 7,500, acid solubility); (*c*) binding to specific carrier proteins in serum; (*d*) binding to specific cell surface receptors; (*e*) weak cross-reaction with insulin receptors; (*f*) immunological cross-reactivity. Based on the known amino acid sequences of IGF-I and -II, it seems probable that these five polypeptides are highly homologous but not identical. They differ in the extent of growth hormone dependence, possibly indicating that only some members of the group are true mediators of growth hormone action (somatomedins). The different peptides may serve different biological functions in vivo. Preliminary characterization indicates that additional polypeptides from rat and human serum have properties similar to the insulinlike growth factors. By contrast, nerve growth factors and relaxin, despite weak sequence homology to IGF-I and insulin, differ significantly from the insulinlike growth factors: They exhibit different reactivities with receptors, antibodies and carrier proteins; they are synthesized by different tissues; and they have different biological activities in different target organs.

METHODS

Materials. D-[U-^{14}C]glucose (12.5 Ci/nM sp act) and [*methyl*-^{3}H]thymidine (6.7 Ci/mM sp act) were purchased from New England Nuclear, Boston, Mass; trypsin (lot 1899), crude collagenase (CL545KB7), and papain (37.4 mg/ml; 19.7 U/mg), from Worthington Biochemical Corporation, Freehold, N. J.; bovine serum albumin (fraction V, lot NS-3309), from Armour and Co., Helena, Ark.; protein A-Sepharose, from Pharmacia, Inc., Piscataway, N. J.; dinonyl phthalate and glycine, from Eastman Organic Chemical Company, Rochester, N. Y.; concanavalin A, soybean trypsin inhibitor, spermine, L-cysteine, and iodoacetamide, from Sigma Chemical Company, St. Louis, Mo.; and vitamin K_5, from ICN Pharmaceuticals, Inc., Irvine, Calif. Porcine insulin (27.5 U/mg) was purchased from Elanco Products Company, Indianapolis, Ind. ^{125}I-Insulin was prepared by a modification of the chloramine-T method at specific activities of 100–200 μCi/μg (21, 22).

Polypeptides. MSA was purified from serum-free media conditioned by the BRL 3A cell line as previously described (4, 19). The conditioned medium was adsorbed onto Dowex (Dow Chemical Co., Midland, Mich.) at neutral pH and eluted at pH 11. The Dowex eluate was then chromatographed on Sephadex G-75 in 1.0 M acetic acid. Assayed by the stimulation of [^{3}H]thymidine incorporation into DNA in chick embryo fibroblasts, MSA eluted from Sephadex G-75 in three broad regions, designated I, II, and III. There are four MSA species in the peak II region. Each of these MSA species has equal specific activity in chick embryo fibroblast bioassay, radioreceptor assay, and radioimmunoassay; therefore, fractions from G-75 Sephadex containing these four species were pooled and used as standard in the radioreceptor assays. Peak II MSA was subjected to further purification by preparative polyacrylamide gel electrophoresis (pH 2.3, 9 M urea), and a homogenous polypeptide, MSA II-1, was isolated and has been used for radioiodination. ^{125}I-MSA was prepared by a previously published modification of the chloramine-T procedure at specific activities of 42–80 μCi/μg (20).

Isolation of antireceptor immunoglobulin(Ig)G and Fab fragments. The source of antireceptor IgG was serum from patient B-2 with the type B syndrome of extreme insulin resistance and acanthosis nigricans (17, 23). This patient's serum is known to contain a high titer of antibody to the insulin receptor. To purify antireceptor IgG, 3 ml of serum was absorbed for 24 h at 4°C into a column containing 3 g of protein A-Sepharose (24), gel volume of 6 ml, equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was then washed three times with 6 ml (per wash) of 0.1 M sodium phosphate buffer, pH 7.0. The IgG was eluted with 9 ml of 0.1 M glycine-HCl buffer, pH 2.8. The eluate was concentrated in an Amicon ultrafiltration cell (10 ml) (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) using a Diaflo membrane PM 10 (lot AE 0920B, Amicon Corp.) to a final volume of 3 ml and then dialyzed overnight against 0.1 M sodium phosphate buffer, pH 7.0. This IgG preparation had a protein concentration of 15 mg/ml determined by the method of Lowry et al. (25), using bovine serum albumin as standards.

Fab fragments of antireceptor IgG were prepared as fol-

lows: 2 ml of IgG preparation in 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 4 mM cysteine, 3 μg/ml of papain, and 1 mM EDTA at 37°C for 10 h. Iodoacetamide was then added to a final concentration of 5 mM. The reaction mixture was applied to a protein A-Sepharose column equilibrated in 0.1 M sodium phosphate buffer, pH 7.0, and allowed to adsorb overnight. Any remaining intact IgG or Fc fragments bind to the protein A column. Fab fragments do not bind to protein A (24) and so were eluted with 10 ml of 0.1 M sodium phosphate buffer, pH 7.0. The eluate was then concentrated to 2 ml by ultrafiltration. Fab fragments were purified by filtration on a Sephadex G-200 column (1.5 × 70 cm, bed volume 143 ml) in 0.1 M sodium phosphate buffer, pH 7.0. The major protein peak has an elution volume consistent with the molecular weight of Fab fragments and blocks insulin tracer binding in a variety of cells.

Preparation of isolated adipocytes. Male Sprague-Dawley rats of 100–160 g were used for all experiments. All studies were performed in the morning on animals that had free access to Purina rat chow (Ralston Purina Co., St. Louis, Mo.). Animals were decapitated, and the epididymal fat pads removed. The fat pads were cut into several pieces and digested at 37°C in Krebs-Ringer bicarbonate buffer containing 2 mg/ml collagenase as described by Rodbell and others (26, 27). The Krebs-Ringer bicarbonate buffer used contained 118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 2.5 mM $NaHCO_3$; the pH was adjusted to 7.4 with NaOH after equilibrating with 95% O_2:5% CO_2. After 50 min incubation at 37°C, the isolated adipocytes were separated from undigested tissue by filtration through a silk screen. The cells were washed three times in a Krebs-Ringer bicarbonate buffer, pH 7.4, and resuspended in the same buffer containing 2% bovine serum albumin.

Fibroblast culture. Cultures were initiated from punch biopsies of skin from the volar surface of the mid-forearm of normal adult volunteers and grown at 37°C in modified Eagle's Minimum Essential Medium supplemented with 20% fetal bovine serum (Flow lot 1150). Cultures were trypsinized, divided 1:3 each week, cultured in 75-cm^2 Falcon (Falcon Industries, Inc., Russell, Ky.) flasks, and used between the 3rd and 15th weekly passage (7, 28).

Hormone binding and glucose oxidation with adipocytes. All studies of hormone binding were performed in the Krebs-Ringer buffer with 20 mg/ml albumin, pH 7.4. The incubation volume was 0.5 ml. The final concentration of cells was 2.4×10^5 cells/ml. The labeled ligand was either ^{125}I-insulin (125 pg/ml) or ^{125}I-MSA (250–300 pg/ml). Unlabeled peptides were added as indicated in the text or figures. Incubation time and temperature required for steady-state binding were 20 min and 37°C for ^{125}I-insulin binding studies and 40 min and 24°C for ^{125}I-MSA binding studies. The incubation was terminated by removing 200-μl aliquots from the cell suspension and rapidly centrifuging the cells in 400-μl plastic microfuge tubes to which 100 μl of dinonylphthalate had been added as described by Gammeltoft and Gliemann (29). All binding studies were performed in duplicate or triplicate.

Glucose oxidation was studied by measuring the conversion of [U-^{14}C]glucose to $^{14}CO_2$, as previously described (26, 27), with an incubation period of 1 h. All glucose oxidation assays were performed in duplicate.

Insulin and MSA binding to human fibroblasts. To prepare suspensions of human fibroblasts for use in binding experiments, human fibroblasts were trypsinized, diluted 1:10, and plated in 100-mm dishes with Eagle's Minimum Essential Media contained 20% fetal bovine serum. 5 d after the cells reached confluence, they were released from the plates by incubation with 0.01% trypsin and 0.5 mM EDTA at 37°C for 8 min. Trypsinization was terminated by the addition of an equal volume of 0.1% soybean trypsin inhibitor. The cells were collected, centrifuged, and resuspended in Hepes buffer (pH 8.0, 0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 10 mM/ml bovine serum albumin).

^{125}I-Insulin (150 pg/ml) or ^{125}I-MSA (250 pg/ml), unlabeled polypeptides as indicated, and 5×10^6 cells were incubated in a plastic tube in Hepes buffer with a total volume of 0.5 ml for 2 h at 15°C. At the end of the incubation period, duplicate 0.2-ml aliquots were layered over 0.2-ml cold Hepes buffer in a microfuge tube and centrifuged for 1 min in a Beckman model 150 microfuge (Beckman Instruments Inc., Fullerton, Calif.), as previously described (28, 30).

[^{3}H]Thymidine incorporation into DNA. Human fibroblasts were grown to confluence at 37°C in 25-cm^2 plastic flasks. The growth media was then replaced with serum-free, modified Eagle's Minimum Essential Media (at pH 7.4 and containing 2.5 mg/ml bovine serum albumin) for 7–10 d. The indicated peptide hormones and proteins were added to each flask, to a final volume of 3 ml of serum-free media. After 18 h incubation, the cells were pulsed with [^{3}H]thymidine, 2 μCi/ml, for 30 min at 37°C. The cells were then solubilized in 0.1% sodium dodecyl sulfate and precipitated with 10% cold trichloroacetic acid as previously described (7).

RESULTS

Characterization of insulin and MSA receptors on rat adipocytes and human fibroblasts. Human fibroblasts (28, 30) and rat adipocytes (9, 29) have separate receptors for insulin and the insulinlike growth factors exemplified by MSA. The insulin receptors can be demonstrated by the specific binding of ^{125}I-insulin and by the specific competition of unlabeled insulin. With both cell types, unlabeled insulin competed for insulin binding over the concentration range of 1–1,000 ng/ml (Fig. 1). The concentration of unlabeled insulin producing 50% inhibition of ^{125}I-insulin binding was 10 ng/ml for rat adipocytes and 5 ng/ml for human fibroblasts, in good agreement with previously reported values for these cells (9, 28).

Insulin analogues compete for ^{125}I-insulin binding in proportion to their insulinlike bioactivity with both fibroblasts and adipocytes (28, 29). Unlabeled MSA competed for ^{125}I-insulin binding, but a 500-fold higher concentration was required. This corresponds to the relative potency of this preparation of MSA in stimulating glucose oxidation in rat adipocytes (see Fig. 5A).

Rat adipocytes (31) and human fibroblasts (30) also have receptors for insulinlike growth factors, as demonstrated by specific binding of ^{125}I-MSA (Fig. 2). Unlabeled MSA competed for ^{125}I-MSA binding to rat adipocytes and human fibroblasts over the range of concentration of 10–1,000 ng/ml. Half-maximal inhibition of specific binding with ^{125}I-MSA was observed at 50 ng/ml of unlabeled MSA in fibroblasts, and 100 ng/ml in adipocytes. When unlabeled insulin was used to compete with the ^{125}I-MSA, however, greatly different patterns were observed. With human fibroblasts,

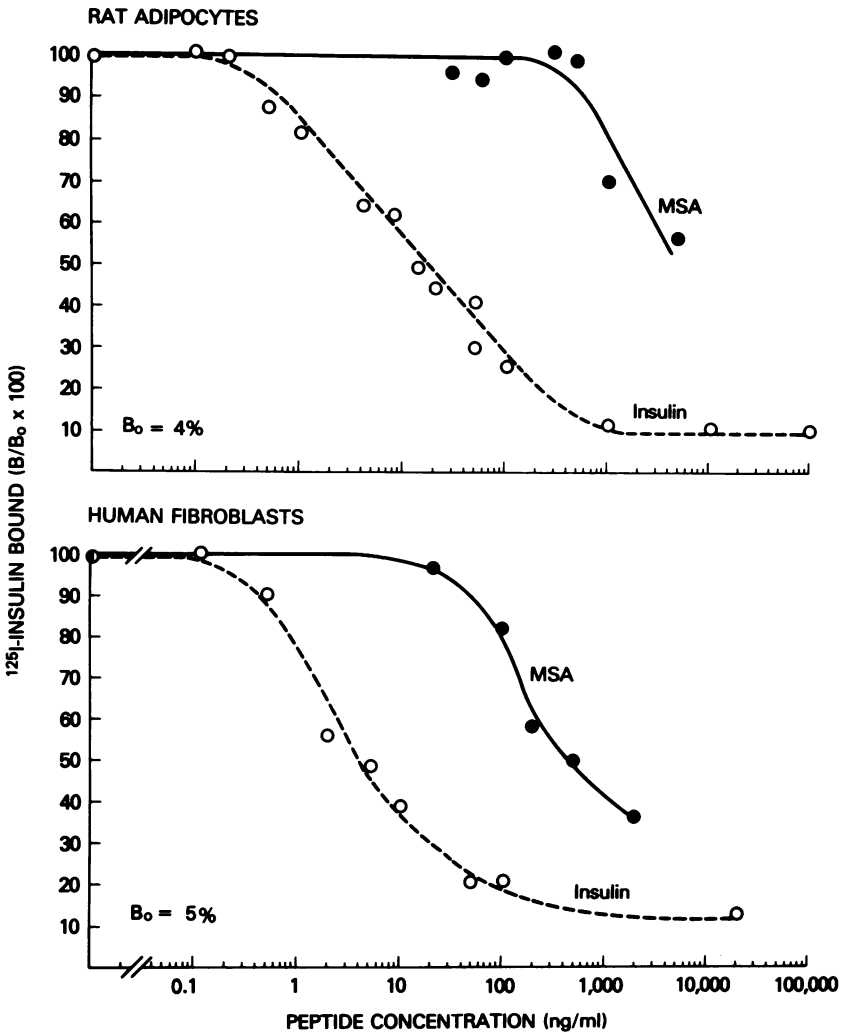

FIGURE 1 Competition by unlabeled MSA (●) and insulin (○) for ^{125}I-insulin binding to rat adipocytes (top) and human fibroblasts (bottom). Binding assays were performed in Krebs-Ringer phosphate buffer, pH 7.4, at 37°C for 20 min for adipocytes and in 0.1 M Hepes buffer for 2 h at 15°C for fibroblasts, as described in Methods. Each point represents the average of duplicate determinations. The data shown are for a representative experiment. Interassay variation is ~10%.

unlabeled insulin competed for ^{125}I-MSA binding with a potency almost equal to that of unlabeled MSA: 50% inhibition of ^{125}I-MSA binding by insulin occurred at a concentration of 40 ng/ml.[3] By contrast, with rat adipocytes, the presence of unlabeled insulin produced an increase in ^{125}I-MSA binding (see Fig. 2). This increase occurred in a dose-dependent fashion between 0.5 and 2 ng/ml of unlabeled insulin. Even with insulin concentrations as high as 10 μg/ml, there was no inhibition of ^{125}I-MSA binding to adipocytes. A similar enhancement of ^{125}I-labeled IGF-I, IGF-II, and MSA binding by insulin has been reported previously (31, 32).

Effects of Fab fragment of antireceptor antibody on ^{125}I-insulin and ^{125}I-MSA binding. Fab fragments of the antibody to the insulin receptor produced a dose-dependent inhibition of ^{125}I-insulin binding to rat adipocytes and human fibroblasts (Fig. 3). A Fab fragment concentration of 10 μg/ml (as designated by the arrows in Fig. 3) inhibited ~95% of ^{125}I-insulin binding and <5% of ^{125}I-MSA binding to rat adipocytes. Similarly, with human fibroblasts, 10 μg/ml of Fab inhibited ^{125}I-insulin binding 90%, whereas MSA binding was reduced only ~15%, indicating that the anti-

[3] Insulin is ~2% as potent as IGF-I in inhibiting ^{125}I-labeled IGF-I binding to human fibroblasts (M. M. Rechler and E. E. Schilling, unpublished results).

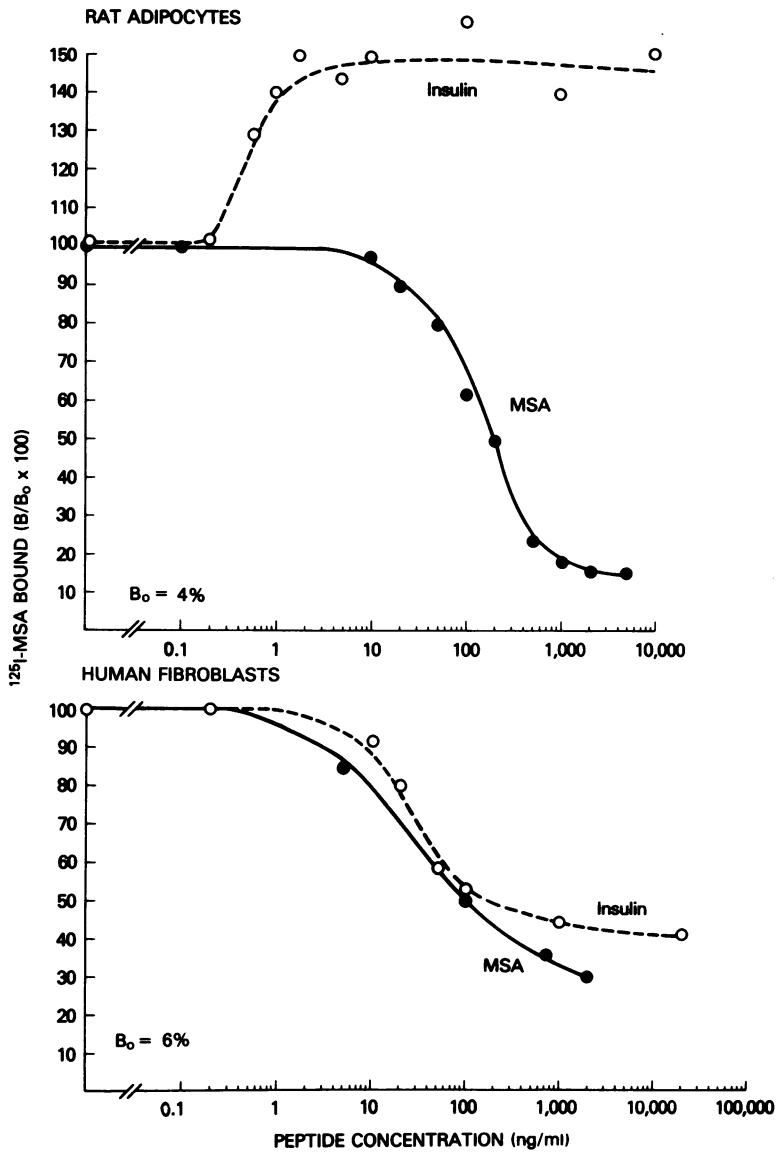

FIGURE 2 Competition by unlabeled MSA (●) and unlabeled insulin (○) for ^{125}I-MSA binding to rat adipocytes (top) and human fibroblasts (bottom). Binding assays for rat adipocytes were performed in Krebs-Ringer phosphate buffer, pH 7.4, at 24°C for 40 min, whereas human fibroblasts were assayed in 0.1 M Hepes buffer for 2 h at 15°C. Each point shows the average of duplicate determinations in one typical experiment of three. Interassay variation was ~10%.

receptor Fab does have some weak affinity for MSA receptors in human fibroblasts. Thus, at higher concentration of Fab, significant blockade of MSA binding did occur. This cross-reaction is limited to the MSA receptor of the human fibroblast (33). Using chicken embryo fibroblasts as the target cell, neither anti-insulin receptor antibody (33) or its Fab fragments inhibited MSA binding, although ^{125}I-insulin binding was inhibited (data not shown). Thus, antireceptor Fab fragments are highly selective inhibitors of insulin receptors at concentrations ≤ 10 μg/ml.

Biological effect of antibody Fab fragment and IgG to insulin receptor. As previously shown, antireceptor antibody stimulated glucose oxidation in rat adipocytes (Fig. 4A) to the same extent as insulin, although higher protein concentrations were required. The Fab fragment of the antireceptor antibody, however, had no biological effect even at 100 μg/ml, 100 times the concentration of IgG that produced a maximum biological effect (Fig. 4A).

Interestingly, in contrast to the ability of antireceptor IgG to stimulate glucose oxidation in rat adipocytes,

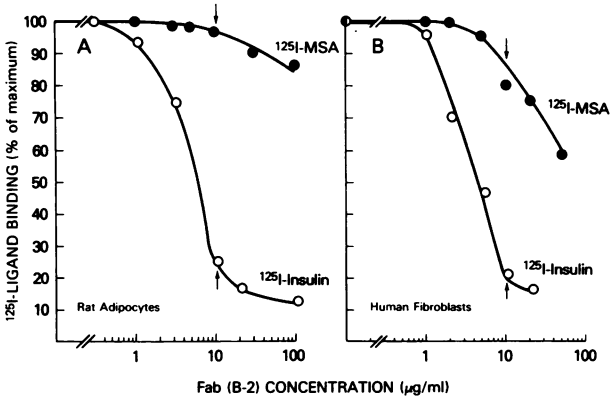

FIGURE 3 (A) Inhibition of ^{125}I-insulin (○) or ^{125}I-MSA (●) binding to rat adipocytes by Fab fragments of antireceptor antibody. ^{125}I-Insulin binding and ^{125}I-MSA binding were measured as described in the legend to Fig. 1 in the presence of the indicated concentrations of antireceptor Fab. Each point is the average of duplicate determinations in a typical experiment. (B) Inhibition of ^{125}I-insulin (○) or ^{125}I-MSA (●) binding to human fibroblasts. Assays were performed as described in the legend to Fig. 2 with 0.2 ng/ml ^{125}I-insulin or 0.25 ng/ml ^{125}I-MSA and the indicated concentrations of Fab. Arrows in both graphs indicate the highest concentration of Fab that was used in biological assays.

neither intact antireceptor IgG nor Fab fragment stimulated [^{3}H]thymidine incorporation into DNA (Fig. 4B).

Effect of Fab fragments on stimulation of glucose oxidation in rat adipocytes by insulin and MSA. Fab fragments of the antireceptor antibody can inhibit ^{125}I-insulin binding to rat adipocytes without inhibiting ^{125}I-MSA binding, and because the Fab fragments do not stimulate glucose oxidation in adipocytes, they may be used selectively to block insulin receptors and thus the effects of insulin or MSA mediated by the insulin receptor. If the effects of either peptide are mediated by the insulin receptor, inhibition or a rightward shift of the dose-response curve would result, whereas no inhibition or shift would be anticipated if the peptides use the MSA receptor.

Fig. 5A shows the dose-response curves for glucose oxidation in rat adipocytes stimulated by insulin in the presence of 0, 3, 5, and 10 μg/ml of antireceptor antibody Fab fragments. The dose-response curves for insulin were shifted to the right as the Fab concentration was increased. The maximum level of stimulation reached in each curve, however, was the same. Thus, monovalent antireceptor antibody appeared to act as a competitor to insulin action in these cells. A similar effect was seen with the dose-response curves for MSA (Fig. 5B). Again a rightward shift of the dose-response curve was seen as the cells were exposed to increasing concentrations of Fab fragments from 0–10 μg/ml.

The degree of the rightward shift of the glucose oxidation curves caused by the Fab fragments can be estimated quantitatively by the change of MSA or insulin concentration required to achieve 50% maximum stimulation. At Fab fragment concentration of 5 μg/ml, a 10-fold increase was required for both MSA and insulin. When 10-μg/ml Fab fragments were added, the shift of concentration to achieve 50% maximum stimulation is 30- and 40-fold, respectively, for insulin and MSA. Thus, the rightward shift of the glucose oxidation curves caused by the Fab fragments is nearly the same for insulin and MSA. Antireceptor Fab fragments appear to act as competitors to insulin and MSA action on adipocytes (Fig. 5). It remains possible that the maximum level of stimulation would be decreased at higher Fab concentrations, but these experiments are not feasible.

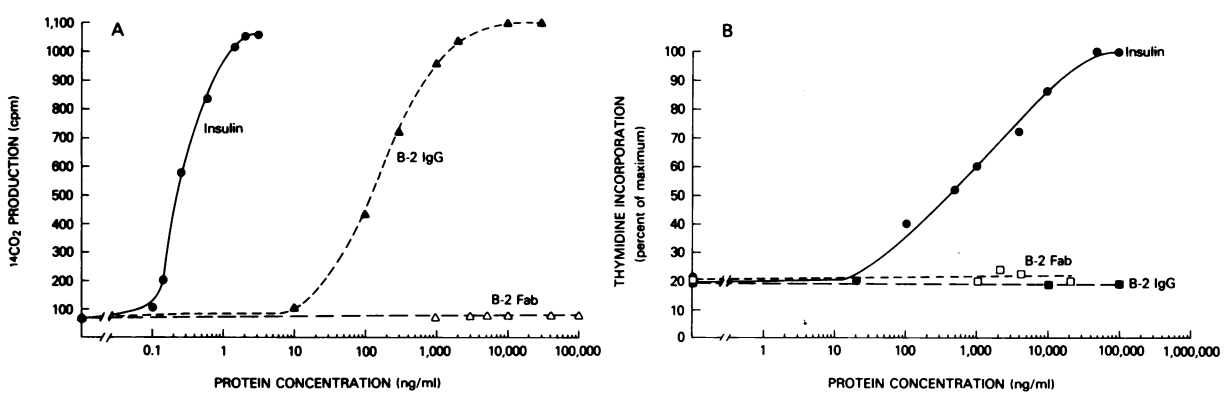

FIGURE 4 (A) Effect of insulin (●), B-2 IgG (▲), or B-2 Fab (△) on glucose oxidation by rat adipocytes. Rat epididymal fat cells were isolated as described by Rodbell (26) and glucose oxidation was measured by the conversion of [U-^{14}C]glucose to $^{14}CO_2$ for 1 h, as described in Methods. Each point shows the average of duplicates or triplicates in a typical experiment. (B) Effect of insulin (●), B-2 Fab (□), or B-2 IgG (■) on [^{3}H]thymidine incorporation in human fibroblasts. The cells were exposed to the ligands for 18 h at 37°C and for 30 min with [^{3}H]thymidine, as described in Methods. Each point is the average of duplicates in a typical experiment.

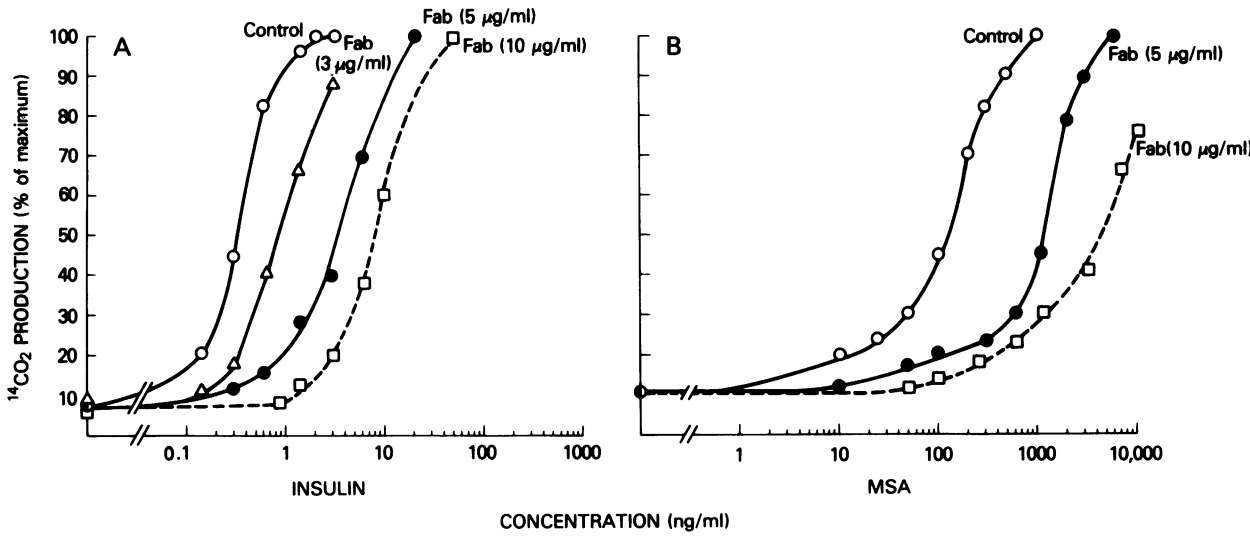

FIGURE 5 Effect of Fab B-2 on the stimulation of glucose oxidation in rat adipocytes by insulin (Fig. 5A) or MSA (Fig. B). Experimental conditions were as described in the legend to Fig. 4A with either insulin or MSA and the indicated concentrations of Fab B-2. Data are shown for no antibody control (○), 3 μg/ml (△), 5 μg/ml (●), or 10 μg/ml of Fab (□). Each point is the average of duplicates or triplicates. Data from several experiments have been combined by expressing glucose oxidation in each case as the percent of maximal insulin stimulation with control (no antibody) cells.

To explore and substantiate the specificity of the inhibition by antibody further, we studied the effect of a high concentration of Fab fragments on glucose oxidation stimulated by either vitamin K_5 or spermine. These substances are known to stimulate glucose oxidation through mechanisms not involving the insulin receptor (34, 35). The addition of antireceptor Fab (10 μg/ml) did not affect the dose-response curves for either of these agents (data not shown). Interestingly, the dose-response curve for stimulation of glucose oxidation by concanavalin A, a lectin that is known to bind to the insulin receptor but not at the insulin binding site, was shifted rightward by the addition of antiinsulin receptor Fab (Fig. 6) (36, 37). The rightward shift produced by the antibody, however, was less than that observed for insulin and MSA.

[^3H]Thymidine incorporation into DNA. [^{3}H]Thymidine incorporation into DNA of cultured human fibroblasts has been considered an example of the growth-stimulating action of insulin and MSA. To see whether the blockade of the insulin receptor would affect this biological response, Fab fragments of antireceptor antibody were added to fibroblasts, and dose-response curves were determined for stimulation of [^{3}H]thymidine incorporation into DNA. Addition of 0, 4, and 10 μg/ml Fab had no significant effect on the dose-response curves for [^{3}H]thymidine incorporation by insulin or MSA (Fig. 7).

To substantiate this finding further, a second variation of this experiment was performed. The insulin or MSA concentration was kept at a constant concentration capable of stimulating thymidine incorporation by 50–70% of maximum stimulation by MSA or insulin and the concentration of antireceptor Fab fragment was varied (Fig. 8); again, no effect of antibody at concentrations up to 20 μg/ml was observed with either MSA or insulin stimulation of thymidine incorporation, although at a concentration of 20 μg/ml,

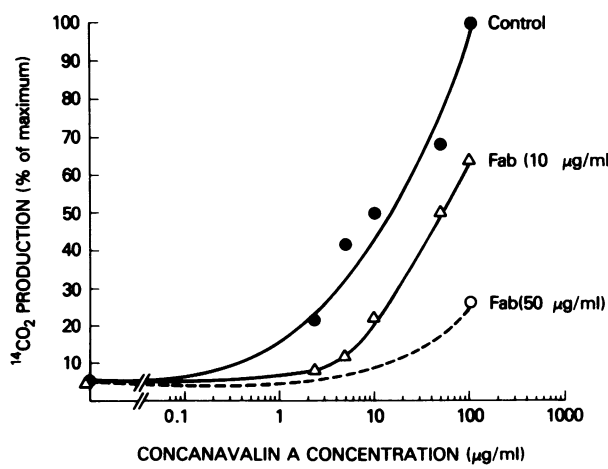

FIGURE 6 Effect of antireceptor Fab on glucose oxidation stimulated by concanavalin A. Experimental conditions were as described in the legend to Fig. 5, except that concanavalin A and Fab (B-2) were added at the indicated concentrations. Each point is an average of duplicates of a typical experiment.

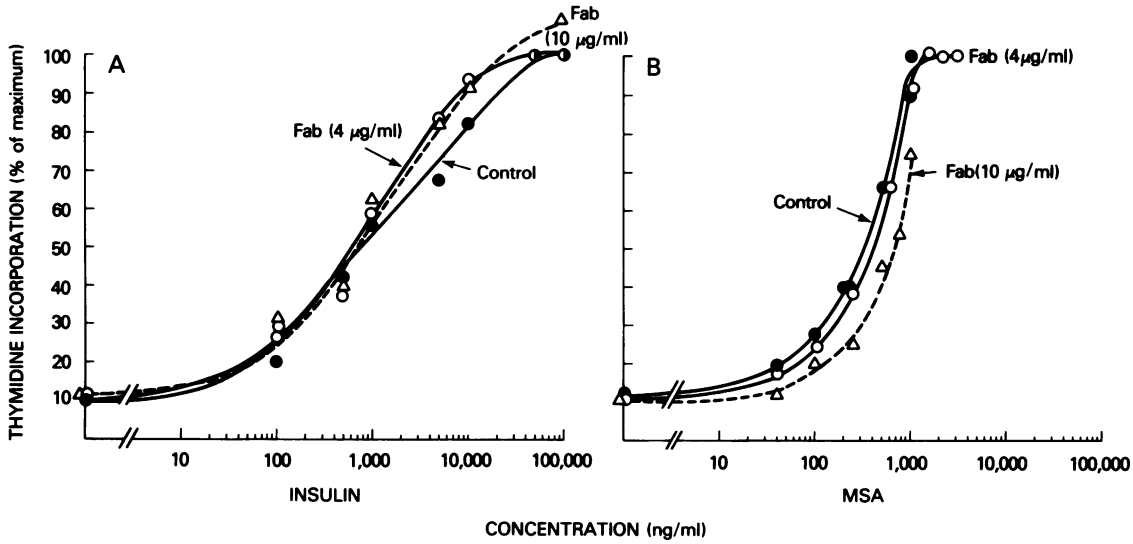

FIGURE 7 Effect of antireceptor Fab (B-2) on [^{3}H]thymidine incorporation stimulated by either insulin (Fig. 7A) or MSA (Fig. 7B) in human fibroblasts. Thymidine incorporation into DNA was measured as described in Methods using the indicated concentrations of insulin or MSA. Each point is an average of triplicates of a typical experiment.

Fab fragment can inhibit ^{125}I-insulin binding in human fibroblasts by 95% or more. Incubation of Fab with human fibroblast monolayers for up to 20 h did not decrease the ability of Fab to inhibit ^{125}I-insulin binding.

Since anti-insulin receptor antibody did not stimulate [^{3}H]thymidine incorporation, and since Fab fragments of anti-insulin receptor antibody did not inhibit insulin- or MSA-stimulated [^{3}H]thymidine incorporation into the DNA of human fibroblasts, it needed to be demonstrated that antireceptor antibody was functional under the experimental conditions. As shown in Table I, antireceptor IgG, insulin, MSA, and fetal bovine serum all increased total protein concentration, whereas antireceptor Fab and control IgG did not. These data indicate that antireceptor antibody is binding to and stimulating an increase of protein in human fibroblasts under experimental conditions for [^{3}H]-thymidine incorporation.

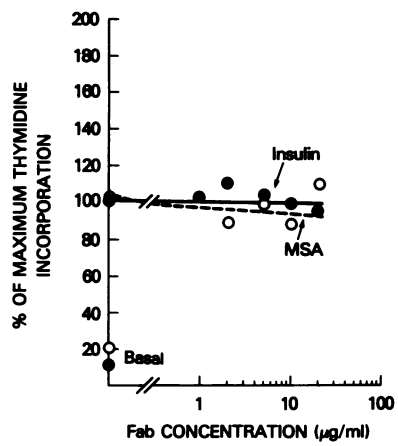

FIGURE 8 Effect of increasing concentration of antireceptor Fab (B-2) on thymidine incorporation stimulated by insulin (●) and MSA (○). [^{3}H]Thymidine incorporation into human fibroblasts was measured as described in Methods using a constant concentration of either insulin (2 μg/ml) or MSA (750 ng/ml) while varying the concentration of antireceptor Fab (B-2). "Basal" indicates the level of thymidine incorporation without addition of insulin or MSA. Each point is an average of triplicates in a typical experiment.

TABLE I
Effect of Various Additives on Protein Content of Human Fibroblasts in Culture

Additions	Number	Total protein (mean±SEM)
		μg/ml
None	8	142±8
Fetal bovine serum (20%)	5	220±18
Insulin (10 μg/ml)	5	174±8
MSA (1 μg/ml)	2	170
Anti-receptor IgG (25 μg/ml)	4	169±10
Control IgG (25 μg/ml)	2	148±11
Anti-receptor Fab (20 μg/ml)	3	140±20

Conditions were identical to those for the thymidine incorporation studies described in Figs. 7 and 8. After the assay, human fibroblasts were solubilized in 0.1% sodium dodecyl sulfate and protein concentration was determined by the method of Lowry et al. (25).

DISCUSSION

Insulin and insulinlike growth factors I and II are structurally similar but immunologically distinct polypeptides that share many common metabolic effects and growth effects (1–10). However, the relative potencies of the two polypeptides for these biological activities are different. For most of the metabolic effects, insulin is 50- to 500-fold more potent than the IGF, whereas the situation is reversed for growth effects (1, 9).

Specific receptors in many cells for insulin and IGF have been reported (1–3, 13, 14, 33). Previous studies had shown that the insulinlike growth factors have weak affinity for the insulin receptor and inhibit insulin binding in proportion to their insulinlike activity (1, 13, 14, 16). Similarly, insulin has a definite affinity for the insulinlike growth factor receptors in many cells (1, 14, 15, 20, 30). These data suggested to us and others that the metabolic effects shared by insulin and insulinlike growth factors may be mediated by the insulin receptor, whereas the growth effects may be mediated by insulinlike growth factor receptors (7, 13, 15, 16, 38). This hypothesis, however, could not be tested without a specific inhibitor for one of the receptors, and it remained a possibility that both receptors were coupled to both types of biological responses. In the present study, we have directly studied this question by taking advantage of Fab fragments of antibodies to the insulin receptor as a specific inhibitor of insulin receptor function.

Two test systems, rat adipocytes and cultured human fibroblasts, were used. Both of these cells have separate receptors for insulin and the insulinlike growth factors, as demonstrated by MSA and insulin binding. MSA is a well-characterized insulinlike growth factor that is similar to human insulinlike growth factor and somatomedins (3, 4, 8, 33, 38). Like the other insulinlike growth factors, MSA has a weak affinity for the insulin receptors on rat adipocytes and human fibroblasts, which is proportional to its insulinlike potency in bioassay. Conversely, insulin has an affinity for the MSA receptor in most cells; and in the human fibroblast, the two peptides compete almost equally for ^{125}I-MSA binding. With the rat adipocyte, however, insulin does not inhibit MSA binding at all. In fact, it actually increases it. This unusual effect of insulin has also been seen with insulinlike growth factor binding to adipocytes (31, 32). Studies on both the mechanism and physiological implication of this finding are in progress. However, it is interesting to note that this effect is observed with physiological concentrations of insulin. Preliminary studies indicate that insulin receptors are involved in this potentiation effect (39).

To inhibit insulin receptor function specifically, we chose to use antibodies to the insulin receptor. These antibodies are IgG in nature and derived from patients with a rare form of insulin-resistant diabetes (17, 23). We have previously shown that these antibodies bind to the insulin receptor (40), block insulin binding, and can immunoprecipitate solubilized receptors (18, 40, 41). This occurs without effect on the binding of other peptide hormones, including glucagon, growth hormone, and epidermal growth factor (41).

The data of the present study provide further evidence both for the specificity and actions of the antireceptor antibody, and for the fact that the receptors for insulin and the insulinlike growth factors are distinct. In these studies, we have shown that the antireceptor antibodies will almost completely inhibit insulin binding to adipocytes, with little effect on MSA binding. Similar results have also been obtained with rat liver and chick embryo fibroblasts (33). Harrison et al. (41, 42) have shown that the antireceptor antibody will quantitatively immunoprecipitate solubilized insulin receptors from human placenta with no precipitation of MSA receptors (41, 42). In human fibroblasts, the antireceptor antibodies also inhibit insulin binding, but some inhibition of MSA binding is observed. Whether this is due to immunologic similarities between the insulin and MSA receptors on human fibroblasts, or to the presence of a second anti-MSA receptor antibody (which is both tissue and species specific) is uncertain. With both adipocytes and fibroblasts, however, it is possible to choose an antibody concentration that will block insulin binding by >90% with <15% inhibition of MSA binding, thus providing a probe for distinguishing which biological effects are mediated through which receptor.

For these experiments, it was essential to use monovalent antireceptor antibody, since the intact IgG is able to mimic insulin's acute and chronic metabolic effects. Thus, antireceptor IgG stimulated glucose oxidation in rat adipocytes and protein synthesis in fibroblasts. It is important to note, however, that antireceptor IgG does not have any effect on thymidine incorporation into DNA. This is the first biological action of insulin we have tested that the antibody IgG to the insulin receptor could not duplicate. Fab fragments, on the other hand, were without significant effect on either biological response.

As one would predict, addition of the Fab fragment of the antibody to the insulin receptor as a specific inhibitor of the insulin receptor caused the dose-response curve for insulin stimulation of glucose oxidation in rat epididymal adipocytes to shift toward the right, consistent with competitive inhibition of insulin action at the receptor level. A similar qualitative and quantitative shift was observed when MSA was used instead of insulin, directly demonstrating that the acute metabolic actions of insulin and MSA are mediated

by the insulin receptors. This effect of the Fab was specific to those ligands acting through the insulin receptor; thus, vitamin K_5 and spermine, which stimulate glucose oxidation but not via the insulin receptor, were unaffected (34, 35). The effect of concanavalin A on glucose oxidation was blocked by Fab, but for any given Fab concentration, the rightward shift of the concanavalin A dose-response curve was less than that observed with insulin and MSA. This suggests that concanavalin A exerts its insulinlike effect by binding to the insulin receptor but not to the same site as insulin, MSA, or antireceptor antibody.

In contrast, antireceptor antibody Fab fragments did not interfere with the ability of either insulin or MSA to stimulate thymidine incorporation into human fibroblasts. This is strong evidence that neither insulin's nor MSA's ability to stimulate growth is mediated by the insulin receptor. More likely, both peptides exert their effect on growth through a receptor for MSA or another insulinlike growth factor. These data do not rule out the possibility that MSA and insulin may have different postreceptor mechanisms.

The data presented provide direct evidence that the insulin receptor mediates the metabolic effects of insulin and the insulinlike growth factors, whereas the growth effect of both is mediated by a different growth receptor. Since most of the metabolic effects shared by insulin and the insulinlike growth factors occur rapidly, the question remains, however, whether the chronic metabolic effects, such as enzyme induction, are mediated by the insulin receptor or the growth receptor. Recently Van Obberghen et al. (43) demonstrated that the insulin anti-receptor antibody can induce lipoprotein lipase, which indicated that the insulin receptor is capable of mediating not only rapid metabolic effects, but also chronic metabolic effects. Additional testing with Fab of other biological activities of insulin and MSA are in progress and should help further to define this concept.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Beverly Knight for her excellent secretarial assistance.

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