Characterization of the Binding of Multiplication-stimulating Activity to a Receptor for Growth Polypeptides in Chick Embryo Fibroblasts*

(Received for publication, July 19, 1976, and in revised form, January 13, 1977)

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Multiplication-stimulating activity (MSA) is the name given to a family of polypeptides with a molecular weight of approximately 10,000 that have been isolated from the culture medium of a rat liver cell line. MSA stimulates growth and DNA synthesis in chick embryo fibroblasts and has weak insulin-like metabolic activity. We have purified MSA and characterized the binding of 125I-labeled MSA to specific receptors in chicken fibroblasts. Binding was rapid and reversible. Competitive binding data obtained at steady state and plotted according to Scatchard were linear. A Kd of 4.2 x 10^-8 M^-1 and a binding capacity of 58,000 MSA molecules/cell were calculated. Only unlabeled MSA or closely related polypeptides competed for the binding of MSA tracer. MSA preparations at different stages of purification inhibited binding in accordance with their relative activities as stimulators of chicken fibroblast DNA synthesis. NSILA-s (non-suppressible insulin-like activity soluble in acid ethanol), a polypeptide purified from human plasma with chemical and biological properties similar to those of MSA, potently inhibited the binding of 125I-labeled MSA to chicken fibroblasts. Insulin and proinsulin were 60 and 25% as potent as MSA by weight, respectively, as inhibitors of MSA binding and DNA synthesis in chick embryo fibroblasts and has weak insulin-like activity, measured by in vitro glucose oxidation in adipose tissue (1) and by competition for insulin binding to specific insulin receptors (5, 6). MSA shares this constellation of properties with some polypeptides that have been purified from human plasma, the somatomedins and NSILA-s (7-10).

Like insulin, MSA is presumed to act by binding to specific cell surface receptors (11). The binding of purified MSA, labeled with 125I, has been reported in plasma membranes from rat liver (12-14) and in chicken fibroblasts (14). The binding sites for MSA in the two tissues appeared to have different specificities. In rat liver membranes, insulin was <10^-4 as potent as MSA in competing for MSA tracer binding, whereas in chicken fibroblasts MSA and insulin were nearly equipotent (14). We have suggested that the binding site for MSA in chicken fibroblasts is a specific "growth" receptor, and that the growth-promoting effects of MSA and insulin are mediated by this receptor (14). We now present a detailed characterization of the binding of MSA to chicken fibroblasts. In particular, we have defined the spectrum of insulin-like and growth polypeptides capable of interacting with the MSA receptor, in order to correlate binding with growth stimulating activity.

EXPERIMENTAL PROCEDURES

Materials - Carrier-free Na125I was obtained from Amersham/Searle, trypsin from Nutritional Biochemicals Corp., and soybean trypsin inhibitor from Sigma. Crystalline zinc porcine insulin (25 units/mg) was purchased from Eli Lilly. The following polypeptides were generous gifts: porcine proinsulin (Lots 615-1112B-278 and 615-984B-99C), R. E. Chance, Eli Lilly and Co.; guinea pig insulin, C. C. Yip, Banting and Best Research Institute; bovine desalane-desasparagine insulin, F. H. Carpenter, University of California, Berkeley; glucagon, M. Rodbell, National Institutes of Health; human growth hormone, A. E. Wilhelmi, Emory, and the National Pituitary Agency; S-sulfonated insulin B chain, Mann Research Laboratories;

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2.5 S nerve growth factor purified from male mouse submaxillary glands (15), A. Liuzzi, National Institutes of Health; epidermal growth factor (16), R. Ledda, Hershey Medical Center; fibroblast growth factor (17), D. Gospodorowicz, The Salk Institute.

NSILA-s purified from pooled human plasma (7) was a kind gift of F. R. Freycht and R. F. Hummel, University of Zurich. The NSILA-A preparations used in this study had insulin-like activities of 2.7, 8, and 31 milliunits/mg, measured as stimulation of glucose oxidation in rat adipose tissue in vitro. These compared with a specific activity of 370 milliunits/mg for the most highly purified preparations of NSILA-s that have been described (7, 8). The 2.7 milliunits/mg NSILA-A preparation contained less than 0.02% immunoreactive insulin and proinsulin by weight (15).

Purification of MSA—We have purified MSA from serum-free culture medium conditioned (1) by a Buffalo rat liver cell line, DRL 3A. This cell line had been originated by H. Coon (18). The conditioned medium was chromatographed on Dowex as described by Duiaak and Temin (1), dialysed against 2% (v/v) acetic acid, and lyophilized. The Dowex MSA, purified approximately 8-fold, then was applied to a Sephadex G-50 (fine) column equilibrated with 1 M acetic acid (21). Most of the protein emerged in the excluded volume, while MSA appeared in two peaks, designated Peak I (closer to the void) and Peak II (21). Sephadex Peaks I and II both had approximately 10 times the specific activity in the chicken fibroblast thymidine incorporation bioassay of Dowex MSA, but differed from each other in mobility on acrylamide gel electrophoresis (21). No interconversion of Peaks I and II was observed on rechromatography.

Peak I was purified further by rechromatography on Sephadex G-50 in 1 M acetic acid. Fractions were analyzed by disc electrophoresis at pH 2.7, in 9 M urea (21). The purest fractions, i.e., those showing two closely migrating protein bands, both of which had activity in the thymidine incorporation assay, were used in the binding experiments. Gel filtration of these MSA fractions on a 6% agarose column in 6 M guanidine hydrochloride (22) gave only a single protein peak. The specific activity of purified MSA (relative to the same lot of pure insulin) in the chicken fibroblast thymidine incorporation assay and the fold purification over conditioned media, were similar to those reported by Smith and Temin (4). The insulin-like activity of our purified MSA preparation was 20 milliunits/mg in a glucose oxidation assay in rat adipocytes in vitro (kindly determined by K. Baird). Maximum contamination by insulin and proinsulin was 0.02% (g/g) by radioimmunoassay (kindly performed by C. M. Hendricks, National Institutes of Health).

Inactivation of MSA by Reduction and Carboxymethylation—Peak I Sephadex MSA, 400 µg, was reduced with dithiothreitol and carboxymethylated with recrystallized iodoacetic acid as described by Hirs (23). The incubation mixture was applied to a Sephadex G-25 column equilibrated with 1 M acetic acid, and the carboxymethylated MSA was recovered in the excluded fractions. Carboxymethylated MSA had <0.1% of the biological activity of the untreated preparation in stimulating [3H]thymidine incorporation in chick embryo fibroblasts.

Iodination of MSA—Purified MSA was labeled with Na125I by a modification of the chloramine T method (24). The iodination mixture typically contained 6 µg of MSA in 20 µl of 0.3 M potassium phosphate, pH 7.4, and 1.5 to 2 µCi of Na125I. Freshly dissolved chloramine T, 120 µg/ml, was added in successive 10-µl aliquots. The reaction was monitored by the incorporation of radioactivity into trichloroacetic acid-precipitable material. When the incorporation had reached a plateau, usually after two additions of chloramine T, the reaction was stopped with 1 ml of 0.3 M phosphate buffer containing 1 mg/ml of bovine serum albumin. The mixture was immediately applied to a column of Sephadex G-50 (fine) (50 x 1 cm) equilibrated with 1 M acetic acid containing 1 mg/ml of bovine serum albumin at 4°C. Fractions of 1 ml were collected. 125I-labeled MSA was clearly resolved from 125I-labeled albumin and free iodine. Fractions in the 125I-labeled MSA peak were screened for their ability to bind to the MSA receptors of rat liver membranes (14). The fraction giving the highest total binding, specific/nonspecific binding ratio, and sensitivity was selected for use in binding experiments. The radioiodinated MSA was distributed in plastic tubes in 50-µl aliquots and stored at -20°C. It was stable for approximately 4 weeks. The preparations used had specific activities of 60 to 335 Ci/mg (220 Ci/mg being equivalent to 1 atom of 125I x 10,000 daltons).

125I-labeled MSA co-migrated with unlabeled MSA as a single peak during gel filtration on Sephadex G-50 in 1 M acetic acid (Fig. 1A) and during disc electrophoresis at pH 2.7 (Fig. 2). Despite those indications that the 125I-labeled MSA was physically intact, we were unable to bind more than 50% of the MSA tracer to any MSA receptors, including the high capacity rat liver membrane MSA receptors (14) at high membrane concentrations. The cumulative binding of an aliquot of 125I-labeled MSA, following serial incubations with fresh aliquots of liver membranes, likewise did not exceed 30% (Fig. 3). Specific binding declined as much as 50% between the first and second incubations, and decreased still further in the third and fourth incubations (Fig. 3). This reduction in 125I-labeled MSA binding required the presence of membranes (Fig. 3). Although these results could be due to degradation of the tracer during incubation with liver membranes, it is more likely that they indicate that approximately 70% of the MSA tracer was incapable of being bound.

\[\text{Fig. 1. A, gel filtration of } 125\text{-labeled MSA. A, } 125\text{-labeled MSA was chromatographed on Sephadex G-50 (fine) in } 1 \text{ M acetic acid containing } 1 \text{ mg/ml of bovine serum albumin as described under "Methods." The radioactivity in the individual fractions is plotted. In separate gel filtrations on the same column, the peak of the excluded volume (marked by blue dextran) was in Fraction 15, and the salt peak in Fractions 36 and 37. Several weeks after the iodination of MSA, ~5% of the radioactivity appeared in the void volume (presumably representing aggregates) and ~10% as free iodide. B, gel filtration of eluted } 125\text{-labeled MSA. } 125\text{-labeled MSA was bound to chicken fibroblasts under standard binding assay conditions. Approximately 80% of the total binding was specific. The cell-associated radioactivity was ex-}

\[\text{pected that this represents authentic insulin or proinsulin. The BRL 3A cells were grown for many generations in the absence of serum, making residual contaminating hormone un-}

\[\text{likely, and it is doubtful that the liver cells synthesize insulin } \text{de novo. A more probable explanation would be the release of insulin-}

\[\text{specific proteases into the culture medium. Liver is rich in insulin-degrading activity. Degradation of the insulin tracer would reduce binding to the antibody, and would be indistinguishable from com-}

\[\text{petition by unlabelled insulin.}
per se did not inactivate MSA.

tracer.

were used for binding studies 3 to 5 days after plating. tryptose phosphate broth (1)) containing 10% fetal calf serum and

sheet was solubilized with 2.5 ml of 0.1% sodium dodecyl sulfate, and

the radioactivity in duplicate 1-ml aliquots determined. In control

was at a density of 3.3 x 10^7 cells/l00-mm dish in 10 ml of ET medium

addition of an equal volume of 0.1% soybean trypsin inhibitor. The

from the cell monolayer by incubating with 0.05% trypsin, 0.5 mM

experiments, MSA was bound to confluent tertiary cultures of

day old chick embryo carcass was minced and digested with trypsin.

previously described (19). A 12-day old chick embryo curcass was minced and digested with trypsin. The released cells were dispersed and plated in 100-mm dishes in ET medium (Temin's modified Eagle's medium with 20% by volume trypsin, 0.5 mM EDTA alone bound 80% as much Y-labeled MSA as cells

suspended with EDTA + trypsin. Mechanical scraping after washing

for fibroblasts in situ (Fig. 5) and for fibroblasts suspended by trypsin

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Growth of Chick Embryo Fibroblasts for Binding Experiments— Chick embryo fibroblasts were prepared for binding experiments as previously described for thymidine incorporation studies (19). A 12-day old chick embryo curcass was minced and digested with trypsin. The released cells were dispersed and plated in 100-mm dishes in ET medium (Temin's modified Eagle's medium with 20% by volume trypsin, 0.5 mM EDTA alone bound 80% as much Y-labeled MSA as cells

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Binding of \( ^{125}\)I-labeled MSA to Chicken Fibroblasts in Suspension—The standard binding assay mixture included: \( ^{125}\)I-labeled MSA, 250 pg; 1 to 2 \( \times 10^6 \) chicken fibroblasts in suspension; unlabeled polypeptides as indicated; in a total volume of 0.5 ml of Hepes binding buffer. The incubation was conducted in plastic test tubes at 22°C for 3 h. Over 80% of the fibroblasts were viable at the end of the incubation as judged by trypan blue exclusion. To determine the tracer radioactivity bound to chicken fibroblasts, duplicate 0.2-ml aliquots of the cell suspension were layered over 0.2 ml of cold Hepes binding buffer and centrifuged for 1 min in a Beckman model 152 microfuge. The radioactivity in the cell pellet was determined in a Nuclear-Chicago Autogamma counter at 85% efficiency.

Total binding is the percentage of tracer radioactivity bound in the absence of unlabeled MSA. Nonspecific binding is defined as the radioactivity bound in the presence of an excess of unlabeled MSA (1 \( \mu \)g/ml). Specific binding is the difference between total and nonspecific binding. In general, 80% of the total \( ^{125}\)I-labeled MSA bound to chicken fibroblasts at steady state was specific. The specific binding/10^5 cells decreased with increasing cell concentration (Table I).

Elution of Bound MSA—\( ^{125}\)I-labeled MSA was bound to chicken fibroblasts under standard assay conditions. The cells were collected by centrifugation, and the pellets washed with Hepes binding buffer and the procedure of Terris and Steiner (25). The pellet was resuspended in 0.2 ml of 0.1% trypsin, 0.5 mM EDTA (for the indicated times). Trypsinization was terminated by the addition of an equal volume of 0.1% soybean trypsin inhibitor (see Methods). The suspended cells from each dish were collected by centrifugation, washed with Hepes binding buffer. The incubation was conducted in plastic test tubes at 22°C for 3 h. Over 80% of the initial radioactivity was extracted.

Insulin Binding to Chicken Fibroblasts—The binding of \( ^{125}\)I-insulin to chick embryo fibroblasts was performed as described for cultured human fibroblasts (26), except that Hepes binding buffer, pH 8.0, was replaced with Buffer A (75 mM TrisHCl, pH 7.5, 10 mM glucose; 30 mM NaCl; 1 mg/ml of bovine serum albumin) (27). \( ^{125}\)I-labeled porcine insulin (150 to 230 Ci/mg, 50 pg), unlabeled polypeptides as indicated, and 2 to 4 \( \times 10^5 \) fibroblasts in Buffer A were incubated in a total volume of 0.5 ml for 2 h at 15°C. MSA Binding to Rat Liver Membranes—\( ^{125}\)I-labeled MSA, 300 to 500 \( \mu \)g/ml, was incubated with highly purified rat liver plasma membranes (generously provided by D. M. Neville, Jr., National Institutes of Health) in 0.4-ml microfuge tubes at 22°C (13, 14). The incubation medium consisted of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg/ml of bovine serum albumin and supplemented with unlabeled peptides as specified in a final volume of 0.15 ml. After 90 min, duplicate tubes were centrifuged in a Beckman microfuge, and the radioactivity in the membrane pellets determined.

Results

Specificity of MSA Binding—The binding of MSA to chick embryo fibroblasts was highly specific. MSA preparations at different stages of purification competed for \( ^{125}\)I-labeled MSA binding in proportion to their potencies in the chicken fibroblast thymidine incorporation assay (Fig. 6). Peak I and
Peak II Sephadex MSA had equal specific activities in the thymidine incorporation assay and competed equally well for tracer MSA binding. Dowex MSA had an approximately 10-fold lower specific activity in the thymidine incorporation assay than did Sephadex MSA and competed for the binding of tracer MSA with approximately 10-fold lower potency. Sephadex Peak I MSA that had been reduced and carboxymethylated was virtually inactive in the thymidine incorporation assay, and did not compete for \(^{131}I\)-labeled MSA binding. High concentrations of unrelated polypeptide hormones and growth factors failed to compete for \(^{125}I\)-labeled MSA binding. The polypeptides tested included: human growth hormone, glucagon, insulin B chain, and nerve growth factor at 10,000 ng/ml; epidermal growth factor, 600 ng/ml; and fibroblast growth factor, 200 ng/ml.

**Kinetics of MSA Binding** — The rate of association of \(^{125}I\)-labeled MSA with chicken fibroblasts increased with temperature (Fig. 7). Half-maximal specific binding was observed in 240 min at 4°, in 80 min at 15°, in 45 min at 22°, and in 10 min at 37°. At 22°, a plateau of binding occurred between 3 and 6 h. Steady state conditions, 3-h incubation at 22°, were adopted for the standard binding assay. The maximum total and specific binding of \(^{125}I\)-labeled MSA to chicken fibroblasts also was greater at lower temperatures (4°, 15°, and 22°), than at 37° (Fig. 7).

Binding of MSA to chicken fibroblasts was reversible. Dissociation of bound \(^{125}I\)-labeled MSA was observed after addition of unlabeled MSA at steady state (Fig. 7) or after dilution (Fig. 8). The dissociation rate was not first order. It was more rapid at 37° (75% dissociated in 6 h) than at 22° (50% dissociated in 6 h) (Fig. 8). The \(^{125}I\)-labeled MSA released at both temperatures was physically intact by gel filtration (data not shown).

**pH Dependence of MSA Binding** — MSA binding to chicken fibroblasts was examined over the pH range 6.0 to 9.1 (Fig. 9). Specific binding was maximal at pH 7.5 to 8.0. It was reduced by 50% at pH 6.6, and by 30% at pH 8.3, but was relatively stable at more alkaline pH values. Phosphate buffer inhibited MSA binding: specific binding at pH 7.0 and 7.5 in phosphate buffer was less than 40% of that observed in Hepes buffer. Binding experiments routinely were performed in Hepes buffer at pH 8.0.

**MSA Degradation** — The \(^{125}I\)-labeled MSA remaining in the supernatant medium after incubation with chicken fibroblasts for 3 h at 22° was examined by gel filtration on Sephadex G-50 in 1 M acetic acid (not shown) and by acrylamide gel electrophoresis (Fig. 2). The tracer MSA appeared to be physically intact. \(^{125}I\)-labeled MSA was also undegraded after incubation with chicken fibroblasts at 37° (not shown).

**Table I**

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<th>Treatment</th>
<th>Specific Binding</th>
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<tr>
<td>Preincubation</td>
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<td>None</td>
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<tr>
<td>None</td>
<td>6</td>
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<td>Buffer, 37°</td>
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*The numbers in parentheses represent the percentage of control specific binding, i.e. binding in the absence of preincubation, for each cell concentration.

*NT, not tested.

To test the functional integrity of incubated \(^{125}I\)-labeled MSA, aliquots of the supernatant medium recovered after incubation with chicken fibroblasts were bound to fresh receptors, as previously described for insulin (28). MSA tracer that had been preincubated with fibroblasts at 22° gave 24 to 40% lower specific binding in the rebinding experiments than did unincubated tracer (Table II). These results have been normalized and are presented in the same panel as the 22° association experiment (Δ).

**Fig. 7.** Kinetics of \(^{125}I\)-labeled MSA binding at different temperatures. Chicken fibroblasts, 2 x 10⁶/ml, were incubated with (- - -) or without (○ - ○) 1 μg/ml of unlabeled MSA, and with 200 μg/ml of \(^{125}I\)-labeled MSA at 4, 15, or 37° in one experiment or with 360 pg/ml of tracer MSA at 22° in another experiment. The percentage of tracer radioactivity bound to the fibroblasts at different times is plotted.

Reversibility: in a separate experiment, tracer MSA was incubated with fibroblasts for 3 h at 22°. Total binding was 2.5% and nonspecific binding 0.6%. Unlabeled MSA, 1 μg/ml, was added (indicated by a vertical arrow) and the incubation was continued. The radioactivity remaining bound to the fibroblasts was determined at different times after addition of the unlabeled MSA. These results have been normalized and are presented in the same panel as the 22° association experiment (Δ).

**State of Bound \(^{125}I\)-labeled MSA** — Some polypeptides appear to be degraded while still bound to their receptors (26, 29, 30). To evaluate this possibility, the \(^{125}I\)-labeled MSA bound to chicken fibroblasts was eluted with 0.1% Triton X-100 containing 6 M urea and 3 M acetic acid (25) and examined by gel filtration (Fig. 1B). Over 75% of the applied radioactivity appeared in a single peak in the same elution position as MSA tracer that had not been bound to fibroblasts. Less than 5% of
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FIG. 8. Dissociation of 125I-labeled MSA from chick embryo fibroblasts by dilution at different temperatures. 125I-labeled MSA was incubated with 2.1 x 10^6 fibroblasts/ml at 22°C for 1 h (O, □) or 3 h (○, □). After 1 and 3 h, 3.15 and 4.9% of the tracer radioactivity was bound, respectively, of which 82% was bound specifically. The cells were collected by centrifugation (600 x g, 2 min) and resuspended in the same volume of Hepes binding buffer (without tracer MSA) at either 22°C (O, □) or 37°C (○, □). Cell-associated radioactivity was determined at different times after resuspension and is plotted as percentage of the maximal 125I-labeled MSA bound.

the radioactivity was in fragments smaller than MSA. Similar results were obtained with MSA that had been bound to chicken fibroblasts at 37°C (not shown). Thus, by gel filtration criteria, the 125I-labeled MSA associated with chicken fibroblasts (i.e., either bound to cell surface receptors or internalized) was intact. Sufficient quantities of 125I-labeled MSA could not be obtained by elution to perform rebinding experiments.

Receptor Degradation—In the absence of purified receptors, the stability of receptors during the binding reaction can only be studied indirectly (31). Operationally, receptors are considered to be degraded if the binding of tracer is reduced by preincubation of cells (or membranes) in the absence of tracer. The effects on MSA binding of preincubating chicken fibroblasts for 3 h at different temperatures are shown in Table I. Preincubation of 1 to 4 x 10^5 fibroblasts/ml at 22°C reduced binding by 10 to 20%. At 4°C, binding was not reduced, whereas at 37°C, it was reduced by approximately 50%. These results are consistent with a temperature-dependent loss of MSA receptors. This loss could represent true degradation, shedding to the medium, internalization, etc. and might contribute to the lower specific binding of MSA at 37°C (Fig. 7).

Equilibrium Analysis of MSA Binding Data—The inhibition of 125I-labeled MSA binding by different concentrations of unlabeled MSA under steady state conditions is shown in Fig. 10A. In 23 experiments, the specific binding of 125I-labeled MSA was inhibited 50% by 39 ± 22 ng/ml (mean ± S.D.) of MSA. The concentration giving half-maximal inhibition provides a reasonable approximation of the K_d when the sum of the concentrations of receptor and labeled ligand is much less than the apparent K_d (32, 33). These conditions are satisfied for the chicken fibroblast MSA receptor: tracer concentration (5 x 10^{-11} M) + receptor concentration (2 x 10^{-10} M) ≪ apparent K_d (2.4 x 10^{-8} M).

MSA binding to chicken fibroblast receptors was saturable at high polypeptide concentrations. When bound/free MSA was plotted against bound MSA (Fig. 10B) as described by Scatchard (34), a straight line, consistent with a single class of noninteracting binding sites, was obtained. Assuming that our MSA preparation is homogeneous and has a molecular weight of 10,000, we have calculated a K_d of 4.2 ± 1.8 x 10^{-10} M (mean ± S.D.) from the slopes of the Scatchard plots in 10 experiments, and a binding capacity (R_0) of 17,400 ± 8,500 MSA molecules/cell from the abscissa intercepts. If iodination had damaged 70% of the 125I-labeled MSA tracer so that it no longer could be bound to chicken fibroblasts (see "Methods"), the apparent R_0 would be an underestimate; K_d, however, would not be affected. After correction for possible damage to

![Graph](http://www.jbc.org/fig10.png)
the MSA tracer, a binding capacity of 58,000 molecules of MSA/cell was derived. We have attempted to determine $K_r$, $R_r$, $h_r$, and $h_d$ from the rate of association at 22° at different peptide hormone concentrations (31). The net rate of formation of MSA-receptor complex, $d[H]\,d/t$, equals $k_r[H][R] - k_d[H][R]$, where $[H]$ and $[R]$ are the concentrations of free MSA and receptor, respectively, and $k_r$ and $k_d$ are the association and dissociation rate constants. The rate equation may be integrated and expressed as:

$$\ln \frac{[HR]_{eq}}{[HR]_{eq} - [HR]} = (k_r[H] + k_d)t,$$

where $[HR]_{eq}$ is the concentration of MSA-receptor complex at equilibrium, and $[HR]$ is the concentration at time $t$. The approach to equilibrium was studied at $[H]$ from 0.8 to 5.3 ng/ml of $^{125}$I-labeled MSA in one experiment, and 1.1 ng/ml of $^{125}$I-labeled MSA and 13.4 ng/ml of MSA (3.4 ng/ml of $^{125}$I-labeled MSA + 10 ng/ml of unlabeled MSA) in a second experiment.

The fact that a maximum of 30% of $^{125}$I-labeled MSA radioactivity can be bound to MSA receptors, suggests that the remaining 70% of the $^{125}$I-labeled MSA may be damaged and hence should not be considered part of the total available $^{125}$I-labeled MSA. Let us identify the bound, free, and total $^{125}$I-labeled MSA radioactivity as $B$, $F$, and $T$ if no correction is applied, and as $B'$, $F'$, and $T'$ if we correct for the nonbindable component of the tracer. Then, $B = B'$, $T = T'$, and $F' = (T' - B') = (0.3 T - B)$. The ordinate for the Scatchard plot is $B/F = B/(0.3 T - B)$. The abscissa for the Scatchard plot is obtained by multiplying the fraction of $^{125}$I-labeled MSA bound, $B/T$ or $B'/T'$, by the concentration of MSA (comprised predominantly of unlabeled MSA, which is unaffected). Since $B/T' = B/0.3 T$, the [bound MSA] = [bound MSA]/0.3. The abscissal intercept, $R_o = R_o/0.3$. Since $B < 0.3 T$, $B/F' = B/0.3 T$, and $B/F = B/T$. Therefore,

$$K' = \frac{\Delta B'/F'}{\Delta [\text{bound MSA}]},$$

$$= \frac{\Delta B/0.3 T}{\Delta [\text{bound MSA}]},$$

$$K = \frac{K'}{0.3}.$$

Thus, correction for possible damage to $^{125}$I-labeled MSA increases $R_o$ by 1/0.3, without appreciably changing $K$.

FIG. 10. A, competition for $^{125}$I-labeled MSA binding by different concentrations of MSA. $^{125}$I-labeled MSA, 250 pg/ml, was incubated with approximately 1.3 × 10$^{-6}$/ml of chick embryo fibroblasts in the presence of different concentrations of unlabeled MSA under standard assay conditions. Nonspecific binding, 0.65%, has been subtracted. Temperatures, 22°. B, Scatchard plot of MSA competitive binding data from A. The 200-ng/ml points are subject to large errors and have not been plotted.
Insulin Analogs — Although the MSA receptors of chicken fibroblasts were similar in their reactivity with NSILA-s, they differed strikingly in their reactivity toward insulin (14, 37). Insulin at concentrations as high as 10 μg/ml inhibited 125I-labeled MSA binding to rat liver membranes negligibly, whereas it was almost as potent as MSA by weight in inhibiting MSA tracer binding to chicken fibroblasts (14). In 16 experiments in chicken fibroblasts, MSA binding was reduced 50% by 43 ± 22 ng/ml (mean ± S.D.) of MSA and by 86 ± 48 ng/ml of insulin. Comparing the relative potencies in individual experiments, insulin was 62% ± 32% as potent as MSA, with a range of 20 to 100%. This distinctive competition by insulin also was observed when 125I-labeled MSA was bound to chicken fibroblast monolayers in situ without exposure of the cells to trypsin (Fig. 5).

The ability of different insulin analogues to compete for 125I-labeled MSA binding to chicken fibroblasts next was evaluated (Fig. 12). Porcine proinsulin was approximately 50% as potent as porcine insulin in this experiment, and 39% ± 15% (S.D.) as potent in five experiments. Guinea pig insulin and desalanine-desasparagine insulin were considerably weaker. The same lots of peptides were tested as competitors for 125I-labeled insulin binding to the well characterized insulin receptor of cultured human lymphocytes (Table III). Potency in the insulin receptor assay has been shown to correlate with in vivo insulin-like biological activity (31). Proinsulin, guinea pig insulin, and desalanine-desasparagine insulin were 2 to 3% as potent as porcine insulin against the insulin receptor, and MSA and NSILA-s were 0.2 to 0.7% as potent. Thus, the specificity of the chicken fibroblast MSA receptor for different insulins and insulin-like polypeptides differed markedly from the specificity of the lymphocyte insulin receptor. In particular, MSA, NSILA-s, and proinsulin were considerably more potent competitors for MSA binding to MSA receptors than for insulin binding to insulin receptors.

125I-Insulin Binding to Chicken Fibroblasts — Although the specificity of the chicken fibroblast MSA receptor differed from the specificity of the cultured human lymphocyte insulin receptor, and although the properties of insulin receptors have been highly conserved in nature (6, 31), the possibility remained that chicken fibroblasts possessed an insulin receptor with a unique specificity, and that 125I-labeled MSA was binding to this atypical insulin receptor. To test this possibility, 125I-labeled insulin was prepared and its binding to chicken fibroblasts studied directly.

125I-Labeled insulin bound specifically to chicken fibroblasts. The binding was inhibited by relatively low concentrations of unlabeled insulin (Fig. 13, Table IV). Insulins of different species competed for insulin tracer binding in the order of their insulin-like activities in in vitro bioassays: chicken insulin (not shown) > porcine insulin > porcine proinsulin > guinea pig insulin (Fig. 13). Insulin B chain, glucagon, and human growth hormone did not inhibit 125I-labeled insulin binding. Finally, as consistently observed for insulin receptors (42), a Scatchard plot of the insulin competitive binding data obtained in chicken fibroblasts was curvilinear (Fig. 14).

\[ \text{NSILA-s binding to chicken fibroblasts.} \]

\[ \text{Competition for 125I-labeled MSA Binding by Insulin and Insulin Analogs} \]

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Growth Peptide Receptor in Chicken Fibroblasts

The data for the cultured human lymphocyte insulin receptor were obtained from Gavin et al. (38), De Meyts et al. (39), and Kosmakos. The same preparations of MSA and insulin analogues were used in the MSA and insulin competitive binding experiments. Cesareo fibroblasts. Fibroblasts, 5.6 x 10^6/ml, were incubated with 80 pg/ml of 125I-labeled insulin and the indicated concentrations of unlabeled insulin. The maximum specific binding has been plotted as 100%. The proinsulin Lot 615-984B-99C used in this experiment had a potency (by weight) relative to insulin of 0.16 against turkey erythrocyte insulin receptors (6) and 0.08 against the Balb/cf3 mouse fibroblast insulin receptors (41).

### Table III

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chick embryo fibroblasts</th>
<th>Human fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>4.5 ± 0.8*</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>MSA</td>
<td>83.4 ± 41.9</td>
<td>1010 ± 388</td>
</tr>
<tr>
<td>MSA/insulin*</td>
<td>20.8 ± 8.8</td>
<td>217 ± 164</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M. for five experiments with chicken fibroblasts and five experiments with human fibroblasts.  
* Ratio of concentrations (ng/ml) of MSA and insulin giving half-maximal inhibition in each experiment.

curved Scatchard plot has been attributed to heterogeneity of insulin receptors (31) or negatively cooperative site-site interactions among insulin receptors (39) or both.

Although the preceding results suggested that the chicken fibroblast insulin receptor was a typical insulin receptor, two important differences were noted. First, MSA competed for 125I-labeled insulin binding to chicken fibroblasts better than it competed for the binding of 125I-labeled insulin to other insulin receptors. Specifically, MSA was 5% as potent as insulin in chicken fibroblasts, contrasted with 0.5% as potent in human fibroblasts (Table IV), cultured lymphocytes (Table III), placental cells (5), rat liver membranes (6) and turkey erythrocytes (6). The specificity of MSA (60 milliunits/mg) was 0.2% that of insulin (25 units/mg) in the *in vitro* bioassay for insulin, the stimulation of glucose oxidation in adipose tissue.

A second difference between the chicken fibroblast insulin receptor and other insulin receptors was the effect of insulin on the kinetics of dissociation of bound 125I-labeled insulin. In a number of systems, including cultured human lymphocytes (39, 42), circulating human monocytes (42), cultured human fibroblasts (26), cultured human placenta cells (5), and turkey erythrocytes (6), insulin enhanced the dissociation of labeled insulin from insulin receptors following dilution. De Meyts et al. (39, 42) have interpreted these results as indicating that the affinity of unoccupied insulin receptors for insulin decreased when the fraction of insulin receptors occupied by insulin increased, *i.e.* that negatively cooperative site-site interactions occurred among insulin receptors.

We have performed the analogous dissociation kinetics experiments in chicken fibroblasts (Fig. 14). The dissociation rate following dilution in the presence and absence of 1µg/ml of insulin, a concentration sufficient to fill most of the insulin receptors, was identical. The same result was obtained when the 125I-labeled insulin tracer size was reduced to 25 pg/ml (an insulin concentration that by itself should not induce cooperativity (39), when the dissociation of nonspecifically bound insulin was subtracted, and when the dissociation was performed at 22°C instead of 15°C (data not shown). If the dilution used were insufficient to prevent rebinding of the dissociated 125I-labeled insulin, dilution in the presence of unlabeled insulin would have caused an apparent enhancement of dissociation, which was not observed. Thus, we have been unable to demonstrate any effect of insulin on the dissociation kinetics of the chicken fibroblast insulin receptor, so that the curved Scatchard plot for insulin in these cells does not appear to result from negative cooperativity.

polypeptide, MSA, to one of its target cells, the chick embryo fibroblast. The MSA receptor appears to mediate the stimulation of DNA synthesis by MSA and related polypeptides. The chicken fibroblasts - MSA, insulin, NSILA-s (Fig. 11), proinsulin (Fig. 12), and somatomedin A - stimulated thymidine incorporation in chicken fibroblasts with comparable potency (14). The binding and activity dose response curves for these polypeptides were nearly superimposable, suggesting that there are no spare receptors for closely related polypeptides such as NSILA-s and the somatomedins.

Comparison with Insulin Receptors

The temperature and pH dependence, specificity for insulin analogues, curved Scatchard plot, and negative cooperativity of insulin receptors have been highly conserved in a wide range of species, tissue types, and cultured cells (6, 31, 42). As summarized in Table III, the properties of the chicken fibroblast MSA receptor are quite distinct in most respects from those of a well characterized and representative insulin receptor, that of cultured human lymphocytes. The insulin receptor of chicken fibroblasts differs from the lymphocyte and other insulin receptors in two respects: (a) MSA competed 10 times more effectively for 125I-labeled insulin binding to chicken fibroblasts (Table IV); and (b) the rate of dissociation of insulin from chicken fibroblasts following dilution was not enhanced by diluting in the presence of excess insulin (Fig. 14). Nonetheless, the 125I-labeled MSA binding observed in chicken fibroblasts almost certainly does not represent binding to the insulin receptor.

It is unclear whether the chicken fibroblast insulin receptor differs fundamentally from other insulin receptors, or whether the observed insulin binding was a composite of 125I-labeled insulin binding predominately to MSA receptors and secondarily to insulin receptors. Several points argue against the second interpretation. First, proinsulin should have exhibited the same increased potency as MSA in inhibiting 125I-labeled insulin binding to chicken fibroblasts. It did not. Secondly, the steep slope of the Scatchard plot for insulin (Fig. 14) corresponds to a higher $K_d$ and much lower $R_0$ than those calculated for MSA (Table IV).


Comparison with Insulin Degrading Enzymes

An insulin-specific protease first described by Mirkvay (44) has been purified from rat muscle and characterized by Brush (45). Insulin degradation by a presumably similar proteolytic activity has been described in purified rat liver plasma membrane (38) and in isolated hepatocytes (46). Several considerations make it rather unlikely that the MSA binding observed in chicken fibroblasts was binding to insulinase molecules on the cell surface. First, insulin was degraded more readily at higher temperatures (37), whereas MSA bound more extensively at lower temperatures (15). Secondly, the $K_m$ for insulin degradation was high, $0.2 \times 10^{-4}$ M, compared to the $K_d$ for MSA binding, $0.24 \times 10^{-4}$ M. Thirdly, the specificity of insulin degrading enzymes differed from that of MSA binding. Whereas proinsulin (28, 45, 46) and NSILA-s (47) were potent competitive inhibitors of both insulin degradation and MSA binding, guinea pig insulin and desalamin-desasparagine insulin, poor competitors for MSA binding, were 75% and 220% as potent as insulin, respectively, in inhibiting insulin degradation (31).

Comparison of MSA and NSILA-s Receptors in Rat Liver Membranes and Chicken Fibroblasts

MSA and NSILA-s are closely related polypeptides, and MSA and NSILA-s receptors have been described in rat liver membranes and chicken fibroblasts. The MSA receptor in chicken fibroblasts differs markedly from the MSA and NSILA-s receptors in rat liver membranes. It is more similar to the NSILA-s receptor in chicken fibroblasts, but even these receptors do not appear to be identical.

Temperature Dependence—Steady state binding was maximal at lower temperatures ($4^\circ > 15^\circ > 37^\circ$) for MSA binding to chicken fibroblasts, at higher temperatures ($30^\circ > 12^\circ$) for NSILA-s binding to chicken fibroblasts (48) and at intermediate temperatures ($20^\circ > 4^\circ > 37^\circ$) for NSILA-s binding to rat liver membranes (13).

pH Dependence—The NSILA-s receptor in rat liver membranes had a broad pH optimum (pH 6 to 8) with decreased binding at more alkaline pH (13). NSILA-s binding to chicken fibroblasts had a sharp pH optimum (7.5 to 8.0), with an abrupt decrease on both the acid and alkaline sides (48). MSA binding to chicken fibroblasts had the same pH optimum and lability in acid as NSILA-s binding to chicken fibroblasts, but was more stable at alkaline pH.

Equilibrium Constant and Binding Capacity—Equilibrium constants of association of 0.4 to $1.0 \times 10^{-6}$ M$^{-1}$ were calculated for MSA and NSILA-s (48) binding to chicken fibroblasts and 0.7 to $1.0 \times 10^{-6}$ M$^{-1}$ for MSA and NSILA-s (13) binding to rat liver membranes after correcting for the purity of the NSILA-s preparation. Similarly, after correction for receptor damage by collagenase treatment (48) and for possible damage to the MSA tracer, it was calculated that chicken fibroblasts bound 58,000 molecules of MSA/cell compared with 21,000 molecules of NSILA-s/cell.

Specificity—The MSA receptor of chicken fibroblasts, on the one hand, and the MSA and NSILA-s receptors of rat liver membranes, on the other, present two distinct patterns of reactivity with insulin and its analogues. Insulin, at 10 $\mu$g/ml, did not compete for MSA or NSILA-s binding to rat liver membranes (13, 14, 37, 49); 100 $\mu$g/ml of crystalline zinc insulin, but not more purified insulin preparations, inhibited NSILA-s binding by 30% (13). By contrast, insulin completely inhibited the specific binding of $^{125}$I-labeled MSA to chicken fibroblasts, and had 62% the potency of MSA. Proinsulin did not inhibit MSA binding to rat liver membranes (12). Similarly, proinsulin was 0.1% as potent as NSILA-s in inhibiting NSILA-s binding to liver membranes; 10 $\mu$g/ml gave 50% inhibition (49). By contrast, proinsulin was 27% as potent as MSA in competing for MSA binding to chicken fibroblasts. MSA receptors. Guinea pig insulin at 1 $\mu$g/ml did not inhibit NSILA-s binding to rat liver membranes (49), but inhibited MSA binding to chicken fibroblasts by 43%.

The NSILA-s receptor of chicken fibroblasts more closely resembles the MSA receptor of chicken fibroblasts than the MSA and NSILA-s receptors of rat liver membranes. MSA was approximately 10% as potent as purified NSILA-s in competing for $^{125}$I-labeled NSILA-s binding to chicken fibroblasts. Insulin completely inhibited NSILA-s binding to chicken fibroblasts, but higher insulin concentrations (1 $\mu$g/ml) were required to inhibit NSILA-s binding by 50% (48) than to inhibit MSA binding to chicken fibroblasts (86 ng/ml). Stated differently, insulin was 10% as potent as MSA in inhibiting NSILA-s binding to chicken fibroblasts, contrasted with 60% (range 20 to 100%) as potent as MSA in inhibiting MSA binding to chicken fibroblasts. The significance of this difference is difficult to assess in view of the different sources of embryos, use of primary or tertiary cultures, different methods of preparing cells for the binding assay, different tracer polypeptides, and different assay conditions.

Both the chicken fibroblast type of MSA receptor and the rat liver membrane type of MSA/NSILA-s receptor have been identified in other cultured cells. Human fibroblast cultures, for example, have an MSA receptor that is indistinguishable from the chicken fibroblast MSA receptor in its specificity for insulin and insulin analogues. In contrast, the MSA receptor of the BRL 3A2 rat liver cell line, like the rat liver membrane receptor, was not inhibited by insulin.

Comparison with Receptors for Somatomedins and Other Growth Peptides

Somatomedins, the proposed mediators of pituitary growth hormone action, are a group of polypeptides most closely related to NSILA-s and MSA. Three different somatomedins—somatomedin A, B, and C—have been purified from human plasma (10, 50–53). In radioreceptor assays for MSA in rat liver membranes and chicken fibroblasts, purified somatomedin A was a potent inhibitor of MSA binding; somatomedin B preparations that were 50% pure were inactive. Somatomedin C preparations of high specific activity and unknown purity were 1% as potent as MSA in competing for MSA binding to rat liver membranes (13). In the reciprocal radioreceptor assays for somatomedin A in human placental membranes using $^{125}$I-labeled homogeneous somatomedin A (29, 54), MSA and somatomedin C were potent inhibitors, whereas somatomedin B was inactive. These results suggest that MSA and somatomedin A are closely related, MSA and somatomedin C may be less closely related, and MSA and somatomedin B are unrelated. Since somatomedin A, somatomedin C, and NSILA-s all were purified from human plasma, it must be asked whether these preparations have been purified sufficiently to exclude contamination by the other two polypeptides. The homogeneity of the somatomedin A preparation used in our studies has been well established. Although it possesses nonsuppressible insulin-like activity, this is almost certainly an intrinsic property of the somatomedin A molecule rather than a reflection of contamination with NSILA-s (55). Since the NSILA-s preparations that we used were less than 15% pure, it is possible that their reactivity toward MSA...
receptors resulted from contamination with somatotropin A. Against this, however, is the fact that MSA potently competed for the binding of "labeled homogeneous NSILA-s to chicken fibroblasts."

A number of other polypeptides have been reported to have potent growth promoting activity in cultured cells. These include EGF (16), NGF (15), and FGF (17). Despite the fact that NGF has extensive sequence and structural homologies with proinsulin, neither NGF, EGF, nor FGF appears closely related to MSA. They did not compete for MSA binding to chicken fibroblasts or rat liver membranes (12). Insulin and proinsulin competed weakly for NGF receptors (56, 57) and not at all for EGF receptors (30, 58). Whereas MSA, NSILA-s, and insulin stimulated thymidine incorporation nonadditively in a variety of cultured cells (14, 36) suggesting a common molecular determinants, active sites, and determinants of target organ specificity of this interesting group of polypeptides. Finally, since human fibroblasts have an MSA receptor that mirrors the chicken fibroblast receptor, our studies in chicken fibroblasts should provide a useful model for the interaction of the growth polypeptides in human plasma with human cells.

Acknowledgments—The critical comments of P. De Meyts, C. R. Kahn, A. C. Moses, R. J. Pollet, and J. Roth assisted in the preparation of the manuscript. The superb secretarial assistance of D. E. Beall is gratefully acknowledged.

Note Added in Proof—Raizada and Perdue (63) recently described a mitogen receptor in primary chick embryo fibroblasts that may be related to the MSA receptor.

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