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Insulin-like Growth Factor II Binding to Cultured Human Chondrosarcoma Cells (42065)

JANET L. COOPER AND GARY L. SMITH

School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0118

Abstract. Cultured cells originally derived from a human chondrosarcoma (A1684) were used to investigate somatomedin binding in terms of kinetics and specificity. In this study, the rat somatomedin, multiplication-stimulation activity (MSA) was utilized. While the human chondrosarcoma cells did not exhibit a mitogenic response to MSA, the rate of transport of glucose and amino acids was significantly increased. In competitive binding experiments a specific insulin-insensitive MSA receptor was identified which showed half maximal displacement of tracer at a concentration of 250 ng/ml of MSA using whole cells. This receptor had an affinity constant of $4.8 \times 10^7\ M^{-1}$. Kinetic analysis of MSA binding to membrane preparations and to Triton X-100 solubilized membranes revealed an increase in the binding affinity to $1.28 \times 10^8\ M^{-1}$ and $2.8 \times 10^8\ M^{-1}$, respectively. Of particular significance is the observation that these cells have especially high levels of MSA receptors. Determination of binding capacity revealed that these cells contain approximately 1.9×10^6 MSA receptors per cell and therefore are an excellent model system for the characterization and purification of somatomedin receptors. Affinity labeling of the MSA receptor using the chemical crosslinking reagent, disuccinimidyl suberate, confirmed that this receptor was of the type II class of somatomedin receptors and exhibited a molecular weight of 218,000 under nonreducing conditions.

The somatomedins are a family of growth hormone-dependent polypeptides which possess insulin-like activity on extraskeletal tissue, stimulate proteoglycan synthesis in cartilage, and are potent mitogens for a variety of cultured cell types (1–5). The somatomedin family consists of two classes of polypeptides which share biochemical and biological characteristics. The first is represented by insulin-like growth factor I (IGF-I) or somatomedin C (Sm-C) which have recently been shown by amino acid sequence analysis to be identical molecules (6). The second class includes insulin-like growth factor II and multiplication-stimulating activity (MSA), a rat somatomedin produced by a cultured rat liver cell line (7, 8). MSA displays extensive amino acid sequence homology to human IGF II and is considered to be the rat equivalent (9). While the two classes of somatomedins share many characteristics they differ in potency and physiological function. These differences appear to be mediated by two distinct types of specific receptors on the surface of target cells (10).

Recently, a cell line derived from a human chondrosarcoma (A1684) was reported by other investigators to contain very high levels of MSA receptors (11). This observation is of particular interest since cartilage is a normal target tissue of the somatomedins, *in vivo*. The presence of large numbers of MSA receptors on A1684 cells should provide an ideal system to study the interaction of the somatomedins with their receptor.

In the present study, we have characterized the MSA receptor on the A1684 cell line with regard to its biological activity and kinetic parameters. The results support the view that these cells offer a distinct advantage over other cell lines in investigations aimed at the purification and characterization of somatomedin receptors.

Materials and Methods. *Cell culture.* The A1684 human chondrosarcoma cell line was obtained from Dr. Joseph DeLarco of the Laboratory of Viral Carcinogenesis, National Cancer Institute. Fresh cultures were initiated every 2 months from stocks frozen in liquid nitrogen and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Cells were grown in plastic tissue culture dishes (Lux, Miles Labs.) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Biological activity of MSA. MSA was purified from serum-free medium conditioned

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by the growth of BRL-3A rat liver cells by established procedures (7–9). The MSA used in this study consisted of a single protein staining band in acid and alkaline acrylamide gel systems and corresponded to MSA polypeptide III-1 in the designation system of Moses *et al.* (8).

The rates of DNA synthesis and nutrient transport in A1684 cells were assayed in triplicate in 35-mm tissue culture dishes. Cells were plated at a density of 2.5×10^5 cells/plate in DMEM containing the indicated concentrations of serum and incubated for 3 to 5 days to allow the cells to reach a quiescent state. Cells were then stimulated by the addition of serum or purified MSA and the rates of DNA synthesis, nutrient transport, and sulfate incorporation were monitored at various times thereafter.

DNA synthesis was measured using a 1-hr pulse of [^{3}H]thymidine (0.2 μCi/ml). At the end of the pulse, cells were rinsed and the radioactivity incorporated into TCA precipitable material determined.

The rates of transport of glucose and amino acids were studied using the nonmetabolizable analogs 2-[^{3}H]deoxyglucose and α-[^{14}C]-aminoisobutyric acid (α-AIB). Cultures to be assayed were first rinsed twice with Earle's balanced salt solution (EBSS) and then incubated with 1 ml of EBSS containing 1 μCi/ml of 2-[^{3}H]deoxyglucose for 15 min or 0.5 ml of EBSS containing 1 μCi/ml of α-[^{14}C]AIB for 4 min.

The length of the pulse was determined in control experiments in order to ensure that uptake remained linear during the pulse period. At the end of the pulse period, the cultures were rinsed three times with cold PBS and cells lysed with 0.5 ml of 1% sodium dodecyl sulfate. The cell lysates were assayed for radioactivity by liquid scintillation spectrometry.

The incorporation of [^{35}S]sulfate into acid precipitable material was assayed in duplicate cultures of A1684 cells. Experimental cultures were exposed for 3-hr periods to 10 μCi/ml of [^{35}S]sulfate. At the end of the incubation period, the cultures were rinsed with cold PBS, precipitated with 10% TCA, and lysed with 1% SDS. The cell lysates were analyzed for radioactivity by liquid scintillation spectrometry. All radioactive materials were purchased from Amersham, Arlington Heights, Illinois.

^{125}I-MSA binding. Purified MSA III-1 was radioiodinated to a specific activity of approximately 100 Ci/g by the Iodogen procedure (12).

^{125}I-MSA binding to A1684 cells was performed using monolayer cultures or suspended cells removed from the plates by scraping. Cells were incubated in binding buffer (DMEM with 0.1 *M* Hepes and 10 mg/ml BSA) containing 500 pg ^{125}I-MSA in the presence or absence of various concentrations of unlabeled MSA or insulin. After incubation for 8 hr at 4°C the amount of cell associated radioactivity was determined.

The membrane preparations used in binding experiments were isolated using the procedure of Thom (13). Aliquots of 50 μg of membrane protein were dispensed into 1.5-ml polypropylene centrifuge tubes and these samples were centrifuged at 12,000*g* for 30 min in a Beckman Microfuge 12 (Beckman Instruments, Irvine, Calif.). The pellet was resuspended in 200 μl of Hepes binding buffer with ^{125}I-MSA (500 pg) in the presence or absence of unlabeled MSA. After incubation on ice, the membranes were collected by centrifugation at 12,000*g*. The resulting pellet was lysed with 1% SDS and assayed by γ-ray spectrometry. Binding of ^{125}I-MSA to solubilized membranes utilized the polyethylene glycol (PEG) precipitation assay (14). Aliquots of 50 μg of the particulate membrane preparation were dispensed in 1.5-ml conical propylene tubes and centrifuged at 12,000*g* for 30 min. The pellets were solubilized in 50 μl of 0.5% Triton X-100 (Bio-Rad Labs, Richmond, Calif.) for 50 min. The solubilized membranes were incubated in 0.2 ml of 0.1 *M* phosphate buffer (pH 7.4) containing 0.5% BSA with ^{125}I-MSA in the presence or absence of unlabeled MSA. After an incubation period of 90 min, 0.5 ml of ice-cold sodium phosphate buffer containing 0.1% BSA was added and the samples were placed on ice. An aliquot of 500 μl of sodium phosphate buffer containing 25% PEG was added and the samples were incubated for an additional 15 min on ice. The reaction mixtures were filtered under reduced pressure onto glass-fiber filters. The filters were rinsed with 3 ml of 9% PEG in 0.1 *M* Tris·HCl buffer, pH

7.4, and then transferred to gamma counter tubes for radioactivity determination.

Affinity labeling of the MSA receptor. Membrane preparations isolated as described above were dispensed (100 μg) into centrifuge tubes and centrifuged at 12,000*g* for 30 min. The pellet was resuspended in Hepes binding buffer containing BSA and ^{125}I-MSA (1.14 n*M*) in the presence or absence of unlabeled MSA. Mixtures were incubated for 3 hr at 22°C. At the end of this period, the membranes were centrifuged at 12,000*g* for 15 min and the supernatant was discarded. The membranes were resuspended in 300 μl of cold PBS and placed on ice. The crosslinking reagent disuccinimidyl suberate, dissolved in DMSO, was added to the reaction mixture over a 15-min period in four aliquots to a final concentration of 0.1 and 0.2 m*M* DSS. The reaction was terminated by the addition of 0.5 ml of Tris·HCl, pH 7.4. The membranes were centrifuged at 12,000*g* for 30 min and the pellets were lysed with 2× treatment buffer and prepared for electrophoresis.

The samples of affinity crosslinked membranes were boiled for 5 min and subjected to polyacrylamide gel electrophoresis according to the method of Laemmli (15). The gels contained 5% acrylamide, with a 100:1 acrylamide bisacrylamide ratio. After electrophoresis, the gels were fixed with 50% methanol, 10% acetic acid, and stained with 0.125% Coomassie Blue R-250 (Bio-Rad Laboratories, Richfield, Calif.), dissolved in 50% methanol, 10% acetic acid. The gels were destained with 20% methanol, 7.5% acetic acid, dried and exposed to Kodak X-OMAT XAR5 film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex lightning plus intensifying screen (E. I. DuPont, DeNemours and Co., Wilmington, Del.) at −70°C. The molecular weight standards used to determine molecular weights consisted of a mixture of proteins (Bio-Rad Laboratories) including myosin (mol wt = 200,000), β-galactosidase (mol wt = 116,500), phosphorylase B (mol wt = 92,500), and bovine serum albumin (mol wt = 66,200).

Results. The human chondrosarcoma (A1684) cells had previously been shown to enter a quiescent state upon incubation in the presence of low concentrations of calf serum. Quiescent cultures treated with calf serum showed a dose-dependent increase in DNA synthesis (Table I). The stimulation of DNA synthesis was maximal at 10% calf serum, exhibiting a 90-fold increase in [^{3}H]thymidine incorporation when compared with untreated cultures. The cultures treated with MSA showed only small increases in DNA synthesis at concentrations up to 1 μg/ml. Other peptide hormones, such as insulin and EGF, were also unable to stimulate DNA synthesis in these cells (data not shown).

Since MSA did not significantly stimulate

TABLE I. BIOLOGICAL ACTIVITY OF CALF SERUM AND MSA ON QUIESCENT A1684 CELLS

	CPM per culture ± SEM			
Addition	[^{3}H]TdR incorporation	2-[^{3}H]deoxyglucose uptake	α-[^{14}C]AIB uptake	$^{35}SO_4$ incorporation
Control unstimulated	350 ± 20	3030 ± 270	1950 ± 105	470 ± 70
Calf serum				
0.5%	1620 ± 103	4950 ± 170	2780 ± 100	
2.5%	17,720 ± 358	5300 ± 175	4470 ± 770	
5.0%	26,550 ± 990			
10.0%	31,400 ± 2740			580 ± 50
MSA				
100 ng/ml	1010 ± 95	3970 ± 110	2120 ± 120	
250 ng/ml	910 ± 85	4760 ± 215	2330 ± 120	
500 ng/ml	830 ± 110	5850 ± 105	2500 ± 50	
1 μg/ml	1740 ± 165	4439 ± 150	3325 ± 460	1200 ± 245

Note. Values presented are the means ± standard error. Uptake rates were determined at the following times after stimulation: [^{3}H]TdR, 24 hr; 2-[^{3}H]deoxyglucose, 2.5 hr; ^{14}C-αAIB, 1.5 hr; $^{35}SO_4$, 4 hr. α-[^{14}C]AIB

DNA synthesis in the A1684 cells, other biological parameters associated with somatomedin activity were analyzed. MSA had previously been shown to stimulate the transport of glucose and amino acids (16). Recent work, by other investigators, has shown that the uptake of α-AIB, can be used as an assay to determine the responsiveness of cells to somatomedin C (17). It was therefore of interest to determine if 2-deoxyglucose and AIB transport could be stimulated in the A1684 cell line by MSA.

The rate of uptake of α-AIB and 2-deoxyglucose was examined in quiescent cultures of A1684 cells. Both 2-deoxyglucose and AIB transport were stimulated by low concentrations of calf serum and MSA (Table I).

Another biological activity of the somatomedins is the stimulation of proteoglycan synthesis (5) and sulfate incorporation (4) in chondrocytes. Since chondrocytes are a normal target cell of somatomedins *in vivo,* it was of interest to determine whether MSA was capable of stimulating the incorporation of sulfate by A1684 cells. Cultures treated with 10% calf serum or MSA at a concentration of 1 μg/ml showed an increase in the amount of [^{35}S]sulfate incorporated into TCA precipitable material (Table I). These human chondrosarcoma cells seem to resemble the rat chondrosarcoma cells previously reported to be incapable of eliciting a mitogenic response to MSA, but were capable of responding with an increase in the rate of proteoglycan synthesis (5, 18).

Since our primary interest in this cell line is due to the fact they had previously been reported to contain large numbers of MSA receptors (11), we examined Sm binding using ^{125}I-MSA. The binding of MSA to its receptor was initially shown to be reversible and was time and temperature dependent (data not shown).

The ability of unlabeled MSA and insulin to compete for the binding of ^{125}I-MSA in this cell line was analyzed in competitive binding experiments. Confluent monolayer cultures of A1684 cells were incubated with 500 pg of ^{125}I-MSA in the presence or absence of unlabeled peptide added in amounts ranging from 50 ng/ml to 1000 ng/ml. The binding was performed for 8 hr at 4°C. The amount of tracer MSA bound was approximately 7.1% of total tracer added per plate. Native MSA competed for ^{125}I-MSA binding (Fig. 1), and half maximal displacement was observed at 250 ng/ml. Maximal displacement was observed at 1 μg/ml. Unlabeled insulin did not compete for ^{125}I-MSA binding. The receptor present in the human chondrosarcoma cell line appears to be an insulin-insensitive somatomedin receptor.

A competitive binding experiment was also performed using A1684 cells in suspension. The competitive binding data in Fig. 2 shows half maximal displacement of tracer MSA at 250 ng/ml of unlabeled MSA. This is in good agreement with the dose–response curves observed in stimulation of transport by MSA (not shown). In the transport studies, the concentration of MSA which was found to give half maximal stimulation was between 100 and 300 ng/ml in three separate experiments. The competitive binding data were analyzed by the method of Scatchard (19) and the results are shown in the inset of Fig. 2. The affinity of the receptor demonstrated by these studies exhibited an affinity constant

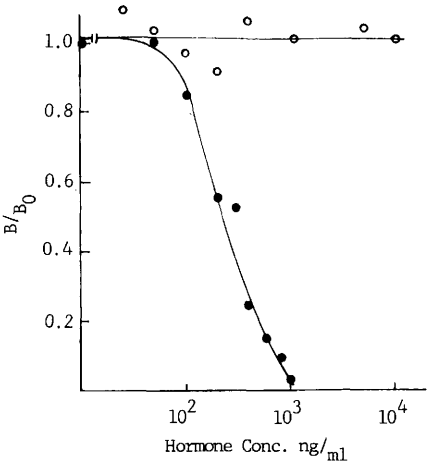

FIG. 1. Specificity of the MSA receptor. Confluent cultures of A1684 cells (2×10^6 cells per 60-mm plate) were incubated with ^{125}I-MSA (500 pg) in the presence of various concentrations of unlabeled MSA (closed circles) or insulin (open circles). Cultures were then incubated for 8 hr at 4°C. The results are plotted as B/B_0, where B represents the radioactivity when the concentration of unlabeled hormone is greater than 0 and B_0 represents the radioactivity when the concentration of unlabeled hormone equals 0.

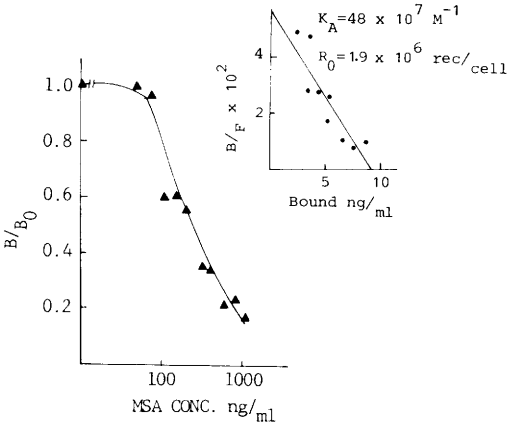

FIG. 2. Competitive binding to A1684 Cells in suspension. Confluent 100-mm cultures of A1684 cells were scraped into Hepes binding buffer and dispensed into centrifuge tubes at 4×10^5 cells per tube. These mixtures were incubated with ^{125}I-MSA (500 pg) and the indicated concentrations of unlabeled MSA. At the end of the incubation period, the tubes were centrifuged and the radioactivity in the cell pellet was determined. The results are plated at B/B_0 with the 1.0 point representing 7.1% of total tracer bound. A Scatchard analysis of this data is shown in the inset.

(K_A) of 4.8×10^7 M^{-1} with a binding capacity (R_0) of 1.9×10^6 receptors/cell. The points generated by the Scatchard analysis were slightly curvilinear (the correlation coefficient was approximately 0.855). This curvilinearity may indicate the ^{125}I-MSA is interacting with two or more distinct receptors of differing affinities.

Binding to membranes. Crude particulate membranes isolated from the A1684 cells were evaluated for their ability to bind ^{125}I-MSA. In competitive binding experiments to the particulate membranes (Fig. 3A), half maximal displacement of ^{125}I-MSA was observed at 65 ng/ml of unlabeled MSA. Maximal displacement was observed at 500 ng/ml of unlabeled MSA. The amount of total binding in this experiment represents approximately 12.8% of the total tracer added per tube. Nonspecific binding represented 5.4% of total tracer added per tube. A Scatchard analysis of this data shown in Fig. 4 demonstrates a receptor with an affinity of 1.28×10^8 M^{-1}. This affinity constant was greater than that observed with intact cells. The increase in affinity constant of the receptor demonstrated by ^{125}I-MSA binding may be due to an alteration of the environment around the receptor during the process of isolation. The binding capacity of the receptor shown in particulate membranes was calculated to be 5 pmole/mg of membrane protein.

The particulate membranes solubilized in Triton X-100 were also analyzed for their ability to bind ^{125}I-MSA. The binding experiment was carried out for 90 min at which time the bound hormone was separated from the free hormone using the polyethylene glycol assay (14). Half maximal displacement was observed at approximately 100 ng/ml of unlabeled MSA. Maximal displacement was observed at 1400 ng/ml of MSA. The total radioactivity bound in this experiment rep-

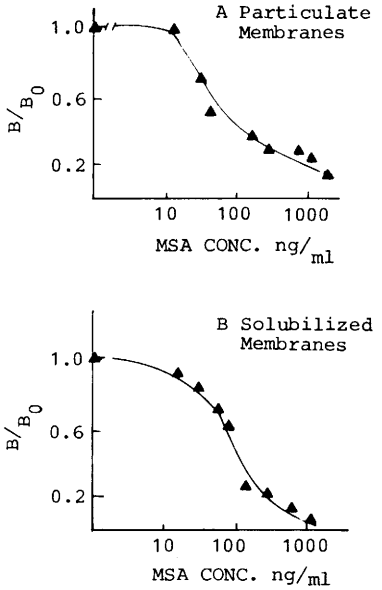

FIG. 3. Binding of MSA to particulate and solubilized membranes. (A) Membranes (50 μg) prepared from A1684 cells were incubated in Hepes binding buffer with ^{125}I-MSA (120 pg) and the indicated concentrations of unlabeled MSA. The membranes were incubated for 4 hr at 22°C. The results are plotted as B/B_0 with the 1.0 point representing 12.8% of total tracer added. (B) Particulate membranes (50 μg) solubilized with 0.1% Triton X-100 were incubated with ^{125}I-MSA (120 pg) and the indicated concentrations of unlabeled MSA. The solubilized membranes were incubated for 90 min at 22°C and were assayed using the PEG Assay. The results are plotted as B/B_0 with the 1.0 point representing 19.0% of total tracer added.

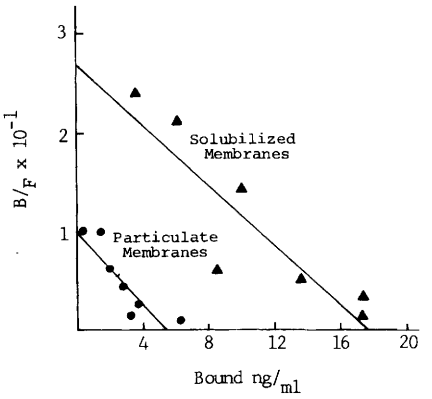

FIG. 4. Scatchard analysis of binding to membrane preparations. Scatchard analysis of the competitive binding curves generated for particulate membranes (3A) and solubilized membranes (3B).

resented 18.9% of total tracer added per tube. Nonspecific binding represented 3.1% of total tracer added. A comparison of these competitive binding curves showed that upon solubilization, the amount of specific binding increased due to a larger amount of total binding and by a decrease in nonspecific binding. This phenomenon may be due to the unmasking of receptor sites on the membrane. A Scatchard analysis of the binding curve demonstrated for solubilized membranes is also shown in Fig. 4 and revealed a somatomedin receptor with an affinity constant of $2.8 \times 10^8\ M^{-1}$ and a binding capacity of 18 pmole/mg. A comparison of the kinetic parameters demonstrated for particulate and solubilized membranes reveals that the affinity constant does not appear to differ significantly between the solubilized and particulate membrane preparations and the difference can be attributed to experimental error. The binding capacity of the receptor did, however, show a threefold increase upon solubilization. This increase may be due to the unmasking of specific receptor sites previously unavailable for binding in particulate membrane preparations and in whole cells.

To confirm that the MSA receptor on A1684 cells was of the type II insulin-insensitive class, affinity labeling experiments were performed using the chemical crosslinking reagent, disuccinimidyl suberate. Crosslinking of ^{125}I-MSA to its receptor on membranes followed by SDS–polyacrylamide gel electrophoresis and autoradiography revealed a band with a molecular weight of approximately 220,000 under nonreducing conditions. The band was not present when binding was carried out in the presence of unlabeled MSA (Fig. 5). When the crosslinked receptor was subjected to electrophoresis under reducing conditions a band was visualized in excess of 260,000 Da, confirming the lack of subunit composition (data not shown).

Discussion. Previous studies with the A1684 cell line had shown that these cells contain large numbers of MSA receptors (11). In the studies described here, the MSA receptor on the A1684 cells was analyzed

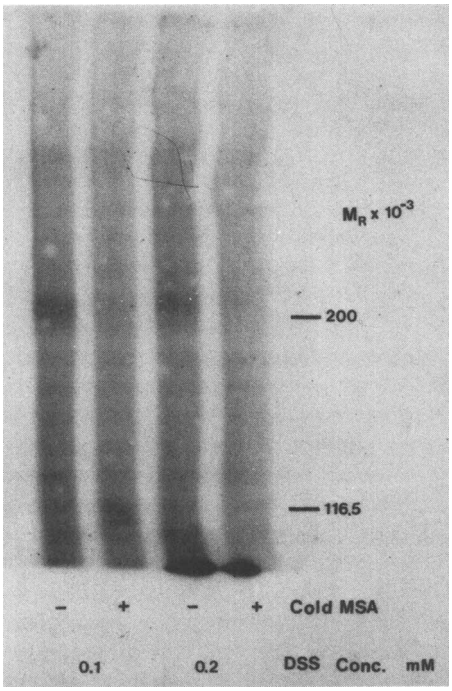

FIG. 5. Affinity labeling of the MSA receptor. Plasma membranes isolated from the human chondrosarcoma cells were incubated with ^{125}I-MSA (1.14 nM) in the absence (−) or presence (+) of unlabeled MSA (2 μg). The crosslinking was performed with disuccinimidyl suberate (DSS) added by the sequential addition of crosslinker until a final concentration of 0.1 and 0.2 mM was reached. The membranes were solubilized with 2× treatment buffer (2% SDS, 62.5 mM Tris·HCl, pH 6.8) in the absence of reducing agents, analyzed by electrophoresis on a 5% polyacrylamide gel (100:1 acrylamide:bisacrylamide) and visualized by autoradiography.

with regard to its binding characteristics and biological function.

No significant mitogenic response was found upon treatment of A1684 cells with the MSA. However, MSA did stimulate significantly the rate of nutrient transport and sulfate incorporation into acid precipitable material. This cell line appears to resemble rat chondrosarcoma chondrocytes (5) previously reported to be incapable of responding to MSA with regard to DNA synthesis, but which did respond by increasing the rate of synthesis of proteoglycans. This biological activity was determined to be mediated by MSA binding to the insulin receptor (18). Similar results were also observed in rat hepatoma cells (20). However, this does not appear to be the case with the A1684 human chondrosarcoma cells. The increases in the transport of 2-deoxyglucose and α-AIB correlated with the competitive displacement of ^{125}I-MSA binding by unlabeled MSA. Half maximal stimulation of transport was observed with 250 ng/ml of MSA. Half maximal displacement of tracer MSA was also observed at 250 ng/ml of unlabeled MSA. In studies examining ^{125}I-insulin binding, unlabeled MSA was only 1% as potent as unlabeled insulin in competing for insulin receptors. Half maximal displacement was observed with 800 ng of unlabeled MSA. This supports our contention that MSA is acting via the MSA receptor in the human chondrosarcoma cell line. Also, insulin was incapable of competing for ^{125}I-MSA binding (Fig. 1). It remains a possibility that the type I somatomedin receptors contributed to the biological activity seen in Table I. However, IGF-I was no more effective than MSA in stimulating nutrient transport (data not shown).

The binding parameters determined by analysis of the competitive binding data showed an affinity constant of $4.8 \times 10^7\ M^{-1}$ and a binding capacity of 1.9×10^6 receptors/cell. The affinity constant calculated for the MSA receptor in A1684 cells is similar to that calculated for rat embryo fibroblasts (21). It is interesting to note that these cells required additional serum factors in the culture system to produce the mitogenic response to MSA. A similarity then exists between the rat embryo fibroblasts and the human chondrosarcoma cells. Both cell types require additional serum factors for the maintenance of growth and both contain somatomedin receptors exhibiting a relatively low affinity for MSA.

While the affinity constant of the MSA receptor in intact A1684 cells was calculated to be $4.8 \times 10^7\ M^{-1}$, the particulate membranes isolated from A1684 cells demonstrated an affinity constant of $1.28 \times 10^8\ M^{-1}$. This greater affinity constant shown in particulate membranes may be due to an alteration in the membrane environment during the isolation of the membranes. Solubilization of the A1684 cell membranes resulted in a large increase in the number of MSA receptors. This may be due to a recruitment of MSA binding sites upon solubilization of the membranes that were previously unavailable for binding in the particulate membranes.

Since chrondrocytes are a normal target cell of the somatomedins *in vitro,* the A1684 human chondrosarcoma cell line is a convenient model to use in the study of the mechanism of action of these hormones. Of greater significance, however, is the demonstration in this report that these cells contain as many as 2×10^6 MSA receptors per cell. The insulin-insensitivity of the receptor suggests that it is of the type II class of somatomedin receptors (22). Affinity crosslinking experiments (Fig. 5) confirm that the MSA receptor in A1684 cells is composed of a single protein which migrates upon electrophoresis in SDS gels with a molecular weight greater than 220,000 under nonreducing conditions. In contrast, the type I somatomedin receptor is reported to exhibit a molecular weight of >300,000 under similar conditions and is characterized by its insulin sensitivity (22–24). The presence of such a high concentration of receptors, which retain ligand binding activity upon detergent solubilization of membrane preparations, encourages the view that this cell line can be utilized to facilitate the purification and characterization of this somatomedin receptor.

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