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Inhibition of biological activity of multiplication-stimulating activity by binding to its carrier protein

(somatomedin/cell growth/somatomedin binding protein)

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ABSTRACT   Multiplication-stimulating activity (MSA) produced by Buffalo rat liver cells (BRL-3A) in culture is related to the somatomedin family of growth regulatory polypeptides. MSA will stimulate glucose transport and DNA synthesis in normal chicken embryo fibroblasts (CEF) at concentrations of 10–200 ng/ml. MSA found in BRL-3A-conditioned medium, like the somatomedins in serum, does not exist as the free hormone but is bound to a specific high molecular weight carrier protein. In this report we demonstrate that purified MSA carrier protein (MCP) inhibits the biological activity of MSA on CEF as measured by the stimulation of glucose transport and DNA synthesis. In addition, purified MCP competitively inhibits the binding of ^{125}I-labeled MSA to these cells. In control experiments in which insulin was used as the mitogenic agent, MCP had no effect on these biological responses. These results indicate that the inhibitory effect of MCP is the result of specific interaction with MSA and support the hypothesis that cells may be unresponsive to somatomedins bound to their serum carrier proteins.

The somatomedins are small polypeptide hormones that are the proposed mediators of the action of growth hormone on peripheral tissue (1–3). They are unique among polypeptide hormones in that they circulate in plasma bound to larger specific carrier proteins (4–9). Two forms of somatomedin–carrier protein complexes have been identified in both human and rat serum ($M_r \approx 60,000$ and $M_r \approx 200,000$), the larger of which is growth hormone dependent (7, 8). Both forms of the complex are pH dependent and readily dissociate at pH values below 5.5 (5, 6, 8, 9).

Highly purified multiplication-stimulating activity (MSA) is a somatomedin analog purified from serum-free medium conditioned by the growth of buffalo rat liver cells (BRL-3A) in vitro (10–15). Studies utilizing ^{125}I-labeled MSA (^{125}I-MSA) have demonstrated that MSA specifically binds to somatomedin carrier proteins present in both human and rat serum (8, 9). In addition to producing MSA polypeptides in culture, BRL-3A cells also produce an MSA carrier protein (MCP) that appears to be strikingly similar in size to the low M_r carrier ($M_r \approx 60,000$) present in serum (9). We have utilized purified MSA and purified MCP from BRL-3A-conditioned medium as a model system to address the question of the biological role of somatomedin carrier protein(s). In this report we demonstrate that MCP binds to MSA in such a way as to biologically inactivate it. When equimolar quantities of MCP and MSA were added to quiescent cultures of chicken embryo fibroblasts (CEF), there was a 95–100% reduction in the stimulation of DNA synthesis that normally is seen when MSA alone is added. When the concentration of MSA was held constant, this inhibition was directly dependent on the concentration of purified

MCP added to the culture. Receptor-binding experiments demonstrated that this inhibition was solely caused by the inhibition of MSA binding to cell-surface growth receptors. Insulin-stimulated DNA synthesis, which is probably mediated through the same set of receptors (13, 16–19), was unaffected by the presence of MCP, indicating that the inhibition of the biological activity of MSA was highly specific.

MATERIALS AND METHODS

Purification of MCP. Details of the purification and characterization of MCP from BRL-3A-conditioned medium have been presented in a preliminary report (20). Briefly, the source used for the purification of MCP was a previously discarded fraction from the purification scheme of MSA. The first step in this procedure is ion-exchange chromatography with Dowex 50W-X8 resin in the sodium form. MSA binds to the column at neutral pH and subsequently is eluted at pH 11. Dulak and Shing (14) first noted from amino acid composition data that MSA is too acidic to behave in this manner itself and probably binds to the resin indirectly through more basic proteins in the conditioned medium. Both MSA and MCP are eluted in the pH 11 fraction and subsequently are dissociated by dialysis against 1 M acetic acid and then separated by Sephadex G-50 chromatography in 1 M acetic acid. MSA elutes from the column in the retarded fractions, whereas MCP is found in the void volume. The void-volume fractions are pooled, concentrated by lyophilization, and resuspended in phosphate-buffered saline. In the major purification step, this preparation is applied to an affinity column containing a mixture of immobilized MSA polypeptides covalently linked to the N-hydroxysuccinimide ester of Sepharose 4B prepared as described (21). MCP associates with the immobilized MSA and, after thorough washing, is eluted with 0.1 M acetic acid. The eluted MCP is concentrated by lyophilization and rechromatographed over Sephadex G-50 in 1 M acetic acid to ensure removal of residual MSA polypeptides.

The purified MCP migrates as a single band upon electrophoresis in acetic acid/urea (Fig. 1) and specifically binds ^{125}I-MSA, generating a complex with an apparent M_r of 60,000–70,000 as determined by Sephadex G-200 gel filtration in saline (data not shown). MCP also migrates as a single band in NaDodSO$_4$/polyacrylamide gels under nonreducing conditions with an apparent M_r of 31,500. In the presence of 2-mercaptoethanol, two closely migratory bands are observed with M_rs between 30,000 and 32,000.

The MSA used in this report was purified by established procedures using preparative-scale disc gel polyacrylamide electrophoresis in acetic acid/urea at pH 2.7 (13–15). Purified

Abbreviations: MSA, multiplication-stimulating activity; BRL, Buffalo rat liver; MCP, MSA carrier protein; CEF, chicken embryo fibroblasts.

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MSA consisted of a single species, M_r 9000, which probably corresponds to MSA polypeptide II-1 in the designation system of Moses *et al.* (15). MSA was active in stimulating DNA synthesis in quiescent CEF at concentrations of 10–200 ng/ml.

Cell Culture. Primary cultures of CEF were prepared from the body walls of 9- to 10-day-old chicken embryos as described (12, 22). The cultures were maintained in Dulbecco's modified Eagle's medium supplied with 10% calf serum, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) in a 5% CO_2/95% humidified atmosphere at 37°C. Experimental cultures were prepared by trypsinizing primary cultures and replating at a density of 3×10^5 cells per plate or 1×10^6 cells per plate in the medium described above with 0.25% calf serum in 35-mm or 60-mm cell culture dishes (Lux), respectively. These cultures were used for experiments 3–4 days later, when they had reached quiescence.

2-[^{3}H]Deoxyglucose Uptake. Rates of glucose uptake were determined in 35-mm cultures 4 hr after exposure to mitogens (22). Triplicate cultures were pulse-labeled with 1 μCi (1 Ci = 3.7×10^{10} becquerels) of 2-[^{3}H]deoxyglucose (8 Ci/mmol; New England Nuclear) in glucose-free Earle's balanced salt solution for 15 min at 37°C. Uptake rates were linear for 4 times the pulse period used. At the end of pulse, the cultures were rapidly rinsed two times, drained, and lysed with 1 ml of 1% NaDodSO$_4$. Aliquots were assayed for ^{3}H.

Electrophoresis. Protein samples subjected to electrophoresis were first lyophilized to dryness and resuspended in 0.05 ml of 4 M acetic acid/8 M urea containing 0.01% methylene blue as tracking dye. Samples were applied to tube gels (0.5 $\times$ 8.0 cm) of the following composition: 4M acetic acid/8M urea/7.5% (wt/vol) acrylamide/0.25% N,N'-methylenebisacrylamide, 0.5% N,N,N',N'-tetramethylethylenediamine and 0.21% ammonium persulfate. Electrophoresis was carried out toward the cathode at a constant current of 1.5 mA per tube for 4–6 hr at 22°C. Gels were stained in a solution of 0.05% Coomassie blue (R-250) in methanol/acetic acid/H$_2$O, 5:1:5 (vol/vol), for 2.0 hr at 37°C. The gels were destained in 7.5% (vol/vol) acetic acid containing 20% (vol/vol) methanol. All electrophoresis reagents were purchased from Bio-Rad.

^{125}I-Labeled MSA Binding. ^{125}I-Labeled MSA (^{125}I-MSA) was prepared to a specific activity of 60–100 Ci/g by a modification of the chloramine-T procedure as described (16). Binding to growth receptors in cultures of quiescent chicken embryo fibroblasts (2.5×10^6 cells per 60-mm plate) was determined as described (22). Briefly, triplicate cultures of cells were rinsed four times with phosphate-buffered saline and 1 ml of 0.1 M Hepes-buffered Dulbecco's modified Eagle's medium, pH 7.8, containing bovine serum albumin (10 mg/ml) was added. ^{125}I-MSA (10^5 cpm; $\approx$1 ng) was added, and cultures were incubated for 3 hr at 22°C in the absence or presence of various concentrations of highly purified or partially purified MCP. Cultures were then rinsed four times with cold saline and lysed by the addition of 2 ml of 1.0% NaDodSO$_4$. Aliquots were assayed for cell-associated radioactivity by liquid scintillation spectrometry.

Competitive Binding Assay. Competitive binding of ^{125}I-MSA to purified MCP was performed using fatty acid-free bovine serum albumin/activated charcoal to separate bound from free MSA as described (9). Purified MCP (40 ng) was incubated with 500 pg of ^{125}I-MSA and concentrations of unlabeled MSA ranging from 5–800 ng/ml for 3 hr in 0.4 ml of saline containing 5 mg of albumin per ml. At the end of the incubation period, bound MSA was separated from free MSA and the supernatants were assayed for radioactivity by liquid scintillation spectrometry.

RESULTS

Effect of MCP on MSA-Stimulated DNA Synthesis in Quiescent Cultures of CEF. The effect of highly purified MCP and partially purified MCP was investigated. The relative complexities of the MCP preparations are shown in Fig. 1, lanes A–D, which are gel profiles of the different stages of MCP purification. The material designated as partially purified MCP (Fig. 1, lane C), was shown to be free of MSA polypeptides but included several high M proteins. Highly purified MCP (Fig. 1, lane D) appeared as one protein band in this gel system. In NaDodSO$_4$/polyacrylamide gels, this band was further resolved into two distinct proteins with apparent M_rs of 30,000 and 31,500.

It has been reported that MCP in BRL-3A-conditioned medium specifically binds MSA with a higher affinity than does the somatomedin receptor on CEF (9). Therefore, we examined the effect of MCP on the biological activity of MSA (Fig. 2). Quiescent cultures of CEF were simultaneously exposed to MSA (150 ng/ml, the maximally active dose) and concentrations of partially purified or highly purified MCP ranging from 0 to 10 μg/ml (Fig. 2A) or from 0 to 1.6 μg/ml (Fig. 2B), respectively. In both cases, [^{3}H]thymidine incorporation at 12 hr was reduced in a dose-dependent fashion. At a concentration of 4 μg/ml of partially purified MCP or a concentration of 1.0 μg/ml of highly purified MCP, there was a 96% reduction in [^{3}H]thymidine incorporation. In the reverse experiment in which increasing concentrations of MSA were added to cultures containing purified MCP (500 ng/ml), MSA could overcome the inhibition at concentrations greater than 200 ng/ml.

The data from Fig. 2 was replotted as percent inhibition of [^{3}H]thymidine incorporation relative to the concentration of MCP added (Fig. 3). First, this replot shows that the highly purified MCP preparation was approximately 6 times more pure than the partially purified preparation, based on the relative concentrations of each required to cause a 50% inhibition
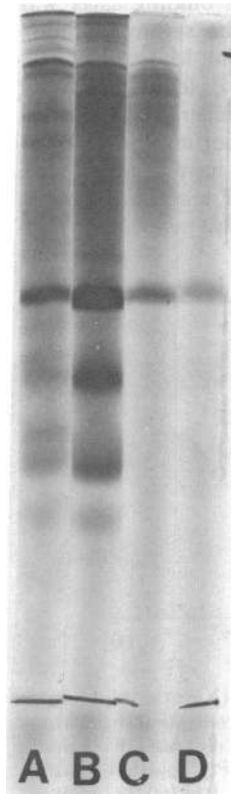

FIG. 1. 4 M acetic acid/8 M urea gel electrophoresis of MCP at different stages of purification. Lanes: A, BRL-3A-conditioned medium proteins, 30 μg; B, Dowex-50 MSA, 30 μg; C, partially purified MCP, 10 μg; and D, highly purified MCP 5 μg.
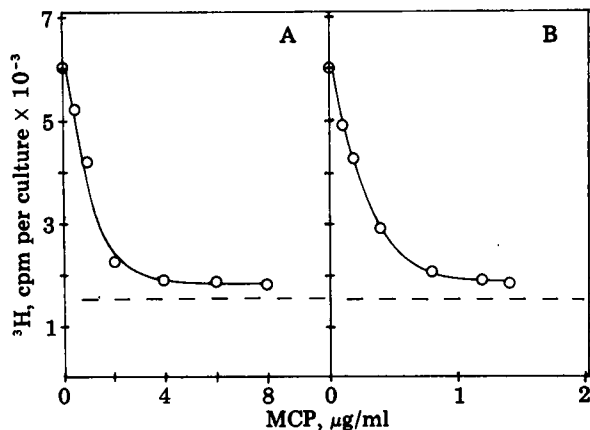
FIG. 2. Effect of MCP on MSA-stimulated DNA synthesis in cultures of quiescent CEF as a function of MCP concentration. Cultures of quiescent CEF were incubated in Dulbecco's modified Eagle's medium with MSA (150 ng/ml) and various concentrations of partially purified MCP (A) or highly purified MCP (B). Rates of DNA synthesis in duplicate cultures were determined at 12 hr by using a 1-hr pulse of [^{3}H]thymidine (0.2 μCi/ml). ---, Background levels of DNA synthesis in quiescent cultures with media changed to serum-free Dulbecco's at the start of the experiment.

of [^{3}H]thymidine incorporation. Second, maximal inhibition was seen at a purified MCP concentration of 1 μg/ml. The MSA polypeptide used in these experiments had a M_r = 9000 and MCP had a $M_r \approx$ 60,000. With the assumption that MSA and MCP bind in a 1:1 molar ratio, the experimentally determined concentration of MCP required to achieve maximal inhibition (1 μg/ml) at an MSA concentration of 150 ng/ml is close to what one would predict theoretically if MSA bound to MCP is biologically inactivated.

Scatchard Analysis of MSA Binding to Purified MCP. Because the data in Figs. 2 and 3 suggested that MSA and MCP bind in a 1:1 molar ratio, a competitive binding assay was performed using the fatty acid-free bovine serum albumin/activated charcoal procedure described by Moses *et al.* (9). Scatchard analysis (23) of the results (Fig. 4) yielded an affinity constant of 0.23 × 10^9 M^{-1}. This value is similar to that reported for the binding of MSA to normal rat serum and to BRL-3A2-conditioned medium (9). As others have reported (16), MSA tracer prepared by the chloramine-T procedure contains a significant amount of damaged unbindable molecules. When

FIG. 4. Scatchard analysis of MSA binding to MCP. Equal quantities of MCP (40 ng) were incubated with 500 pg of ^{125}I-MSA and increasing concentrations of unlabeled MSA. The slope of the line was determined by linear regression analysis.

this is taken into account, the data in Fig. 4 is also consistent with the conclusion that MSA and MCP bind in a 1:1 molar ratio.

Inhibition of ^{125}I-MSA Binding to Somatomedin Receptors on CEF by MCP. To more critically evaluate the molecular nature of MSA inactivation by MCP, receptor binding studies were performed. Concentrations of highly purified and partially purified MCP ranging from 1 to 500 ng/ml and 0.02 to 2 μg/ml, respectively, were tested for their effect on receptor binding. A representative experiment (Fig. 5) shows that both MCP preparations were able to completely inhibit the specific binding of ^{125}I-MSA to somatomedin receptors on CEF. Concentrations of 10 and 90 ng/ml of highly purified and partially purified MCP, respectively, were required to effect a 50% decrease in specific binding. This 9-fold difference correlates well with the biological data in Fig. 3, in which a 6-fold higher concentration of the partially purified MCP preparation was required to cause a 50% decrease in DNA synthesis. These results strongly suggest that MCP exerts its action by binding MSA and blocking its association with somatomedin receptors on CEF, rather than interacting with the cells or receptor sites per se.
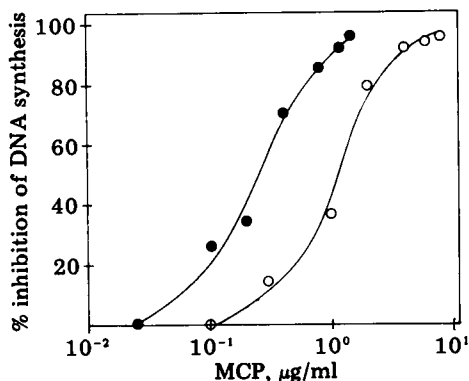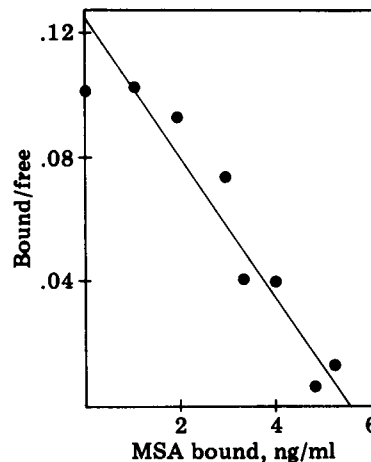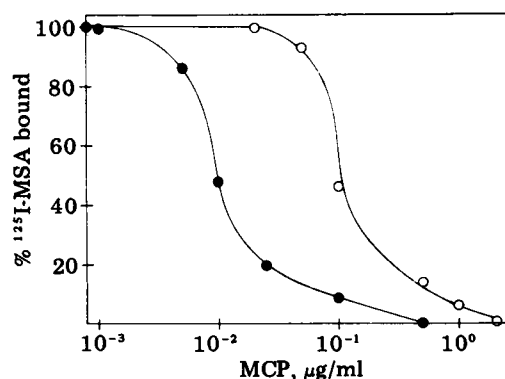

FIG. 3. Inhibition of MSA-stimulated DNA synthesis in cultures of quiescent CEF as a function of MCP concentration (logarithmic scale). The data shown in Fig. 2 *A* and *B* were replotted to obtain the sigmoid curves shown. ●, Highly purified MCP; O, partially purified. The 50% inhibition points are 0.2 μg/ml and 1.2 μg/ml for highly purified MCP and partially purified MCP, respectively.

FIG. 5. MCP-mediated inhibition of ^{125}I-MSA specific binding to somatomedin receptors on quiescent CEF. Cultures of quiescent CEF (60-mm dishes; 2.5 × 10^6 cells per dish) were incubated for 3 hr at 22°C with 10^5 cpm of ^{125}I-MSA ($\approx$1 ng/ml) and various concentrations of highly purified MCP (●) or partially purified (O) MCP. Cultures were processed for cell-associated ^{125}I-CPM and data was corrected for nonspecific binding and plotted as % ^{125}I-MSA bound vs. MCP concentration (log scale). The 100% point (cultures that received ^{125}I-MSA only) corresponds to 1.2% of the total input tracer that was bound (1.2 × 10^3 cpm). Specific binding was 70% of the total bound tracer.

Effect of MCP on Insulin-Stimulated DNA Synthesis and Glucose Uptake. Insulin and MSA elicit similar biological responses when added to quiescent cultures of CEF and presumably mediate their action through the same cell-surface receptors (12, 13, 16–19). A higher concentration of insulin is usually required to elicit the same level of response as MSA. Thus, any agent that affects the cellular receptor sites would be expected to alter the subsequent biological responses, whether insulin or MSA is used as the mitogenic agent. To demonstrate that the action of MCP is not mediated through nonspecific inhibition of cellular processes or through receptor-site alteration or blockage, two types of experiments were performed using MSA or insulin as mitogenic agents, in which the biological responses were measured. In the first of these experiments, quiescent cultures of CEF were mitogenically stimulated with insulin (1 μg/ml) and concentrations of MCP far in excess of that required to completely inhibit the mitogenic response of these cells to a maximally active dose of MSA (150 ng/ml). Fig. 6 clearly shows that MCP has little or no effect on the ability of insulin to stimulate DNA synthesis in these cultures. At the highest concentrations of MCP tested, which were respectively 2.5- and 5-fold excesses of the amounts of partially purified and highly purified MCP required to completely inhibit the MSA response, only a slight and insignificant reduction in the stimulation of DNA synthesis was observed.

In the second type of experiment, with a protocol nearly identical to that in Fig. 6, the effect of MCP on MSA- and insulin-stimulated uptake of 2-[^{3}H]deoxyglucose was tested. Glucose uptake was chosen because it is an event that is stimulated early after the addition of mitogen, in contrast to DNA synthesis, which peaks 12 hr after mitogenic stimulation. MCP had no effect on insulin-stimulated glucose uptake, but was able to cause more than 70% reduction in MSA-stimulated cultures at identical concentrations (Fig. 7).

Effect of Preincubation with MCP. When quiescent cultures were preincubated for up to 12 hr with purified MCP and then mitogenically stimulated with MSA, a 100% inhibition of [^{3}H]thymidine incorporation was still observed. In contrast,

FIG. 7. Effect of MCP on insulin- and MSA-stimulated glucose uptake in quiescent cultures of CEF. Triplicate cultures of CEF were incubated in Dulbecco's modified Eagle's medium containing insulin (1 μg/ml; *Left*, bars A–C) or MSA (150 ng/ml; *Right*, bars A–C). Control-culture (con.) media were changed to serum-free Dulbecco's only at the start of the experiment. To cultures B and C (*Left* and *Right*), highly purified MCP was added at the indicated concentrations. 2-[^{3}H]Deoxyglucose uptake was determined at 4 hr with a 15-min pulse (1 μCi/ml) in a glucose-free Eagle's balanced salt solution at 37°C. The percent inhibitions of 2-[^{3}H]deoxyglucose uptake in cultures that received MCP (*Left* and *Right*, bars B and C), relative to cultures that received only insulin (*Left*, bar A) or MSA (*Right*, bar A) are indicated above the bars. Each bar represents the mean ± SD.

when the cells were preincubated for 12 hr with MCP and then washed prior to MSA addition, there was no inhibition of [^{3}H]thymidine incorporation. These results (Table 1) demonstrate that preincubation with MCP has no direct inhibitory effect on the ability of the cells to respond to MSA.

DISCUSSION

In these studies we demonstrated that the mitogenic action of MSA on quiescent cultures of CEF is strongly inhibited in a dose-dependent fashion by the presence of MCP in the culture medium. At a MSA concentration where the mitogenic response was maximal, an equimolar amount of purified MCP caused a 96% reduction in the stimulation of DNA synthesis. In control experiments in which insulin was substituted for MSA as the mitogenic agent, concentrations of MCP far in excess of those required to completely inhibit the MSA mitogenic response had little or no effect on insulin-stimulated DNA synthesis or glucose transport. Because MSA and insulin effect their action on CEF through the same set of cell-surface receptors (13, 16–19), this
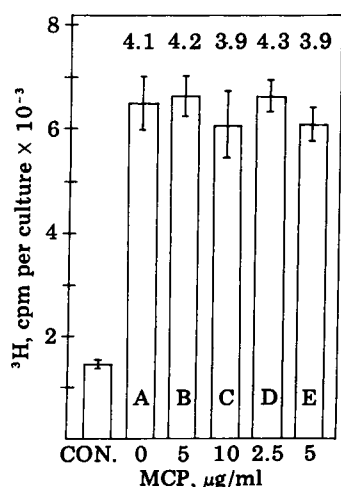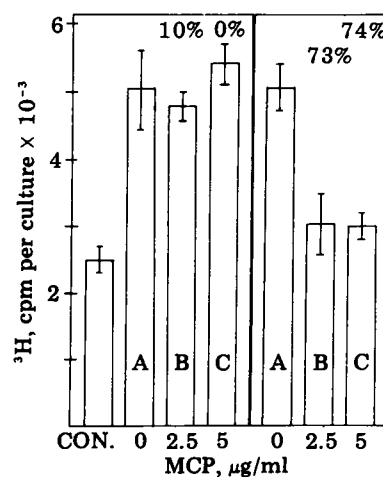

FIG. 6. Effect of MCP on insulin-stimulated DNA synthesis in cultures of quiescent CEF. Triplicate cultures of quiescent CEF were incubated in Dulbecco's modified Eagle's medium containing insulin (1 μg/ml, bars A through E) and partially purified MCP (bars B and C) or highly purified MCP (bars D and E) at the indicated concentrations. Cultures represented by bar A received insulin only. Control-culture (con.) media were changed to serum-free Dulbecco's only at the beginning of the experiment. Rates of DNA synthesis were determined as described in Fig. 2. Fold increases in [^{3}H]thymidine incorporation relative to control cultures are indicated above the bars. Each bar represents the mean ± SD.

Table 1. Effect of preincubation of quiescent cultures of CEF with MCP prior to MSA stimulation

| MSA stimulation | MCP presence | | ^{3}H-thymidine, cpm per culture | % inhibition |
	Pre-incubation	MSA stimulation		
−	−	−	4,791 ± 718	—
+	−	−	11,646 ± 275	—
+	−	+	3,617 ± 586	100%
+	+	+	2,033 ± 177	100%
+	+	−	11,892 ± 781	0%

Triplicate cultures of quiescent CEF were stimulated with MSA (150 ng/ml) in the presence (+) or absence (−) of MCP (5 μg/ml). Some of the cultures had also been preincubated for a 12-hr period with MCP at the same concentration. Levels of [^{3}H]thymidine incorporation were determined 12 hr after MSA addition and the percent inhibition determined. Data shown is the mean ± SD.

suggested that the inhibitory action of MCP was due to a specific interaction between MSA and MCP directly, rather than an interaction between MCP and cell-surface receptors per se. Support of this hypothesis was obtained in subsequent experiments, in which it was demonstrated that MCP was able to completely block the specific binding of ^{125}I-MSA to CEF somatomedin receptors. Because we and others (9) have shown that MCP specifically binds MSA with a high affinity, our results strongly suggest that MCP binds to MSA in such a way as to block its ability to interact with cell-surface receptor sites by either binding to the active site or sterically masking its availability.

The only documented function of somatomedin carrier protein(s) is that it increases the circulating half-life of injected somatomedin activity from a few minutes to several hours (7). In studies similar to this one, designed to investigate the effect of somatomedin carrier protein(s) on the biological activity of somatomedins, it was shown that nonsuppressible insulin-like activity bound to its partially purified carrier was ineffective in stimulating glucose transport and lipogenesis in rat fat cells (24) and insulin-like effects on perfused rat heart (25). Somatomedin-binding proteins from amniotic fluid have also been shown to inhibit the stimulation of thymidine uptake by human fibroblasts and sulfate incorporation by rabbit chrondrocytes (26, 27). In contrast, it was suggested by Froesch *et al.* (28) that fibroblasts and chondrocytes may possess a unique mechanism that allows them to utilize somatomedins in their carrier-bound form. The results of the present study clearly demonstrate that this is not the case with CEF. It should also be pointed out that our results are not an artifact due to an alteration of MCP during the purification procedure. Other investigators have shown that MCP in BRL-3A-conditioned medium binds MSA with the same affinity before and after exposure to 1 M acetic acid (9), which is the harshest step used in our purification procedure. We feel that the use of highly purified MSA carrier protein from BRL-3A-conditioned medium provides a unique opportunity to investigate the biological role of somatomedin carrier protein(s), and should greatly facilitate further studies.

Since we have demonstrated that MSA bound to its carrier protein is biologically inactive in stimulating DNA synthesis in CEF, it appears paradoxical that these cells are stimulated by BRL-3A-conditioned medium or serum, in which the MSA or somatomedin exists primarily in the bound form. In the case of conditioned medium, it is apparent that free MSA is in excess. This is shown by the fact that when ^{125}I-MSA is incubated with conditioned medium and then fractionated on Sephadex G-200 at neutral pH, all of the radioactivity remains as free MSA. Serum, however, has specific MSA-binding sites available in two high M_r complexes (8, 9). The MSA complex described in the report is similar in size to the low M_r complex in serum, which may be a precursor to the higher M_r form (6). It is conceivable that the larger M_r complex is active and serves as a delivery system for cell utilization. Alternately, CEF may respond to other insulin-like growth factors in serum similar to that described by Poffenbarger (29).

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