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Purification and Characterization of Multiplication-Stimulating Activity (MSA) Carrier Protein

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The rat liver cell line, BRL-3A, is known to produce a family of polypeptides referred to as multiplication-stimulating-activity (MSA). Serum-free conditioned medium from this cell line is a rich source for the purification of these somatomedin-like molecules. Somatomedins in serum, as well as MSA produced by BRL-3A cells in culture, exist primarily as a high molecular weight complex bound to specific carrier proteins. This study describes the purification of the MSA carrier protein (MCP) from conditioned medium using affinity chromatographic procedures. The purified carrier protein is shown to specifically bind labeled MSA and generates a complex with an apparent molecular weight of 60,000–70,000 daltons. Characterization of the carrier protein indicates that it consists of two different noncovalently linked protein chains with apparent molecular weights of 30,000 and 31,500 daltons. The availability of a pure carrier protein should provide a unique opportunity to investigate the functional significance of the carrier protein in the biological activity of the somatomedins.

Key words: MSA, somatomedin, carrier protein

The somatomedins are a family of low molecular weight polypeptide hormones (mol wt $\simeq$ 7,500) that are the proposed mediators of the peripheral action of growth hormone on skeletal tissue [1, 2]. As a group, the somatomedins also have weak insulin-like activity and promote the growth of a variety of cell types in culture [1–5]. Several small polypeptides that fit the current definition of somatomedins have been purified from human serum. These include somatomedins A and C and the insulin-like growth factors IGF I and IGF II [3, 6–8]. A unique property of the somatomedins is that they circulate in plasma bound to high molecular weight protein(s) referred to as somatomedin carrier proteins (SM carrier proteins) [9–11]. While the role of the large carrier protein(s) in the biological activity of the somatomedins is still speculative, it has been established that the presence of at least one form of SM carrier protein(s) is also growth hormone dependent,

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and association of somatomedin with this carrier greatly prolongs the circulating half-life of somatomedin activity in vivo [12, 13].

The somatomedin activity in rat serum is also complexed to a somatomedin carrier protein(s), analogous to the situation that exists for human somatomedins [3]. Although it is at this time difficult to predict structural similarities between human and rat SM carrier protein(s), highly purified Somatomedin A, IGF I, and IGF II all bind to rat SM carrier protein in a highly specific manner [14]. The precise chemical nature of the interactions of these molecules with rat SM carrier protein(s) is not known, but it is probably a weak noncovalent interaction since treatment at low pH is routinely used to dissociate the molecules [9–14].

In addition to the human somatomedins described above, purified multiplication-stimulating-activity (MSA) has also been shown to bind to SM carrier proteins in rat serum [14]. MSA refers to a family of low molecular weight somatomedin-like polypeptides purified from serum-free medium conditioned by the growth of a line of buffalo rat liver cells (BRL-3A) [15–18]. MSA appears to be structurally and biochemically similar to the human somatomedins. This similarity is strongly supported by the demonstration that somatomedin A, IGF I, and IGF II compete effectively with MSA for cell surface mitogenic receptors on a variety of cell types and tissues [4, 18], as well as for the same SM carrier proteins in rat serum [14].

At present, the molecular events involved and the physiological significance of the interactions between somatomedins and their serum carriers is a complex and largely unresolved issue. When highly purified ^{125}I-MSA is added to whole, untreated rat serum at neutral pH, two molecular weight forms of carrier protein can be demonstrated by Sephadex G-200 gel filtration [13, 14]. Only the low molecular weight form can be demonstrated in serum exposed to acid conditions (1 M acetic acid) and subsequently returned to neutral pH prior to the addition of ^{125}I-MSA. All attempts to generate the high molecular weight form from the low molecular weight form have been unsuccessful. That the relationship between the high and low molecular forms may be physiologically significant is indicated by experiments showing that only the low molecular weight form is demonstrable in serum from rats that have been hypophysectomized [13, 14].

In an attempt to better understand the molecular basis of the interactions between somatomedins and their carrier protein(s), we have taken advantage of the fact that BRL-3A cells produce SM carrier protein as well as MSA under serum-free conditions in culture and have utilized this as a source from which to purify a rat SM carrier protein. In this report, we demonstrate the purification of MSA carrier protein (MCP) from serum-free BRL-3A-conditioned medium and show that purified MCP specifically binds ^{125}I-MSA and generates a ^{125}I-MSA–carrier protein complex with a molecular weight similar in size to the low molecular weight form seen after the addition of ^{125}I-MSA to rat serum [13, 14]. We feel that this represents an important step in understanding the biochemistry and biological significance of SM carrier protein(s).

MATERIALS AND METHODS

MSA Purification and Separation From Carrier Protein

Serum-free conditioned medium was prepared from roller bottle cultures of BRL-3A rat liver cells as described by Dulak and Shing [19] MSA was purified from the conditioned medium using established procedures [16–18]. Briefly, 4-liter batches of conditioned medium were subjected to ion exchange chromatography using Dowex 50W-X8 resin in

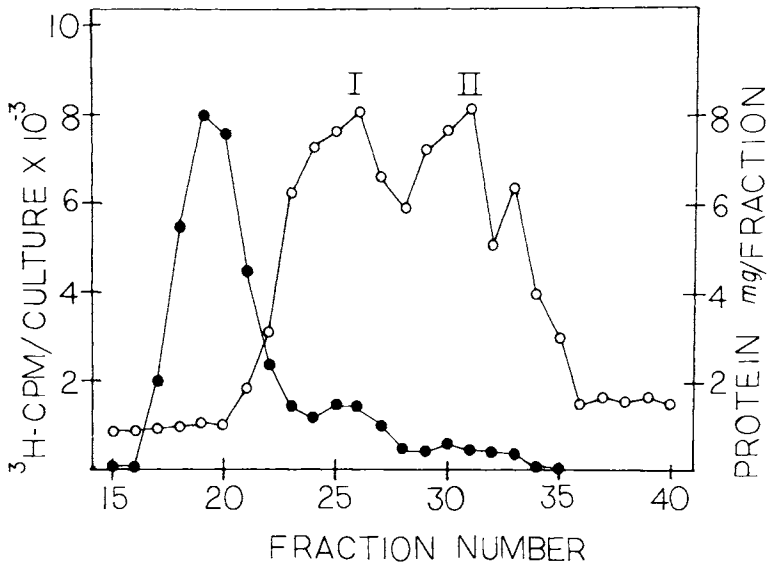

Fig. 1. Sephadex G-50 chromatography of Dowex-50 MSA in 1 N acetic acid. Fifty milligrams were chromatographed over a 2.5 × 100 cm column at 4°C. Ten-milliliter fractions were collected and assayed for protein content (•) and for multiplication-stimulation activity (○).

the Na^+ form. MSA absorbs to the resin at neutral pH (apparently indirectly via its carrier protein [19]) and is eluted at pH 11. This Dowex-50 MSA was then chromatographed on Sephadex G-50 in 1 N acetic acid. Most of the protein elutes from the G-50 column in the void-volume region, including the MSA carrier protein(s) that have been dissociated from the MSA by the acid treatment (Fig. 1). The pooled void-volume fractions from this G-50 step served as the starting material for the subsequent isolation of the carrier protein. MSA elutes from the G-50 column in two peaks of activity as measured by the stimulation of labeled-thymidine incorporation into quiescent cultures of chicken embryo fibroblasts. Both peaks have comparable activity in this assay and together consist of a family of seven or eight biologically active polypeptides. The MSA affinity column (to be described later) was prepared using a mixture of these two peaks of MSA since it was not feasible to purify enough of a single molecular weight species of MSA for this purpose.

For studies characterizing the binding properties of the carrier protein using radioactively labeled MSA, a single species of MSA was purified. The first peak of MSA activity to elute from the G-50 column was pooled and subjected to preparative scale disc polyacrylamide gel electrophoresis in acetic acid:urea at pH 2.7 as previously described [18]. This MSA preparation consisted of a single protein-staining band in acid and alkaline acrylamide systems (mol wt 9,000) and was active in stimulating DNA synthesis in chicken embryo fibroblasts at concentrations of 10–20 ng/ml and was maximally active at concentrations of 150–200 ng/ml. This preparation of purified MSA probably corresponds to MSA polypeptide II-1 of the designation system of Moses et al [20].

MSA Radioiodination

Six μg of highly purified MSA (mol wt 9,000) was radioiodinated to a high specific activity (~ 80 Ci/g) by a modification of the chloramine-T procedure [21]. The reaction

mixture was chromatographed over a 1 × 50 cm column of Sephadex G-50 resin (fine) equilibrated at 4°C in 1 M acetic acid containing 1 mg/ml of bovine serum albumin (BSA). One-milliliter fractions were collected and assayed for specific binding to MSA receptors on chick embryo fibroblasts. Fractions containing ^{125}I-MSA that showed the highest degree of specific binding were pooled, dispensed in 0.2 ml aliquots and stored at -20°C. ^{125}I-MSA was stable for approximately 4 weeks.

Affinity Chromatography

The activated *N*-hydroxysuccinimide ester of Sepharose 4B was prepared as described by Parikh et al [22]. To 8 ml of packed, activated Sepharose 4B equilibrated in 0.1 M phosphate buffer, pH 7.2 at 4°C, was added an equal volume of the same buffer containing 10 mg of a mixture of MSA polypeptides from the G-50 column (see above). The reaction mixture was gently stirred overnight at 4°C. The resin was then extensively washed with distilled deionized water and then incubated for 2 h at room temperature with 0.1 M glycine, pH 9.0, in 0.1 M bicarbonate buffer. This was done to mask residual reactive ester groups. The resin was then washed with several volumes of 0.1 M acetate buffer (pH 3.5), followed by several volumes of distilled deionized water, and was finally equilibrated in Mg^{2+}- and Ca^{2+}-free Dulbecco's phosphate buffered saline (PBS, pH 7.4). This yielded a product consisting of Sepharose 4B containing a succinylaminoethyl arm to which MSA was covalently linked presumably via the α amino group. The slurry was transferred to a 10 ml plastic syringe yielding a column with a packed bed volume of approximately 6 ml.

Purification of MSA Carrier Protein

The beginning material used as the source for the purification of MSA carrier protein (MCP) from rat liver cell conditioned medium was a pool of the fractions comprising the void volume eluted from the Sephadex G-50 gel filtration step in the MSA purification scheme described above. It is during this step that MSA is separated from the high molecular carriers by gel filtration under acidic conditions. Fractions comprising the void volume were pooled, concentrated by lyophilization and rechromatographed over an identical G-50 column in 1 N acetic acid to insure complete separation of the MSA. Again the fractions comprising the void volume were pooled, dried by lyophilization, and then resuspended in PBS, pH 7.4, to a final concentration of 500 μg/ml. Ten milliliters of this solution was applied to the affinity column at 22°C at a flow rate of 5 ml/h. The protein content of the column effluent was continuously monitored by measuring the absorbance at 280 nm. Two-milliliter fractions were collected. After sample loading, the column was washed with PBS, pH 7.4, until the effluent was free of protein. The column was then eluted with 0.1 M acetic acid, pH 3.5, at a flow rate of 15 ml/h. Fractions comprising the eluted protein peak were pooled, dialyzed extensively against 1 M acetic acid, and concentrated by lyophilization. The protein residue was resuspended in 1 M acetic acid and chromatographed over a 1 × 50 cm Sephadex G-50 column as previously described. One-milliliter fractions were collected and analyzed by acetic acid-urea (4M:8M) polyacrylamide gel electrophoresis. Fractions free of MSA peptides, (V_0 fractions) were pooled as purified MCP.

Electrophoresis

Samples subjected to electrophoresis in acetic acid-urea (4M:8M), were first dried by lyophilization and resuspended in 0.05 ml of acetic acid-urea (4M:8M) containing 0.01% methylene blue as tracking dye. The samples were applied to 0.5 cm × 8.0 cm tube gels of

the following composition: 4M acetic acid:8M urea, 7.5% acrylamide (w/v), 0.25% methylene bisacrylamide (w/v), 0.5% *N,N,N',N'*-tetramethylene diamine (TEMED) (v/v) and 0.21% ammonium persulfate (w/v). Electrophoresis was carried out toward the cathode for 4–6 h at 22°C. Gels were stained in a solution of 0.05% coomassie blue in methanol-acetic acid-H_2O (5:1:5). The gels were destained in 7.5% acetic acid (v/v) containing 20% methanol (v/v).

When protein bands were to be eluted from gels and assayed for biological activity, a pH 4.4 electrophoretic system was used in 8 M urea. Samples were lyophilized and resuspended in 0.05 ml of acetic acid-urea (0.5 M:8 M) with 0.01% methylene blue as tracking dye. Samples were applied to 0.5 × 8.0 cm tube gels of the following composition: 7.5% acrylamide (w/v), 8 M urea, 0.75 M acetic acid (pH 4.4), 0.2% methylene bisacrylamide (w/v), 0.5% TEMED (v/v), and 1.25 μg riboflavin. An unstained gel was sliced into 2 mm slices and the slices subjected to electrophoretic elution. Samples derived from each slice were dialyzed, lyophilized, and resuspended in 400 μl of PBS, pH 7.4, containing 5 mg/ml fatty acid-free BSA. The ability of the eluted protein to specifically bind ^{125}I-MSA was determined using the fatty acid-free BSA activated charcoal assay described by Moses et al [14].

Electrophoresis in 10% SDS-polyacrylamide tube gels (0.5 × 8.0 cm) was carried out as described by Weber et al [23]. Samples were denatured and reduced in buffer containing 2-mercaptoethanol by heating for 10 min at 65°C prior to electrophoresis. Where appropriate, molecular weight standards were included. The gels were stained and destained as described for acetic acid-urea gels.

Binding of ^{125}I-MSA to Purified MCP and Rat Serum

^{125}I-MSA (7×10^5 CPM) was incubated with 0.5 ml of rat serum, acid dialyzed rat serum, Dowex-50-treated rat serum or 5.0 μg of purified MCP (from BRL-3A conditioned medium) in 0.5 ml of 0.1 M Hepes-buffered DMEM (pH 7.4) containing 10 mg/ml of BSA. The reaction mixtures were incubated for 5 h at 22°C. To determine binding specificity, 10 μg of unlabeled MSA was added to a parallel reaction mixture. At the completion of the binding reaction, the mixtures were immediately diluted 1:1 with cold PBS, pH 7.4, and fractionated by chromatography over Sephadex G-200. The BSA used in the reaction mixtures had no detectable ^{125}I-MSA binding activity (data not shown).

Sephadex G-200 chromatography was performed using a 2.5 × 40 cm column of Sephadex G-200 (fine) equilibrated in PBS, pH 7.4. The column was poured and eluted under 12 cm constant head pressure in the descending mode. Fractions of 2.25 ml were collected at a flow rate of 5 ml/h. To calibrate the column with protein markers, 0.5 ml of calf serum diluted 1:1 with PBS, pH 7.4, was filtered over the column. Aliquots of the fractions were assayed for protein content by the method of Lowry et al [24]. When ^{125}I-MSA binding reaction mixtures were passed over the column, aliquots (0.1 ml–0.25 ml) of the fractions were assayed for ^{125}I-CPM by liquid scintillation spectrometry. Approximately 70% of the applied radioactivity was recovered.

Competitive Binding Assay for MSA

Specific binding of ^{125}I-MSA to purified MCP for Scatchard analysis [25] and for determination of binding activity in protein samples eluted from gel slices was performed using fatty acid-free BSA activated charcoal to separate bound from free MSA as described by Moses et al [14]. For the Scatchard analysis, 40 ng of purified MCP was incubated with 0.5 ng of ^{125}I-MSA and various concentrations of unlabeled MSA for 3 h in 0.4 ml of PBS

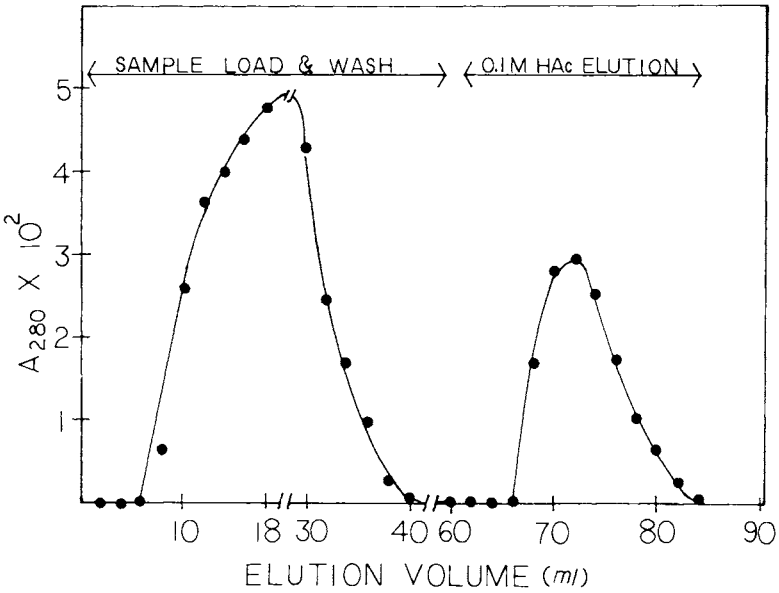

Fig. 2. Affinity chromatographic purification of MSA carrier protein. Five milligrams of a mixture of high molecular weight proteins, obtained from the void-volume region following chromatography of Dowex-50 MSA on Sephadex G-50 in 1 N acetic acid, were applied to a column of Sepharose 4B resin containing covalently immobilized MSA. Following extensive washing with PBS to remove unbound proteins, the column was eluted with 0.1 M acetic acid. Protein content in the eluant was continuously monitored by absorbance at 280 nm.

containing 5 mg/ml BSA. At the end of the incubation period, bound MSA was separated from free MSA and the supernatants assayed for radioactivity. Binding of ^{125}I-MSA to BRL-3A2 conditioned medium (a generous gift from M. Rechler) was also performed in a similar manner substituting 100 μl of medium in place of the purified MCP.

Binding of ^{125}I-MSA to samples eluted from gel slices was performed by resuspending protein samples in 0.4 ml of PBS containing 5 mg/ml BSA. Four assay tubes were used for each gel slice sample and contained 0.1 ml of the protein sample, 0.3 ml assay buffer, and 1×10^5 CPM of ^{125}I-MSA. Two of the tubes also contained 1 μg of unlabeled MSA for determination of specific binding. Separation of bound from free MSA was done as described above.

RESULTS

MSA polypeptides in BRL-3A conditioned medium exist in a high molecular weight form bound to a specific carrier protein [14]. During the purification of MSA, conditioned medium is first subjected to ion exchange chromatography using Dowex 50W-X8 resin in the Na^+ form. MSA is known to absorb to the resin at neutral pH and is then eluted at pH 11. Dulak and Shing [19] first suggested that MSA does not bind to the resin directly but does so via more basic proteins in the medium, presumably the carrier. After the Dowex-50 step, the MSA preparation is chromatographed on Sephadex G-50 in 1 N acetic acid. The detectable MSA activity elutes from the column as two peaks in the low molecular weight region clearly separated from the bulk of the protein, which appears near the

void volume of the column (Fig. 1). This procedure dissociates the MSA-carrier protein complex and the MSA carrier protein (MCP) now elutes along with the majority of the protein in the void-volume region. BRL-3A conditioned medium thus provides a rich source of MCP as well as MSA polypeptides.

Because of the simple pH dependence of the binding of MSA peptides to their carrier protein(s), it was reasoned that MSA peptides immobilized on a solid support should provide a highly specific and simple method with which to purify BRL-3A-produced MSA carrier protein (MCP). The *N*-hydroxysuccinimide active ester of Sepharose 4B was prepared as described by Parikh et al [22]. A mixture of MSA polypeptides (peaks I and II, Fig. 1) were reacted with the activated Sepharose 4B to prepare the affinity column. For details of this procedure refer to Materials and Methods. The void-volume proteins obtained from Sephadex G-50 chromatography of Dowex-50 MSA (Fig. 1) were pooled and concentrated by lyophilization for use as the source for the purification of carrier protein. The Sephadex G-50 step was repeated to insure removal of the MSA polypeptides and the protein preparation was resuspended in PBS at a concentration of 500 μg/ml. Ten milliliters of this solution were loaded onto the affinity column at a flow rate of 5 ml/h. Protein content in the eluant was monitored by absorbance at 280 nm. After sample loading, the column was washed extensively with PBS, pH 7.4, until the protein content in the eluant was negligible (Fig. 2). The column was then eluted with 0.1 M acetic acid, pH 3.5, at a flow rate of 15 ml/h. Two-milliliter fractions were collected. One major peak of protein was released from the column during the acidic elution (Fig. 2). The fractions comprising this peak (68–86 ml total elution volume) were pooled, dialyzed against 1,000 volumes of 2% acetic acid (v/v) to remove salt, and concentrated by lyophilization. The residue was resuspended in 1 ml of 1 M acetic acid and chromatographed over a 1 × 50 cm column of Sephadex G-50 in 1 N acetic acid at 4°C (data not shown). This step was necessary to remove MSA peptides that were released during the acidic elution of the affinity column. The void-volume fractions were analyzed by acetic acid-urea (4M:8M) polyacrylamide gel electrophoresis. Fractions free of MSA polypeptide contamination were pooled as purified carrier protein (data not shown). To test the purity of this preparation and to examine its presence throughout the purification procedure, 5 μg were electrophoresed in the same acetic acid-urea system (Fig. 3). Only one protein band was observed for the purified carrier protein preparation. There were no detectable MSA peptides present. Shown for reference in Figure 3 are protein samples of BRL-3A conditioned medium (40 μg), and Dowex-50 MSA (40 μg). No additional protein bands were evident when as much as 30 μg of the purified MCP preparation was applied to similar gels.

To show that the purified protein band appearing in the gels corresponded to binding activity, a similar gel was sliced prior to staining, and protein was extracted from the slices by electrophoretic elution. Samples were then assayed for binding capacity using the fatty acid-free BSA activated charcoal procedure for the separation of bound and free MSA described by Moses et al [14]. As can be seen in Figure 4, binding activity in the gel coincided with the observable stained protein band in an identical gel. This proves that the protein purified by the affinity chromatographic procedure is indeed the MSA carrier protein.

To determine the relationship of purified MCP to the MSA or somatomedin binding proteins in rat serum, the experiments detailed in Figures 5 and 6 were performed. MSA labeled with iodine 125 was incubated with normal rat serum, acid dialyzed rat serum, Dowex-50-treated rat serum, or purified MCP. Identical reaction mixtures contained excess unlabeled MSA for determination of specific binding. After 5 h of incubation, the mixtures

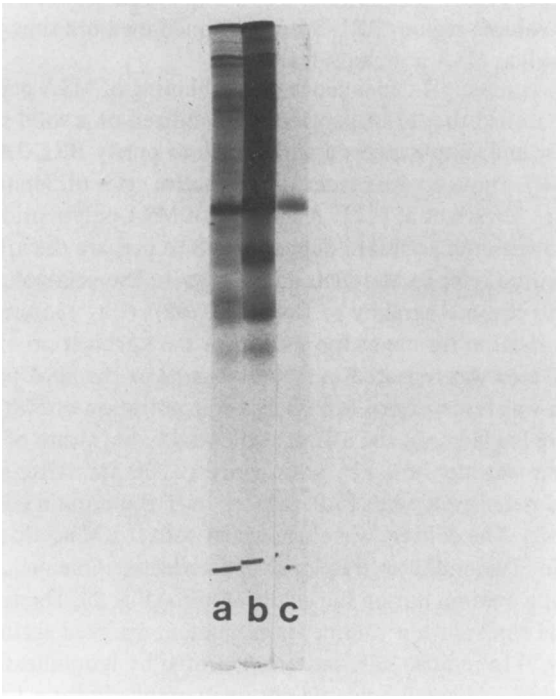

Fig. 3. Electrophoresis of purified carrier protein in acetic acid-urea gels. Carrier protein, 5 μg, purified by affinity chromatography (c) was subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. For comparison, 40 μg samples of whole conditioned medium proteins (a) and Dowex-50 MSA (b) are shown.

were individually applied to the same 2.5 × 40 cm column of Sephadex G-200 equilibrated in PBS at 4°C. Aliquots of the 2.25 ml fractions were assayed for radioactivity. The results shown in Figure 5 confirm those previously reported by others [13, 14]. MSA binds to normal rat serum and after chromatography on Sephadex G-200 at neutral pH, radioactivity appears in five peaks. Binding to peaks 2 and 3 is specific and can be eliminated in the presence of an excess of unlabeled MSA. Binding to peak 1 is nonspecific, peak 4 is free ^{125}I-MSA and peak 5 is free ^{125}I. Acid dialysis of normal rat serum eliminates binding activity in the region of peak 2 (Fig. 5B).

When MCP purified by affinity chromatography was incubated with ^{125}I-MSA and the mixture chromatographed on Sephadex G-200, specific binding was detected in a region corresponding in size to the peak 3 binding activity in normal and acid-dialyzed rat serum (Fig. 6A). This complex elutes in a position similar in size to that of calf serum albumen and has an approximate molecular weight of 66,000–68,000 daltons.

Dowex-50 chromatographic treatment of normal rat serum followed by acid dialysis also destroys binding in the region of peak 2 (Fig. 6B). These results show that the complex generated when purified MCP is incubated with ^{125}I-MSA appears to be equivalent to the lower molecular weight (peak 3) complex found in normal or acid-dialyzed rat serum [13, 14].

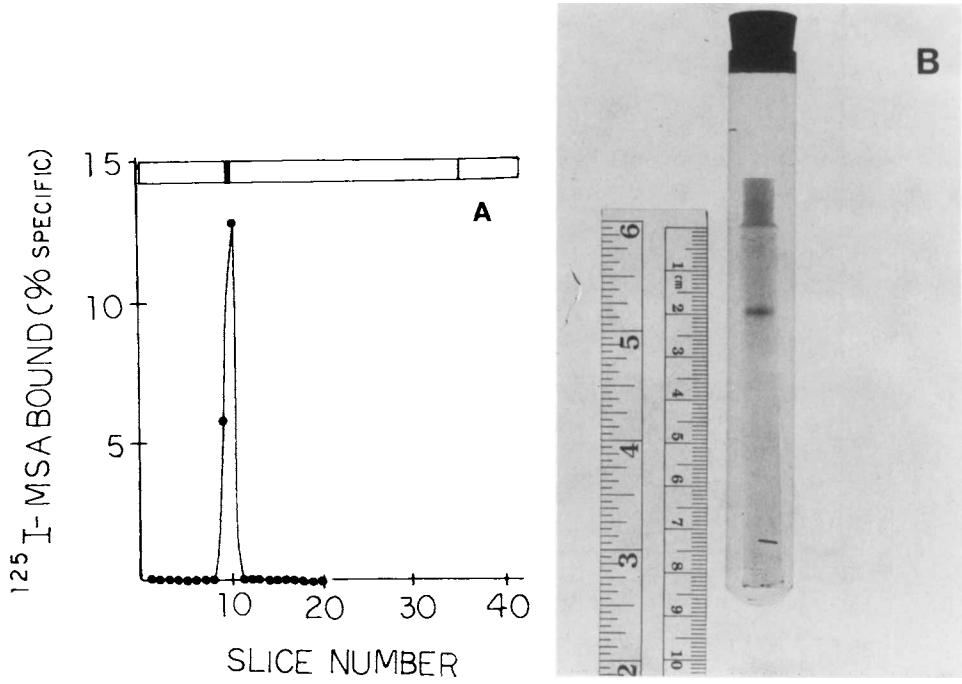

Fig. 4. Localization of MCP activity in acetic acid-urea gels. Samples of purified MCP (5 μg) were subjected to electrophoresis in acetic acid-urea gels at pH 4.4. The lower gel consisted of 7.5% acrylamide and a 1 cm upper gel was applied with an acrylamide concentration of 2.5% acrylamide. Following electrophoresis, one gel was sliced into 2 mm slices, and the slices were individually subjected to electrophoretic elution. Eluted samples were then assayed for carrier protein activity in the fatty acid-free BSA activated charcoal binding assay (A). An identical gel was stained for visualization of protein bands (B). A schematic diagram of the stained gel appears over the profile of carrier protein activity in panel A.

The approximate molecular weight of purified MCP obtained by affinity chromatography was determined using SDS polyacrylamide gel electrophoresis according to the method of Weber et al [23]. Samples were prepared for electrophoresis in the presence and absence of 2-mercaptoethanol. Under reducing conditions, the purified MSA carrier protein ran as two closely migrating bands with apparent molecular weights of 30,000 and 31,500 daltons (Fig. 7B). The faster-migrating band stained much lighter than the slower band. In the absence of 2-mercaptoethanol, a single sharp band with an apparent molecular weight of 31,500 was observed (Fig. 7A). It should be noted that these molecular weight determinations may be subject to error due to the possible glycoprotein nature of these proteins. This may also explain the difference in staining intensity of the two bands appearing under reducing conditions.

To determine the binding affinity of purified MCP to MSA, a competitive binding assay was performed using the fatty acid-free BSA activated charcoal procedure described by Moses et al [14] for the separation of bound and free MSA (Fig. 8). Scatchard analysis of the results yielded an affinity constant of $0.23 \times 10^{9} M^{-1}$. In addition, the affinity constant was determined for the binding of ^{125}I-MSA to BRL-3A2 conditioned medium.

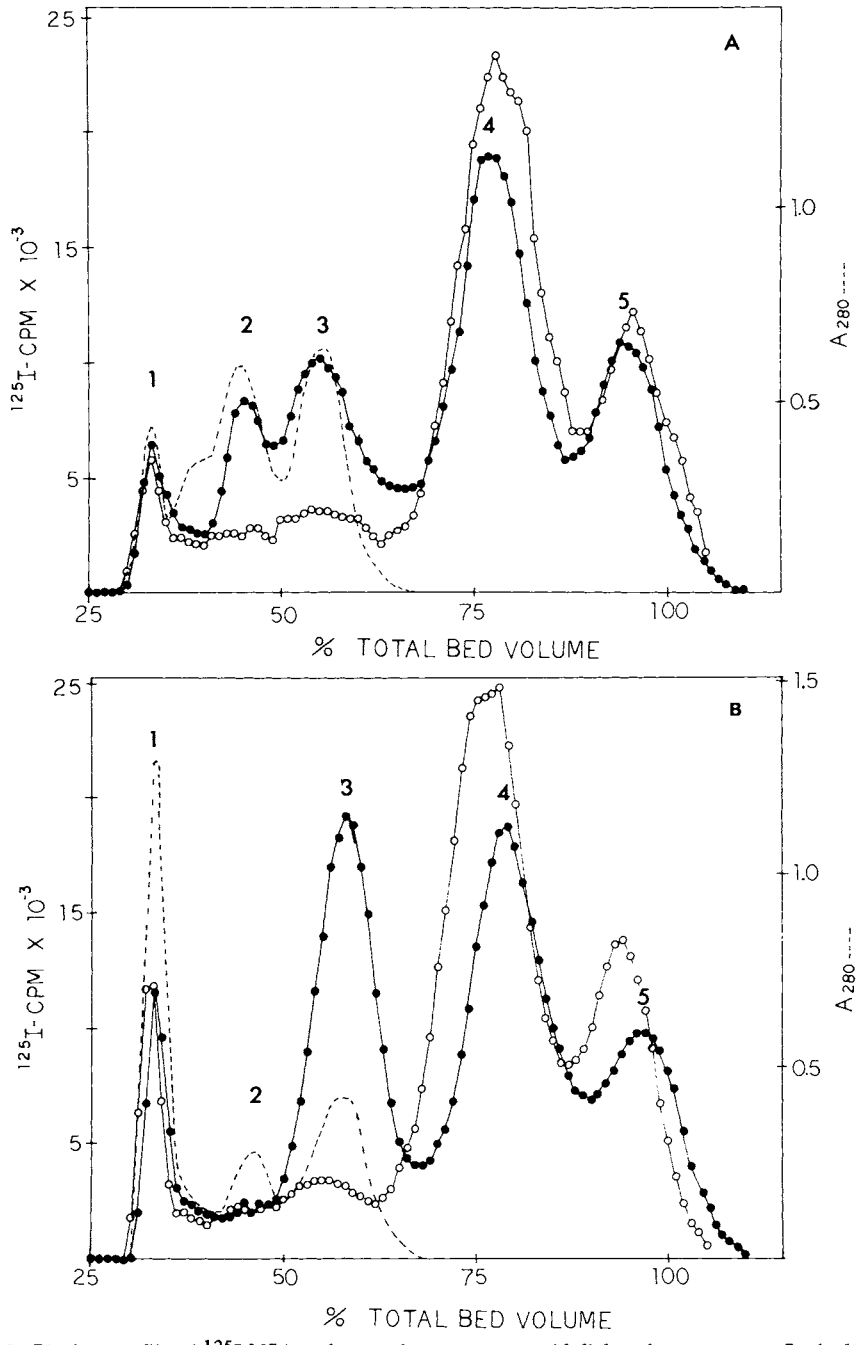

Fig. 5. Elution profile of ^{125}I-MSA and normal rat serum or acid-dialyzed rat serum on Sephadex G-200. ^{125}I-MSA was incubated with normal rat serum (A) or acid-dialyzed rat serum (B) for 5 h at 22°C. The reaction mixtures were applied to a 2.5 X 40 cm column of Sephadex G-200 equilibrated in PBS at 4°C. Fractions of 2.25 ml were collected and assayed for radioactivity (•). Identical mixtures contained 10 μg of unlabeled MSA for determination of specific binding (○). The dotted line represented the elution profile of 0.5 ml of calf serum monitored by absorbance at 280 nm, which was applied prior to each run to calibrate the column.

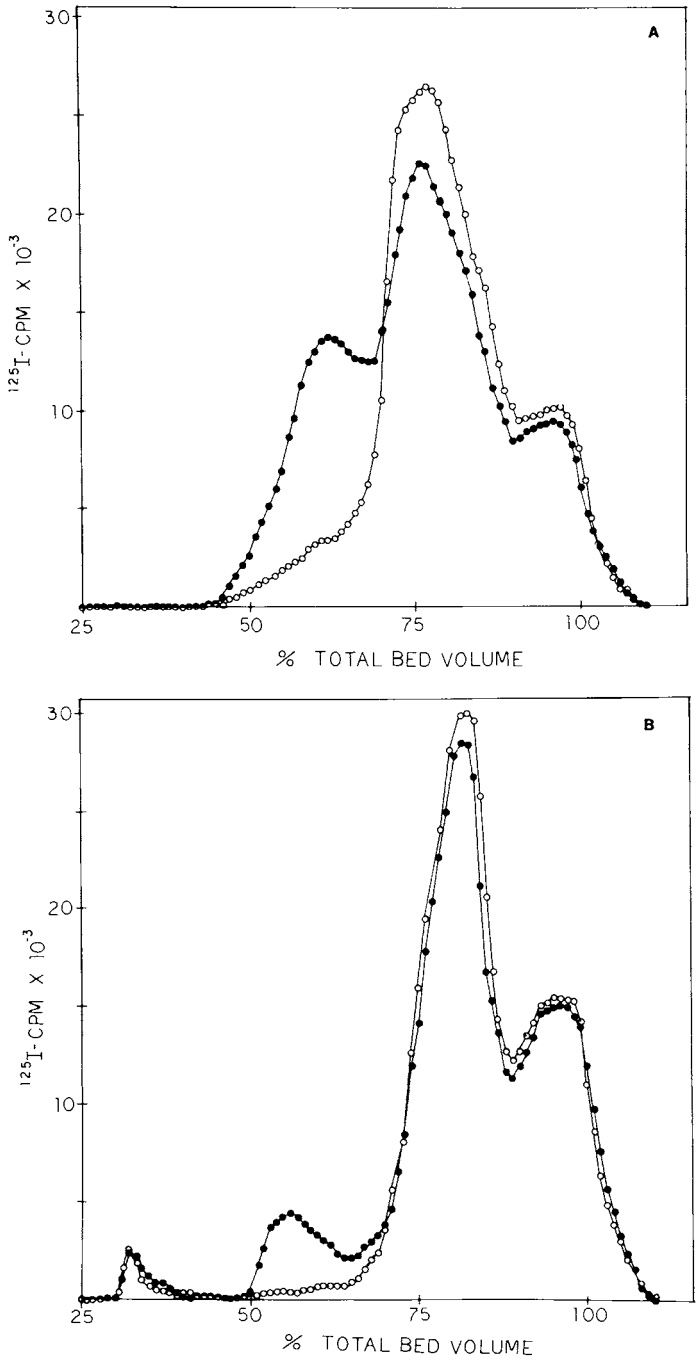

Fig. 6. Elution profile of ^{125}I-MSA and purified MCP or Dowex-50-treated rat serum. ^{125}I-MSA was incubated with purified MCP (A) or a Dowex-50 preparation of normal rat serum (B) for 5 h at 22°C. The reaction mixtures were then applied to the same Sephadex G-200 column described in Figure 5. Fractions were assayed for radioactivity (•). Identical reaction mixtures contained 10 μg of unlabeled MSA for determination of specific binding (○).

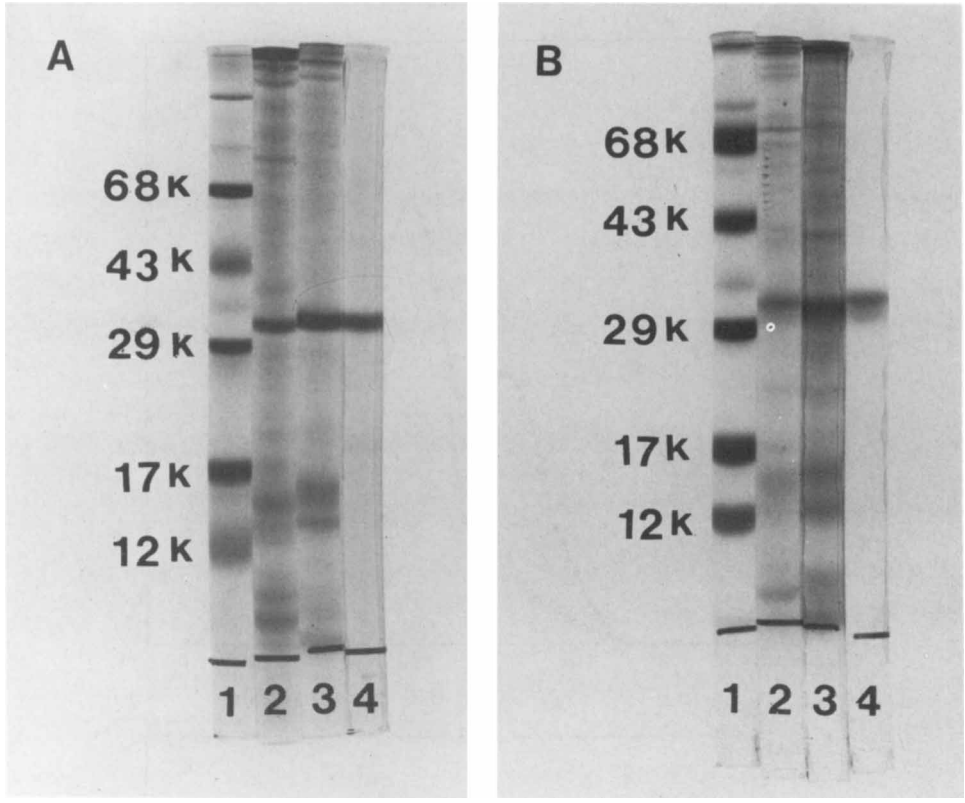

Fig. 7. Molecular weight determination of purified MCP. Samples containing (1) a mixture of standard molecular weight markers, (2) 30 μg of whole BRL-3A conditioned medium proteins, (3) 30 μg of Dowex-50 MSA, and (4) 5 μg of MCP purified by affinity chromatography were electrophoresed on 10% SDS polyacrylamide gels in the absence (A) or presence (B) of 2-mercaptoethanol.

BRL-3A2 cells are a subclone of BRL-3A cells that produce MSA carrier protein but do not produce MSA [14]. This allows a direct comparison of purified MCP to carrier protein in its native state in conditioned medium to determine if the MCP has been altered in any way by the purification procedure. The affinity constant for BRL-3A2 medium was determined to be $0.3 \times 10^9 M^{-1}$. These values are not significantly different. It was not possible to use BRL-3A medium for this purpose since MSA is produced in apparent excess, and all of the MCP is complexed with MSA. When ^{125}I-MSA is incubated with BRL-3A conditioned medium followed by fractionation on neutral Sephadex G-200, all of the radioactivity appears as free MSA (data not shown and personal communication with M. Rechler).

DISCUSSION

In this report, we have described the purification of the MSA carrier protein from BRL-3A rat liver cell condition medium. The purification scheme involved ion exchange chromatography using Dowex 50W-X8 resin in the Na^+ form. MSA is known to absorb to the resin at neutral pH and is then eluted at pH 11. Based on the amino acid composition of

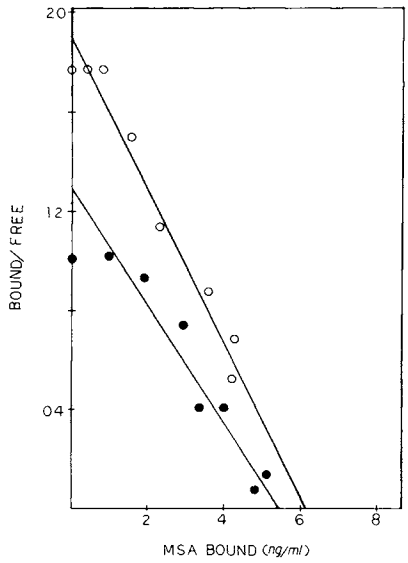

Fig. 8. Scatchard analysis of MSA binding to purified MCP or to BRL-3A2 conditioned medium Equal amounts of MCP (40 ng, •) or conditioned medium (100 μl, ○) were incubated with 500 pg of ^{125}I-MSA and increasing concentrations of unlabeled MSA (5-800 ng/ml) for 3 h in 0.4 ml of PBS containing 5 mg/ml fatty acid-free BSA. Bound MSA was then separated from free MSA by the fatty acid-free BSA activated charcoal procedure. The slopes of the lines were determined by linear regression analysis.

purified MSA, Dulak and Shing [19] previously suggested that MSA probably does not bind to the Dowex column directly but does so via more basic proteins in the conditioned medium. We now know that the carrier protein accounts for this behavior of MSA. Next the carrier protein-MSA complex is dissociated under acidic conditions and the components separated by chromatography on Sephadex G-50 in 1 N acetic acid. The carrier proteins, appearing in the void volume of the column can then be purified by specific binding to a column containing covalently bound MSA. It should be noted that the procedures used for the preparation of the affinity column involved the synthesis of the *N*-hydroxysuccinimide ester derivative of Sepharose 4B as described by Parikh et al [22]. This is a simple and mild technique to immobilize proteins essential via the α amino group to yield a ligand that is separated from the support by lengthy hydrocarbon extensions. This facilitates binding that might be hindered sterically in other methods of immobilization. In addition, since coupling is via primary amino groups, the possibility of inducing protein configuration changes is minimal.

The carrier protein purified by these procedures migrates as a single band in acetic acid-urea and in SDS polyacrylamide gel electrophoresis systems with an apparent molecular weight of approximately 31,500 daltons. In the SDS system under reducing conditions (2-mercaptoethanol), a second minor band appears with an apparent molecular weight of 30,000 daltons. Thus, the carrier protein appears to be composed of two different protein chains. The possibility that the components we have purified consist of a single chain with heterogeneity in the degree of glycosylation cannot be excluded at this time. This would require the separation and analysis of both species for amino acid and carbohydrate content. The molecular weight determinations may be subject to error since the carrier protein may

be a glycoprotein. This data is consistent with a report by Fryklund et al [26] that a somatomedin binding protein purified from human serum is a glycoprotein and is composed of two dissimilar protein chains with molecular weights between 35,000 and 45,000 daltons. Unfortunately, these investigators reported rather low total binding of somatomedin A by their purified preparation and further details were not given.

The MSA carrier protein purified by affinity chromatography bound labeled MSA and formed a complex that eluted from Sephadex G-200 with a molecular weight in excess of 60,000 daltons. This binding was specific and could be inhibited in the presence of unlabeled MSA. It is tempting to speculate that the complex is composed of one each of the carrier protein subunits and one MSA molecule; however, this must await more careful study. The size of the MSA-carrier protein complex seen in this report corresponds to the low molecular weight complex observed upon the addition of labeled somatomedin, NSILA or MSA to rat serum [14]. Moses et al [13, 14] showed that when labeled MSA is incubated with rat serum and then fractionated on Sephadex G-200 at neutral pH, radioactivity could also be detected in five peaks. Only binding to peaks II (mol wt $>$ 150,000) and III (mol wt $>$ 50,000) as also shown in this report were specific and could be inhibited by the presence of an excess of unlabeled MSA. The high molecular weight complex (peak 2) shows a striking sensitivity to acid treatment. When serum is treated with 1 N acetic acid and returned to neutral pH prior to mixing with the labeled MSA, binding in the region of peak 2 disappears [13, 14]. Attempts to regenerate the high molecular weight form following dissociation of the complex with acid have been unsuccessful [14]. While the relationship between these two forms of carrier is not yet clear, it has been suggested that the high molecular weight form is the functionally significant one and the lower molecular weight form may be a precursor [12–14]. First, Kaufmann et al [11] have shown that when labeled NSILA is injected into normal rats, radioactivity first appears in the low molecular weight complex followed by conversion into the high molecular weight form. In addition, Moses et al [13] demonstrated that the appearance of radioactivity in the high molecular weight complex is growth hormone dependent and does not occur in hypophysectomized rats. Chronic treatment with growth hormone restored the normal binding pattern.

The protein band observed here in acetic acid-urea gels was shown to be the carrier protein by detection of binding activity in extracts from gel slices that corresponded to the stained band in identical gels. Using these procedures approximately 300–500 μg of purified MCP were obtained from one liter of BRL-3A conditioned medium. Purified MCP was shown to be similar to native carrier protein in BRL-3A2 conditioned medium in that they had similar affinity constants. These values are similar to those previously reported by others [14] and encourage that assertion that purified MCP has not been altered significantly by the isolation techniques used.

In a separate report [27], we have shown that purified MCP will inhibit the biological activity of MSA on chicken embryo fibroblasts. The stimulation of glucose transport and thymidine incorporation by MSA are inhibited 70% and 96%, respectively, when equimolar quantities of MSA and MCP are present in the medium. MCP had no effect on insulin stimulation of these events in the same cells. These results suggest that the MSA-carrier protein complex described in this report is inactive on cells and that the free hormone must be released to exert its activity on responsive cells. The availability of purified components will facilitate studies to determine the nature of the high molecular weight complex (peak 3) in serum and the biological significance of these carrier protein complexes in the mechanism of somatomedin action. The isolation in pure form of the MSA carrier protein from BRL-3A cell conditioned medium is an important step in characteriz-

ing these carrier protein complexes and will provide the opportunity to understand their functional significance. The fact that the purified carrier protein described in this report, as well as the carrier protein from the BRL-3A2 clone that does not produce MSA [14], does not spontaneously form the high molecular weight complex when mixed with MSA, should not diminish the importance of this endeavor. The opportunity to utilize these purified components to search for the conditions or factors necessary for the conversion to the high molecular weight form of the complex now exists. The acid-sensitive, growth-hormone-dependent component of the larger complex does not appear to be the carrier protein itself since Moses et al [14] have shown that impure carrier protein preparations from serum and BRL-3A CM exposed to 1 N acetic acid have the same affinity for labeled MSA as the native carrier protein in BRL-3A2 CM.

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