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Partial Purification of HLAMP-1 Provides Direct Evidence for the Multicomponent Nature of the Particulate Matrix Associated With Cardiac Mesenchyme Formation

Allan R. Sinning*

Department of Anatomy, University of Mississippi Medical Center, Jackson, Mississippi 39216

Abstract H-LAMP-1 is a 283 kDa protein that is involved in the transformation of endothelial cells into mesenchyme within the AV canal and proximal outflow tract of the heart. This protein is part of the particulate matrix that has been suggested to be composed of multicomponent complexes that have been termed cardiac adherons. However, to date no direct evidence has been provided that these proteins are complexed into an adheron-like particle. This report provides the first such evidence by showing that purification of hLAMP-1, under gentle conditions, results in the isolation of multiple bands of similar molecular weight within the fractions that contain anti-hLAMP-1 activity. J. Cell. Biochem. 66:112–122, 1997. © 1997 Wiley-Liss, Inc.

Key words: cardiac cushion tissue; extracellular matrix; heart development; induction; epthelial-mesenchymal interactions; chick embryo

It has been hypothesized that the formation of cardiac mesenchyme within the atrioventricular canal and proximal outflow tract regions of the heart is initiated by the binding of a particulate form of extracellular matrix to the endothelial cell surface [Markwald et al., 1990a]. This matrix, which is produced by the myocardial layer of the heart in these areas, has been shown to contain at least 7–10 major proteins or groups of proteins which range from 30–283 kDa [Krug et al., 1985, 1987; Mjaatvedt et al., 1987; Mjaatvedt and Markwald, 1989; Sinning et al., 1992].

The particulate matrix was first identified as a subset of proteins within an EDTA extract of the embryonic heart [Krug et al., 1987]. These particulates were confirmed by immunostaining with various fibronectin antibodies. These studies revealed the presence of particulates within a more diffuse fibrillar matrix staining pattern in the atrioventricular canal and proximal outflow tract of the heart [Krug et al., 1987; Mjaatvedt et al., 1987; Shiraishi et al., 1995]. The fact that these particles were embedded within a heavily staining fibrillar matrix has made isolation of these particles difficult. The lectin *Glycine max* (soybean agglutinin; SBA) also co-localized with the fibronectin particles and did not stain the fibrillar matrix, suggesting that this molecule could be used to specifically isolate the particulate matrix [Sinning et al., 1992]. This lectin has subsequently been used to isolate the biologically active particulate matrix from both EDTA extract and myocardial conditioned media (MyoCM) [Sinning et al., 1992, 1995; Sinning and Hewitt, 1996]. However, while SBA labels only particles *in vivo*, which are extracted with EDTA, no direct correlation has been made that these proteins are associated together as a multicomponent complex.

The appearance of different groups of protein within this matrix has led to the assumption that the active components of this matrix are organized as multicomponent complexes that have been termed cardiac adherons [Mjaatvedt and Markwald, 1989; Markwald et al., 1990b; Bolender and Markwald, 1991]. Adherons were

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*Correspondence to: Dr. Allan R. Sinning, Department of Anatomy, University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216-4505.
E-mail: ars@anat. umsmed.edu

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first described as a multicomponent complex secreted from L6 muscle cells [Schubert and LaCorbiere, 1980, 1985]. These complexes contain fibronectin, collagen, and several other glycosaminoglycans. The proposed cardiac adherons also contain fibronectin and several glycoproteins that have not been identified [Brauer and Markwald, 1988; Mjaatvedt and Markwald, 1989]. In addition, transferrin, ES130, and a new protein hLAMP-1 have been identified as components of the particulate matrix [Rezaee et al., 1993; Isokawa et al., 1994; Sinning and Hewitt, 1996]. However, no direct evidence to date has shown that these proteins are associated in a multicomponent complex.

HLAMP-1 is a 283 kDa protein that has been isolated from MyoCM and EDTA extract [Sinning and Hewitt, 1996]. This protein was isolated using a monoclonal antibody produced from the SBA bound fraction from MyoCM, labels the particulate matrix in the heart and can block mesenchyme formation in an *in vitro* model of mesenchyme formation [Sinning and Hewitt, 1996]. This report describes the partial purification of hLAMP-1 by size exclusion, ion exchange, and affinity chromatography. One of the consequences of these experiments was the isolation of multiple bands in the fractions that reacted with anti-hLAMP-1. In addition, the extra bands in the different preparations were similar, providing direct evidence that the particulate matrix is composed of multicomponent complexes.

METHODS

Production of Myocardial Conditioned Media

Myocardial cultures were prepared with minor modification as previously described [Krug et al., 1987; Sinning et al., 1988]. Briefly, hearts from Hamburger and Hamilton stage 15–16 chick embryos were removed and washed extensively in calcium and magnesium-free phosphate buffered saline (PBS) to remove any adherent yolk [Hamburger and Hamilton, 1951]. The hearts were then incubated for 1 h in 5 mM EDTA in PBS containing 1 mM phenylmethylsulfonyl flouride (PMSF) at 4°C with gentle mixing. The hearts were pelleted by centrifugation at 50*g* for 5 min and the EDTA removed. Following removal of the EDTA, the hearts were rinsed 5 times in PBS and then incubated for 5 min with 0.5% trypsin at 37°C with occasional mixing. The hearts were repetitively pipetted to ensure adequate formation of a cell suspension and incubated for an additional 2 min. Trypsin activity was suppressed by mixing the cell suspension with 1 ml of chick serum. The cell suspension was then transferred to a 15 ml centrifuge tube with 10 ml of M199 containing 1% chick serum, ITS (5 g/L insulin, 5 g/L transferrin, 5 mg/L selenium) and antibiotics (100 U/ml penicillin and 100 g/L streptomycin). The suspension was then centrifuged at 500*g* for 5 min, the cell pellet resuspended in 10 ml of medium and centrifuged again at 500*g* for 5 min and the supernatant poured off. The pellet was resuspended in medium to provide approximately 17 hearts/ml and 2 ml aliquots were transferred to 35 mm gelatin coated tissue culture plates. The cultures were incubated for 24 h and the nonadherent cells removed. After 3 days, the cultures were placed in EX-CELL 300 without serum. Media was harvested every 2 days and centrifuged at 1,000*g* for 10 min to remove cellular debris. Protease inhibitors were added (1 mM PMSF, 1 µM Leupeptin, 1 µM pepstatin, and 1 mM EDTA) and the medium stored at 4°C. Medium for bioassay experiments was stored without the addition of protease inhibitors.

Size Exclusion Chromatography

MyoCM was subjected to SEC under native conditions. In these experiments MyoCM was dialyzed into 20 mM Tris-HCl and then concentrated 20-fold. A 50-µl aliquot of this solution was injected onto an ultraspherogel 3000 HPLC column (Beckman Inc., Palo Alto, CA) at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected between 4 and 14 min into the run. Fractions were then tested for hLAMP-1 reactivity using a dot blot assay and analyzed for total protein by method of Beardon [1978]. Fractions positive for hLAMP-1 were then separated by SDS-PAGE.

Ion Exchange Chromatography

MyoCM was dialyzed into 20 mM Tris-HCl (pH 7.4) and separated on 4 ml Macro-Prep DEAE column. (Bio-Rad Inc., Richmond, CA). In a typical experiment 20–30 ml of MyoCM was loaded onto the column followed by 5 column volumes of buffer. The bound proteins were eluted using a linear salt gradient from 0–700 mM sodium chloride in 20 mM Tris-HCl. Fractions (1.0 ml) from the run were tested for hLAMP-1 activity by either Western blot or dot blot analysis. Fractions positive for hLAMP-1

were pooled and then separated by SDS-PAGE to determine the proteins present in these preparations. In some experiments the samples were dialyzed against 20 mM Tris-HCl concentrated to 50 µl using ultrafiltration and injected onto the SEC column.

Affinity Chromatography

An anti-hLAMP-1 affinity column was constructed using a 1 ml Avidchrom hydrazide column (Unisys Tech, San Diego, CA) following the manufacturers instructions. Briefly anti-hLAMP-1 was purified from ascites fluid or from hybridoma culture supernatant by passage over a Protein A AvidChrom cartridge (Unisys Tech.), according to the manufacturers instructions. An aliquot of the purified anti-hLAMP-1 (5.0 mg) was exchanged into 50 mM sodium acetate (pH 5.0) and the volume adjusted to 4.5 ml. The purified IgG was oxidized by adding 0.5 ml of 100 mM sodium periodate the vial sealed, wrapped in aluminum foil and allowed to incubate for 30 min at room temperature. The oxidized IgG was then introduced into the cartridge and allowed to incubate for 15 min. The cartridge was then washed with 5 ml of PBS containing 0.5 M sodium chloride to remove the non-covalently bound IgG and then with PBS.

MyoCM dialyzed into PBS was pumped onto the anti-hLAMP-1 column at 0.2 ml/min using a Bio-Rad Econosystem. The cartridge was washed with 10 ml of PBS to remove the unbound protein and eluted using 0.1 M Glycine-HCl (pH 2.5). The eluted peaks were immediately neutralized by passage through a 5-ml desalting cartridge equilibrated with 20 mM HEPES buffer (pH 8.0) and fractions checked for protein by absorbance at A280. Peaks positive for protein were pooled and the concentration of protein in the pooled fractions determined [Beardon, 1978].

Electrophoresis and Western Blotting

Samples for electrophoresis were either precipitated overnight with 10% tricloroacetic acid or concentrated using Amicon (Danvers, MA) ultrafiltration devices. The TCA pellets were washed once in ice cold ethanol, then in ether/ethanol (1:1) and finally in 100% ether and allowed to air dry. The air-dried pellets were resuspended in water followed by sodium dodecyl sulfate (SDS) solubilization buffer containing 40 mM dithiothreitol and boiled for 5 min. The solubilized proteins were separated on a 7.5% SDS polyacrylamide gel following the method of Laemmli [1970]. Concentrated samples were diluted directly into solublization buffer and loaded onto the gel. The separated proteins were either silver stained [Oakley et al., 1980] or further processed for Western blot analysis [Towbin et al., 1979]. Samples for Western blot analysis were transferred to polyvinylidene diflouride (PVDF) blotting membrane (Bio-Rad) using 25 mM tris, 192 mM glycine, and 20% v/v methanol. The resulting blot was blocked with 5% blotto in Tris buffered saline (TBS) for 1 h at room temperature, washed 3 times in TBS containing 0.05% Tween 20 (TBST), and then stained in primary antibody diluted 1:40,000 in TBS containing 1% blotto for 2 h. Following rinses in TBST, the blot was incubated with an alkaline phosphatase labled goat anti-mouse IgG (1:3,000) for 2 h. Following additional rinses in TBST the blot was developed with an alkaline phosphatase development kit (Bio-Rad).

Dot Blot Assay

The presence of hLAMP-1 in the various samples was determined by a dot blot assay. In these runs 100–200-µl aliquots of the fractions or MyoCM were added to individual wells of a Bio-Rad dot blot apparatus. Following passage of the samples through the nitrocellulose, the wells were washed with 300 µl of TBS. The nitrocellulose sheet was then removed from the apparatus and blocked for 1 h in 5% blotto in TBST. The blot was then incubated in anti-hLAMP-1 (1:40,000) for 2 h, rinsed 3–4 times with TBST, and then incubated in an alkaline phosphatase labeled 2nd antibody for 2 h. Finally after several rinses in TBST, the blot was developed with an alkaline phosphatase development kit (Bio-Rad).

RESULTS

Size Exclusion Chromatography (SEC)

In an effort to isolate hLAMP-1, MyoCM was separated on a Beckman 3000 ultraspherogel SEC column. This separation resulted in the identification of 12 separate peaks (Fig. 1). Fractions from these runs were analyzed using a dot blot assay to determine the fractions with hLAMP-1 activity, and then separated by SDS-PAGE. Dot blot assays revealed that hLAMP-1 resided in fractions that corresponded to 5.5–7.0 min into the run (solid circles in Fig. 1).

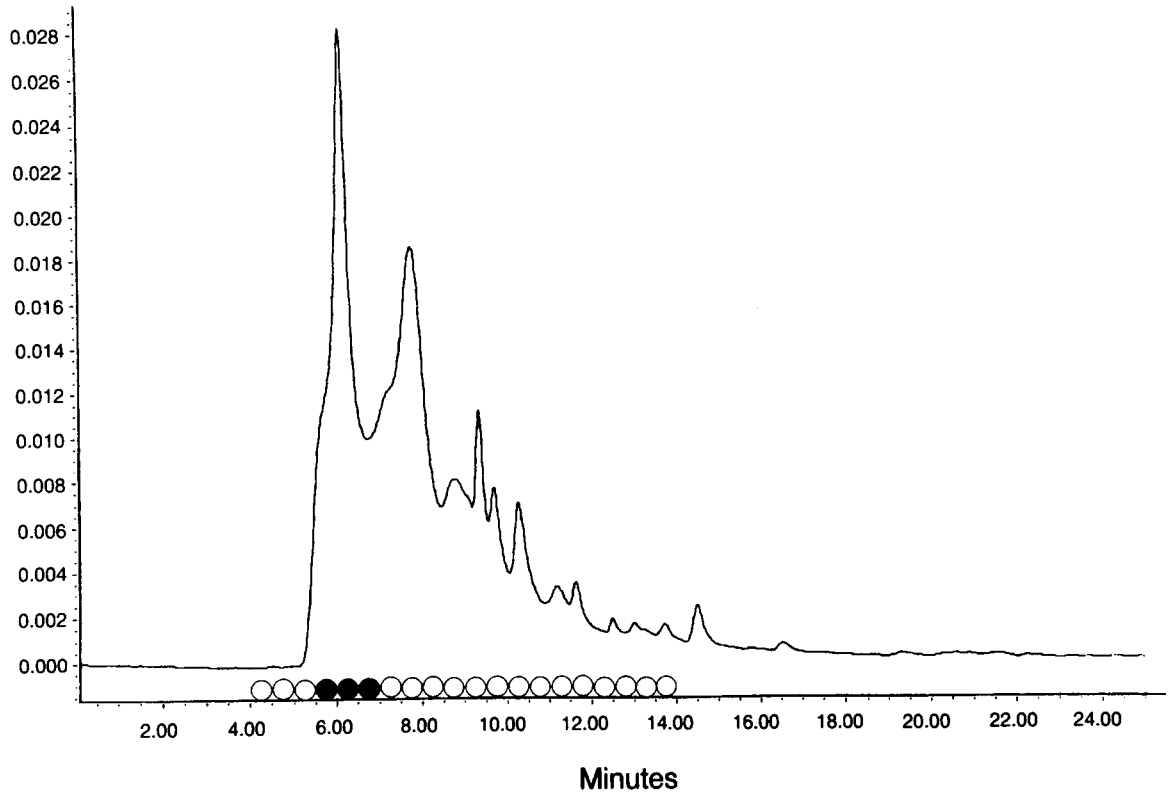

Fig. 1. Chromatogram of MyoCM separated by SEC. SEC resulted in the identification of 12 separate peaks that eluted from 5–15 min into the run. Dot blot assays (circles) revealed that hLAMP-1 activity was restricted to the first peak off the column (black circles). This fraction corresponded to a substance with a molecular weight of greater than 600 kDa.

Based on protein standards run on this column, these fractions represented proteins with an aggregate molecular weight of greater than 600 kDa.

The presence of hLAMP-1 in these fractions was verified by Western blot analysis (not shown) and SDS-PAGE (Fig. 2). In these preparations, a 283 kDa band corresponding to hLAMP-1 was observed in fractions representing 5.5–7.0 min (Fig. 2, lanes C, D, and E) with the highest concentration of hLAMP-1 seen at 6.0–6.5 min (Fig. 2, lane D). In addition to hLAMP-1, these fractions also contained other protein bands, suggesting that hLAMP-1 may be associated in some manner to these other proteins. Included in this group were bands at 200 (Fig. 2, lane C), 160 (Fig. 2, lane E), 130 (Fig. 2, lane E), 90, 80, 70 (Fig. 2, lanes C, D, E), and 53 kDa (Fig. 2, lane C). In addition bands of 90, 80, 70, and 45 kDa were also seen in other fractions, suggesting the presence of multiple proteins at these molecular weights.

Affinity Chromatography (AC)

To aid in the purification of hLAMP-1, an anti-hLAMP-1 affinity column was constructed using a 1-ml AvidChrom hydrazide cartridge (Unisys Tech.) following the manufacturers instructions. In these experiments, more protein was eluted than expected, from previous scanning densitometry studies of hLAMP-1 in conditioned media, suggesting the presence of other proteins in this fraction. This was confirmed by SDS-PAGE of the various fractions (Fig. 3; Fig. 4, lane A). In these gels, a distinct band at 283 kDa representing hLAMP-1 was observed in association with the bound fraction (Fig. 3, lane C; Fig. 4, lane A) and unfractionated MyoCM (Fig. 3, lane A). This band was seen to be greatly reduced or absent in the pass-through fraction (Fig. 3, lane B). In addition to hLAMP-1, additional protein bands were observed within the eluted fraction. While these experiments showed some variability in the runs, presumably because of protein load (compare Fig. 3,

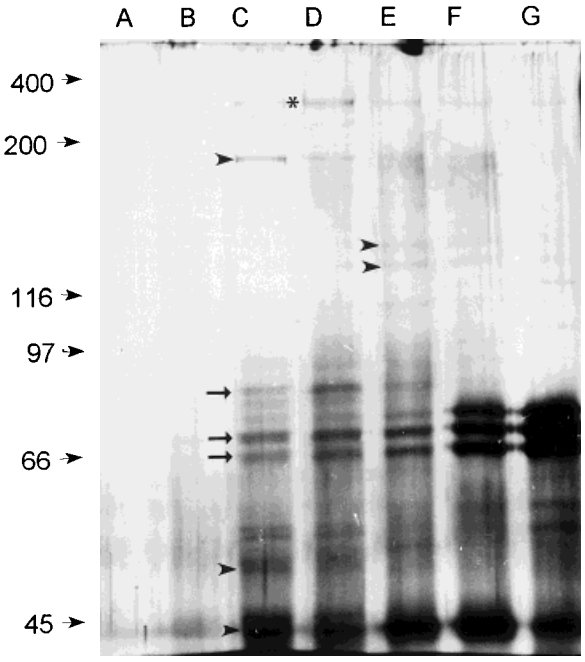

Fig. 2. SDS-PAGE of SEC fractions. Fractions 2–8 (Lanes A-G) from the SEC runs were separated to reveal the protein(s) within these active fractions. A large molecular weight protein corresponding to hLAMP-1 (*) was observed in fractions 4–6 (**lanes C, D,** and **E**). In addition, bands were observed at 90, 80, and 70 kDa (arrows) and at 200, 160, 130, 53, and 45 kDa (arrowheads).

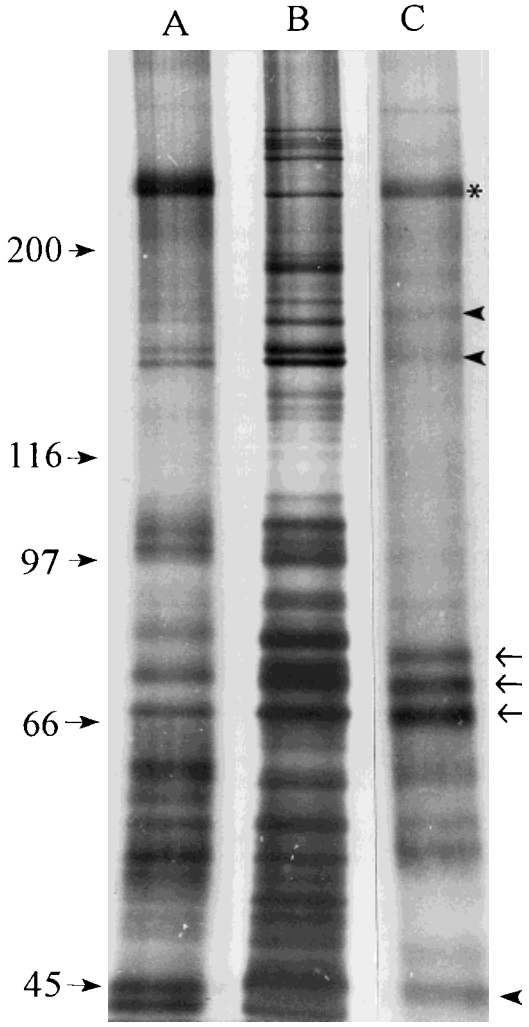

Fig. 3. SDS-PAGE of fractions from affinity coloumn. The eluted fraction (**lane C**) from the anti-hLAMP-1 column was compared to unfractionated MyoCM (**lane A**) and the pass-through fraction (**lane B**). A large band representing hLAMP-1 was present within the unfractionated and eluted fractions (*) but was greatly reduced or absent in the pass-through fraction. In addition, the eluted fraction contained several other major (arrows) and minor bands (arrowheads). These bands had kDa's similar to that seen in the SEC experiments.

lane C and Fig. 4, lane A), these other bands consistently ranged from 35–220 kDa and included major bands at 70, 80, and 90 kDa. Also, bands at 45, 130, and 200 kDa were observed. Interestingly, these bands were similar in molecular weight to those observed in the SEC experiments.

Western blot analysis of these same fractions confirmed that this band was hLAMP-1 (Fig. 4). Note the absence of staining in the pass-through fraction (Fig. 4, lane C) as compared to the bound fraction (Fig. 4, lane B) or unfractionated MyoCM (Fig. 4, lane D). In addition, in some preparations the antibody also appeared to stain a band of slightly lower molecular to hLAMP-1 (Fig. 4, lane B). Again this may be due to increased protein load as it was only variably seen in these preparations (compare Fig. 3, lane C and Fig. 4, lanes A and B).

Ion Exchange Chromatography (IEC)

To determine if the proteins isolated by SEC and AC were held together by ionic interactions that were not disrupted under the gentle conditions used, MyoCM was subjected to IEC using a DEAE-Macroprep column (Bio-Rad). For these experiments 20 ml of MyoCM was dialyzed into 20 mM Tris-HCL (pH 7.5) and pumped onto a 4.0 ml column and eluted with a linear salt gradient of 0–700 mM NaCl. In these experiments, four distinct protein peaks were observed at 25, 75, 93, and 145 min into the run, with the remainder of the protein being unresolved into distinct peaks (Fig. 5). Analysis of these fractions by dot blot analysis revealed that anti-hLAMP-1 activity was restricted to fractions that eluted from 128–144 min into the run. These reactive fractions eluted with an

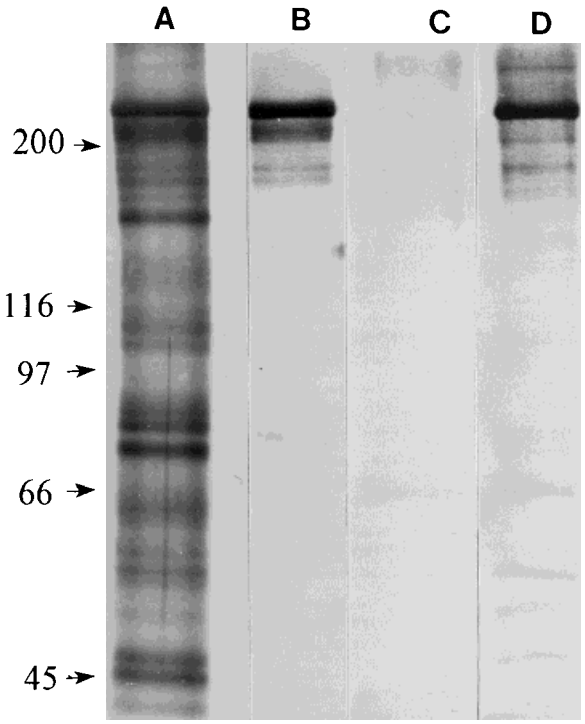

Fig. 4. Western blot of affinity purified hLAMP-1. Western blot analysis revealed that anti-hLAMP-1 recognized one band in the eluted fraction from the anti-hLAMP-1 column (**lane B**) and in unfractionated MyoCM (**lane D**). However, the pass-through fraction (**lane C**) was unreactive, indicating the specificity of the antibody for hLAMP-1. A representative sample separated by SDS-PAGE and silver stained is shown in **lane A** for comparison.

average salt concentration of 443 mM, suggesting a very acidic mixture of protein, and are represented by the darkened region on the chromatograph. Western blot analysis of the pooled fractions also confirmed the presence of hLAMP-1 (not shown). The concentration of protein in these samples was also higher than would be expected for pure hLAMP-1, suggesting that a group of components was eluted at this concentration. SDS-PAGE confirmed the presence of a mixture of proteins in these fractions (Fig. 6, lane A). In addition to hLAMP-1, several bands with kDa's similar to that seen in the other preparations were observed. These included bands at 45, 65–70, 90, and 200 kDa. To determine if these bands were complexed together or just co-eluted because of similar P.I.'s, an aliquot of the pooled fraction was separated by SEC. In these experiments, a large peak similar to that seen in previous experiments utilizing MyoCM was observed at around 7 min into the run (Fig. 7). SDS-PAGE of these fractions revealed hLAMP-1 and most of the protein in this preparation co-eluted from 6.0–6.5 minutes into the run, further suggesting that these proteins were part of a multicomponent complex (Fig. 6).

DISCUSSION

The production of mesenchyme within the atrioventricular canal and proximal outflow tract is a critical event in early heart development. This process is mediated by a particulate form of extracellular matrix that is secreted by the myocardial layer of the heart [Markwald et al., 1990a; Huang et al., 1995; Little and Rongish, 1995]. Previous studies have suggested that the particulate matrix may be composed of multicomponent complexes that have been termed cardiac adherons [Mjaatvedt and Markwald, 1989; Markwald et al., 1990b; Bolender and Markwald, 1991]. This assumption was based on the fact that the active fractions present in these different preparations always consisted of multiple bands when separated by SDS-PAGE [Krug et al., 1985, 1987; Mjaatvedt et al., 1987; Mjaatvedt and Markwald, 1989; Sinning et al., 1992, 1995]. However, while previous studies have identified several proteins that reside in these preparations, they have been unable to clearly distinguish if these proteins actually comprise a multicomponent complex [Rezaee et al., 1993; Isokawa et al., 1994; Sinning and Hewitt, 1996].

The majority of studies investigating the particulate matrix have involved the heart; however, antigens associated with this complex have been localized to other areas of the embryo that undergo a similar type of tissue interaction. Included in these areas are Henson's node, the heart-forming fields, the branchial arch apparatus, underneath the limb ectoderm and associated with trunk neural crest pathways [Brauer and Markwald, 1988; Mjaatvedt and Markwald, 1989; Sinning et al., 1995; Sinning and Hewitt, 1996; Smith et al., 1997]. These studies suggest a more global role for the particulate matrix in development, making the identification and functional role of the individual components of this complex a priority.

HLAMP-1 is a recently described 283 kDa protein component of the particulate matrix [Sinning and Hewitt, 1996]. This protein was isolated using a monoclonal antibody called anti-hLAMP-1, which was made from the SBA binding fraction of MyoCM [Sinning and Hewitt, 1996]. This antibody does not react with other

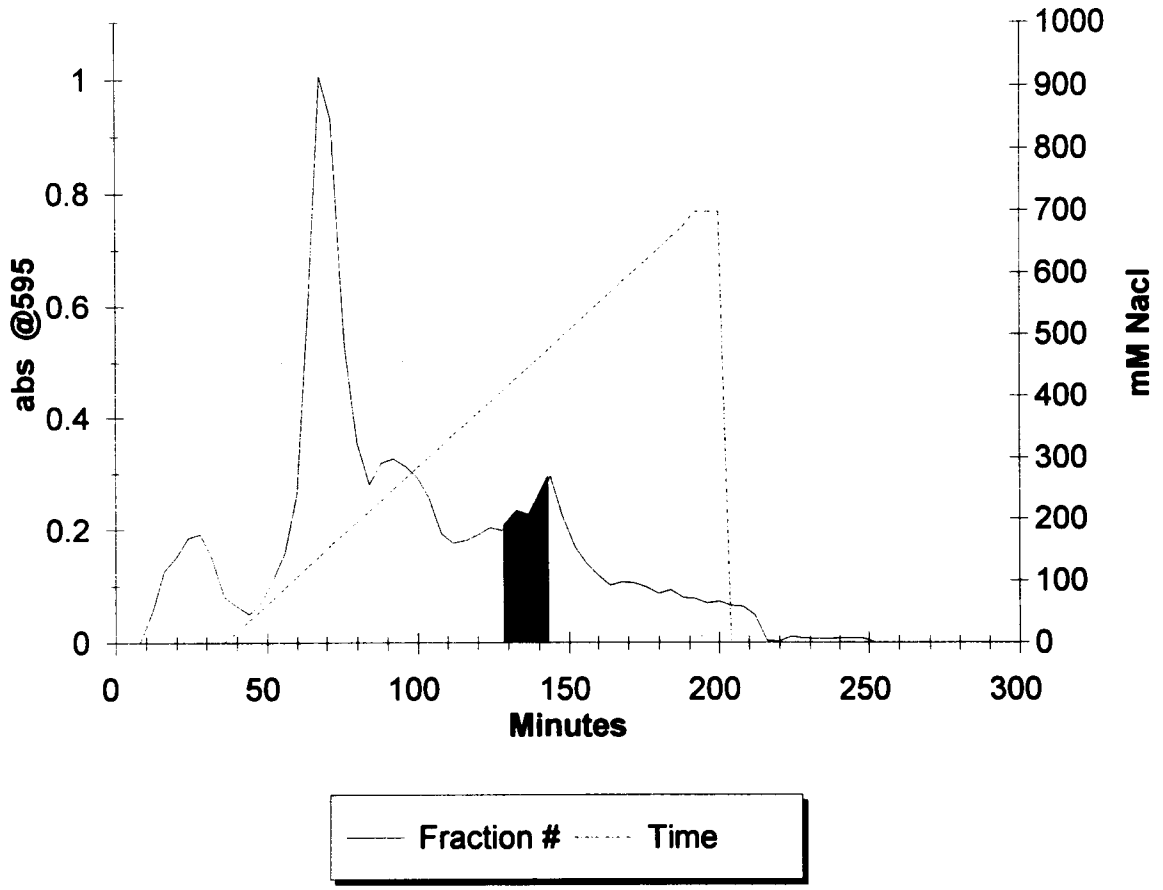

Fig. 5. Chromatogram of MyoCM separated by IEC. Fractions from the IEC run were analyzed for total protein following the method of beardon [Beardon, 1978] and the absorbance at 595 plotted against the time into the run (solid line). A linear salt gradient from 0–700 mM NaCl over 160 min was used to elute the proteins (dashed line). Western blot and dot blot analysis revealed the hLAMP-1 activity was restricted to fractions from 128–144 min into the run (blackened area).

high molecular weight matrix proteins (i.e., fibronectin and cytotactin) present in the myocardial basement membrane prior to mesenchyme formation. In addition the hLAMP-1 antigen has been implicated as an early marker of left-right laterality within the embryo based on its increased expression within the left heart field of the embryo [Smith et al., 1997]. The high specificity of the anti-hLAMP-1 antibody for its antigen has allowed for the localization of hLAMP-1 following chromatographic separation of the particulate matrix. Specifically, anti-hLAMP-1 activity was used to determine the location of the hLAMP-1 protein in chromatography fractions by dot blot or Western blot analysis. These fractions were then analyzed by SDS-PAGE to determine if any other proteins were associated with hLAMP-1 in these fractions.

Separation of MyoCM by size exclusion chromatography resulted in hLAMP-1 activity being restricted to fractions that eluted with an average elution time of 6.25 min. This corresponded to a protein or a group of proteins with a combined molecular weight of around 670 kDa. This suggested that hLAMP-1 was part of a larger complex that co-eluted with other proteins. SDS-PAGE of these fractions revealed that hLAMP-1 was indeed part of a large complex. This observation was verified using an anti-hLAMP-1 affinity column to isolate the hLAMP-1 protein. These experiments also isolated multiple molecular weight species from MyoCM. Similar molecular weight species have been associated with the ES proteins found in EDTA extract and associated with the lectin SBA binding proteins isolated from EDTA extract and MyoCM. Unfortunately, these other methods all involved nonspecific approaches for isolating the components and could not clearly distinguish which if any of these proteins were actually part of the particulate matrix

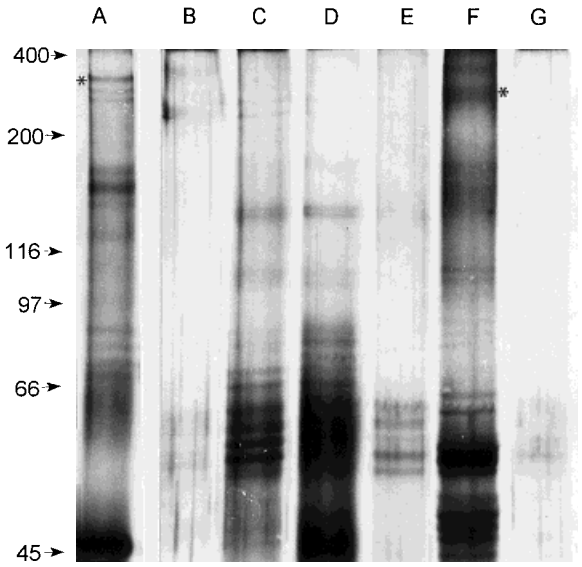

Fig. 6. SDS-PAGE of IEC. The reactive fractions from the IEC experiments were pooled and loaded directly onto a 7.5% gel (**lane A**) or concentrated and loaded onto the SEC column and the separated fractions loaded onto the gel (**lanes B–G**). Separation of the pooled fraction revealed hLAMP-1 (*) and several other proteins, similar to that seen in the other experiments (**lane A**). When this fraction was further separated by SEC, hLAMP-1 was restricted to fraction 5 (**lane F**). In addition, most of the other proteins seen in the IEC fraction were also present in this fraction, indicating that these proteins are tightly bound together.

[Mjaatvedt et al., 1991; Sinning et al., 1992, 1995; Rezaee et al., 1993; Isokawa et al., 1994].

The SEC and anti-hLAMP-1 affinity chromatography experiments verified that these proteins were part of a complex but provided no evidence as to the nature of the interaction between these proteins. Consequently, it was decided to separate the proteins by ion exchange chromatography to determine if these proteins were held together by ionic interaction. The data suggests that some of these proteins may be held together by ionic interaction. In addition, SEC of the IEC separated fractions suggests that some of these proteins are held together by some other type of interaction. The exact nature of this interaction is only now being investigated. However, since the complex is broken up during SDS-PAGE a likely candidate could be intermolecular disulfide bonding. These bonds are typically reduced during SDS-PAGE, allowing the individual components to separate [Laemmli, 1970]. Preliminary experiments in our laboratory support this idea. In these experiments reduced and nonreduced samples were run on the same 6% SDS gel. The nonreduced samples in these preparations were found either within the stacking gel or at the interface being the stacking and running gel, suggesting the presence of a large complex of protein in these samples.

Two other proteins (transferrin and ES130) have been identified as part of the particulate matrix [Rezaee et al., 1993; Isokawa et al., 1994]. Proteins with molecular weight corresponding to these proteins are present in the hLAMP-1 positive fractions, suggesting that these proteins are present in these preparations. While there is no direct evidence that the 70 kDa band in our sample is transferrin, experiments in our laboratory do suggest that ES130 is a component of the bound fraction from the hLAMP-1 column (data not shown). In addition, a latent form of transforming growth factor beta (TGF-β) has been shown to be present in the extracellular matrix of the developing heart [Ghosh and Brauer, 1996]. TGF-β has also been implicated to play a role in mesenchyme formation but has of yet not been associated with the particulate matrix [Choy et al., 1990; Potts et al., 1991, 1992; Runyan et al., 1992; Dickson et al., 1993; Nakajima et al., 1994; Huang et al., 1995]. Latent TGF-β has been described as a 100–110 kDa complex that is composed of a 25 kDa peptide and a 70–80 kDa subunit that remain associated after secretion from the cells [Gentry et al., 1988; Madisen et al., 1990; Miyazono et al., 1990]. A 90–100 kDa protein that may represent latent TGF-β is present in these fractions. We are currently planning experiments to determine if this band is latent TGFβ.

The data presented here confirm that hLAMP-1 is associated with a multicomponent complex that directs mesenchyme formation. Based on data presented in this report and elsewhere the following model is proposed (Fig. 8). In this model, fibronectin acts as a scaffold for the remaining molecules that are associated with hLAMP-1, leaving the cell binding domain of fibronectin exposed for interaction with endothelial cells. This is in agreement with previous studies that localized fibronectin to particles [Mjaatvedt et al., 1987; Mjaatvedt and Markwald, 1989; Sinning et al., 1992]. The remainder of the complex is then composed of the molecules that associate with hLAMP-1. Based on data in this study, those molecules that are held together by strong interactions are overlapped while those held together by weaker

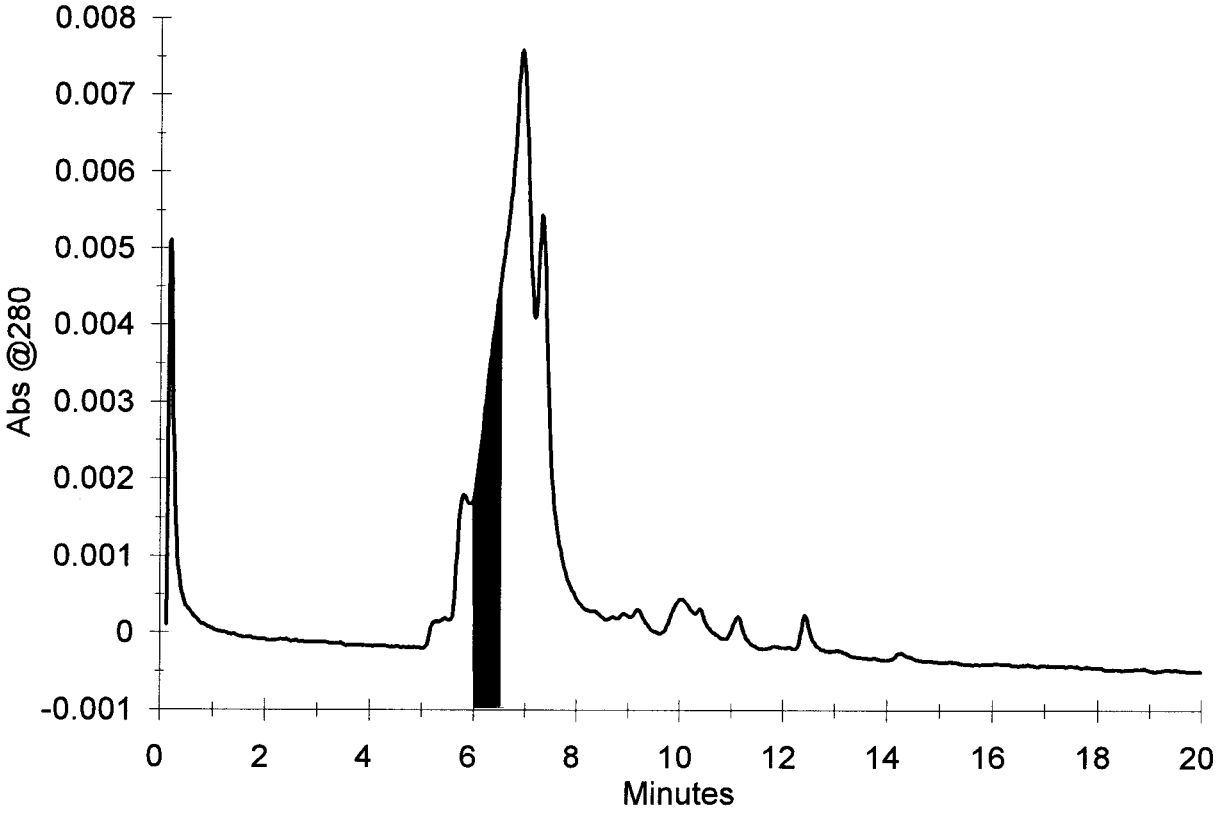

Fig. 7. Chromatogram of pooled IEC fractions separated by SEC. SEC of the IEC fractions resulted in a separation that resembled Figure 1. HLAMP-1 was restricted to a fraction that eluted from 6–6.5 min (darkened region).

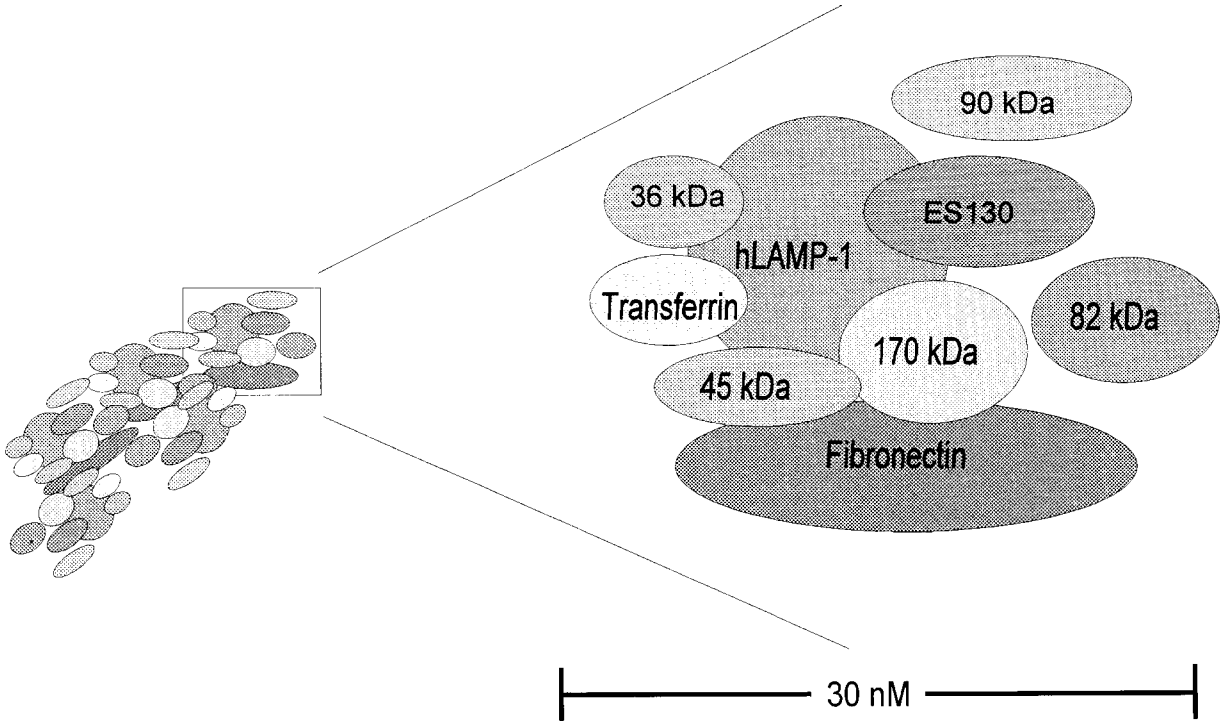

Fig. 8. Model of the particulate matrix. The particulate matrix is composed of multicomponent complexes that involve hLAMP-1 and several other proteins. Based on the work of Mjaatvedt and Markwald [1989], fibronectin is thought to help organize the individual complexes into the particulate matrix that is visible in the light microscope (right side). The individual complexes are composed of proteins that appear to be either strongly or weakly associated with hLAMP-1. In this model, proteins that are strongly associated with hLAMP-1 are depicted as being overlapped while those weakly associated with the complex are left free-standing.

associations appear next to each other. While the specific configuration of the proteins in the complex is unknown this model correlates with current and previous reports on the particulate matrix. For example, fibronectin has been shown to be associated with the particulate matrix in several reports [Mjaatvedt et al., 1987; Mjaatvedt and Markwald, 1989; Sinning et al., 1992] but could not direct the transformation process itself, suggesting a supportive role in this process. The fact that fibronectin is variably seen in the various techniques utilized in this report suggests that fibronectin is either loosely associated with the hLAMP-1 complex or only a minor component of the complex. This model also suggests that multiple hLAMP-1 complexes interact with fibronectin to produce the larger complex seen in electron microscopy [Mjaatvedt and Markwald, 1989].

Finally while this report identifies the proteins that are complexed with hLAMP-1, the role of these proteins in mesenchyme formation has yet to be established. These experiments, however, set the stage for future studies in which the isolated complex or more importantly the individual members of the complex will be tested for biological activity. These future experiments will allow for a determination of which, if any, of these polypeptides are truly the functional components of the particulate matrix.

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