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Membrane-bound heparin binding proteins from HL-60 cells purified in a two-step affinity chromatography differentially eluted with divalent cations

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Abstract

Solubilized membrane proteins from HL-60 cells were separated by two-step affinity chromatography. Proteins eluted with $MgCl_2$ in the first heparin-gel were applied to the second heparin-gel and eluted with $CaCl_2$. The eluted proteins were analysed and purified by electrophoresis. N-terminal amino acid sequences of eight proteins on the characteristic bands were determined. Homology search for the sequences indicated that three microsomal proteins, two nuclear proteins and a glycolytic enzyme were eluted with divalent cations, whereas a nuclear ribonucleoprotein and a membrane–cytoskelton linker protein were not dissociated with divalent cations, but with 2 *M* NaCl. Heparin affinity chromatography combined with differential elution with divalent cations can be a useful method for separation of membrane proteins.
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1. Introduction

Isolation of native proteins from normal cells is important for study of the structure and function of specific proteins. For the membrane proteins to be purified, the choices of a detergent for solubilization and effective resins for subsequent purification are of primary interest.

Heparin shows a high affinity for antithrombin III and other blood-coagulating proteins. Heparin can also bind to such different types of proteins as certain receptors, growth hormones and enzymes involved in nucleic acid metabolism. Based on these interactions, affinity chromatography using heparin-gels has been applied to purify these proteins. Moreover, heparin has been shown to bind to specific proteins on cell surfaces of a number of cell types, including bovine uteri [1], lung carcinoma cells [2], rat brain [3], human neutrophils [4], endothelial cells [5], uterine epithelial cell line [6] and platelets [7,8]. Some of these reports indicated that heparin has the ability to interact not only with water-soluble proteins but also with membrane proteins solubilized with detergents.

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Previously, we observed that guinea pig leucocytes could be fragilized by the treatment with a low concentration of heparin after an extensive washing of the cells with a salt-free solution, while the cells were quite resistant to mechanical disruption without the treatment [9]. The heparin and washing treatment following homogenization disrupted nuclei at the same time, but cytoplasmic organelles including mitochondria and lysosomes remained intact. We concluded that heparin interacted specifically with plasma membranes and nuclear membranes.

In this study, we analysed specific membrane-bound heparin binding proteins (MHBP) in membrane preparations from promyelocytic cells, HL-60. To isolate and identify MHBP, we evaluated the optimum elution conditions for ion compositions of the elution buffer and showed a possibility of isolating specific MHBP by heparin-gel affinity chromatography with $CaCl_2$ or $MgCl_2$ as an eluent. It is suggested that proteins related with membrane functions dependent on Mg^{2+} or Ca^{2+} can be obtained by the present method.

2. Experimental

2.1. Materials

Heparin-Sepharose CL-6B was obtained from Amersham Pharmacia Biotech, and swollen in 0.15 *M* NaCl–10 m*M* phosphate buffer (pH 7.4) before use. Sulfated cellulose gels, Sulfate-Cellulofine, were a product of Seikagaku Kogyo, Tokyo, Japan. Other reagents were of reagent grade.

2.2. Cells

HL-60 cells were supplied by the Japanese Cancer Research Resources Bank. The passage number was 13 when obtained. The cells were maintained and cultured in RPMI-1640 medium (Sigma–Aldrich, Japan) containing 10% heat-inactivated fetal calf serum in a humidified CO_2 incubator at 37 °C.

2.3. Cell fractionation

Cells were propagated in 100 ml medium in a 175-cm^2 dish. Cells grown to a confluency (2.5 to 3.0×10^6 cells/ml) were suspended by shaking the dish vigorously. Usually cells from eight dishes were collected by centrifugation at 300 *g* for 5 min at 4 °C and washed by suspending in 100 ml of 20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$ (TM solution). The washed cell pellets were suspended in 4 ml of TM solution containing protease inhibitors; 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 m*M* pepstatin A, 0.03 m*M* leupeptin, and 10 m*M* E-64. The cells were disrupted with a tight fitting Dounce homogenizer (Kontes, Vineland, NJ, USA). Usually 50 strokes were required for a sufficient disruption of cells by monitoring with a microscope. Immediately after homogenization, an equal volume of 0.5 *M* sucrose was added to the cell homogenate to maintain isotonicity. The homogenate was then centrifuged as above. The precipitates were again suspended in 2 ml of TM solution containing protease inhibitors, homogenized and centrifuged. The supernatants were combined and centrifuged at 5×10^3 *g* for 15 min, yielding precipitates, P5 fractions. The supernatants were finally centrifuged at 4×10^4 *g* for 90 min to obtain precipitates, P40 fractions. Both membrane fractions were suspended in a small volume of water and frozen until use.

2.4. Assay

Protein concentration was determined with a dye reagent, Coomassie brilliant blue G250 (Bio-Rad), according to a procedure of the manufacturer.

2.5. Affinity chromatography

All procedures were carried out at 0–4 °C. Combined membrane fractions amounting to 2-l cultures were solubilized in 1% octyl β-D-glucoside containing 10 m*M* sodium phosphate, pH 7.4 or 10 m*M* Tris–HCl, pH 7.4 (for elution by solution containing $CaCl_2$ in affinity chromatography) containing protease inhibitors as described above. For the rechromatography experiments, membrane fractions amounting to 3- to 4-l cultures were used as starting materials.

Membranes in the detergent solution were kept on ice for 30 min and centrifuged at 4×10^4 *g* for 90 min to obtain extracts. The extracts were first applied to Sulfate-Cellulofine column (0.5 ml bed volume) to remove proteins which bind non-specifically to sul-

fate residues or polysaccharides. The column had been equilibrated with the same buffer used for solubilization. The non-adsorbed proteins were then applied to Heparin-Sepharose CL-6B column (0.5 ml bed volume) with flow-rates of 0.25 to 0.3 ml/min. The column was washed with the buffer containing 1% octyl glucoside and 0.15 *M* NaCl before the elution with $CaCl_2$ or $MgCl_2$. Proteins were eluted successively with 10 column volumes of increasing concentrations of NaCl or $MgCl_2$ in 1% octyl glucoside–10 m*M* sodium phosphate, pH 7.4 or $CaCl_2$ in 1% octyl glucoside–10 m*M* Tris–HCl, pH 7.4. The elution buffer contained 0.15 *M* NaCl throughout chromatography. Each fraction was dialysed against water overnight at 4 °C and concentrated first with Amicon Centriprep 10 (Millipore Corporation, Bedford, MA, USA) and next with Amicon Centricon 10 (Millipore Corporation).

2.6. Electrophoresis

The protein samples prepared as above were denatured in sample buffer at 100 °C for 3 min and analysed by SDS–PAGE under the reducing condition [10]. The gels were stained with silver stain (Plus-One, Amersham Pharmacia Biotech).

2.7. Amino acid sequencing

Pooled membrane fractions from ~25-l cultures were used as starting materials. Solubilized proteins (166 mg of P40 extracts) were purified as above except that larger columns (Econo Column, 15 mm diameter, Bio-Rad) were used. Proteins were eluted with five column volumes of the elution buffer. The proteins (20 to 30 μg) in each fraction from the second heparin-gel chromatography were separated by SDS–PAGE and blotted electrically to a PVDF membrane, Immobilon-P^{SQ} (Millipore Corporation). After staining in Coomassie brilliant blue R250, the specific protein bands were excised and N-terminal amino acid sequences were determined with Applied Biosystems Procise Sequencer, Model 476A. The sequence analysis was performed at Biologica, Nagoya, Japan. Sequence homology was searched by the internet provided by the National Center for Biotechnology Information, NIH, Bethesda, MD, USA.

3. Results

3.1. Heparin binding proteins in membranes

Membrane proteins solubilized with octyl glucoside were applied to a column of Heparin-Sepharose CL-6B. Proteins eluted with increasing concentrations of NaCl were shown by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS–PAGE) (Fig. 1). Protein bands in the electrophoresis gels indicated the presence of MHBP, which required different concentrations of NaCl for the elution, indicating different affinities for heparin. The eluates from P5 fractions contained 1.58, 2.71, 5.04 and 0.64 mg proteins, those from P40 fractions contained 1.80, 1.20, 2.53 and 1.35 mg proteins, respectively, in the order of elution. The results also showed localization of specific proteins in P5 (bands 1–5) and P40 (bands 6 and 7) fractions.

3.2. Elution with $CaCl_2$ or $MgCl_2$

Although a solution containing increasing concentrations of NaCl has generally been employed to elute proteins in chromatography by a heparin-gel, $CaCl_2$ and $MgCl_2$ were tested for their capability to elute proteins from heparin-gels. As shown in Fig. 1, a considerable number of proteins eluted at low concentrations of NaCl, suggesting that there are proteins binding to the gels with low affinity. Therefore, to exclude these proteins, solubilized samples were first passed through a column of Sulfate-Cellulofine, sulfate ester of cellulose gels. The non-adsorbed proteins were used as test samples for subsequent analysis with heparin-gels. The low affinity proteins were also removed by washing the column with the buffer containing 1% octyl glucoside and 0.15 *M* NaCl before the elution with $CaCl_2$ and $MgCl_2$.

Fig. 2 shows that Ca^{2+} or Mg^{2+} was capable of eluting distinct proteins from the gels dependent on their concentrations. The amounts of protein in the P5 extracts were 4.10 mg ($CaCl_2$ elution) and 5.13 mg ($MgCl_2$ elution), respectively, and those in the P40 extracts were 3.15 mg ($CaCl_2$ elution) and 3.94 mg ($MgCl_2$ elution), respectively. Specific protein bands 1, 2, 3, 5, 7, 8, 9, 10 and 14 appeared in the fraction eluted with the buffer containing low concentrations of Mg^{2+}, and proteins in bands 4, 6, 11

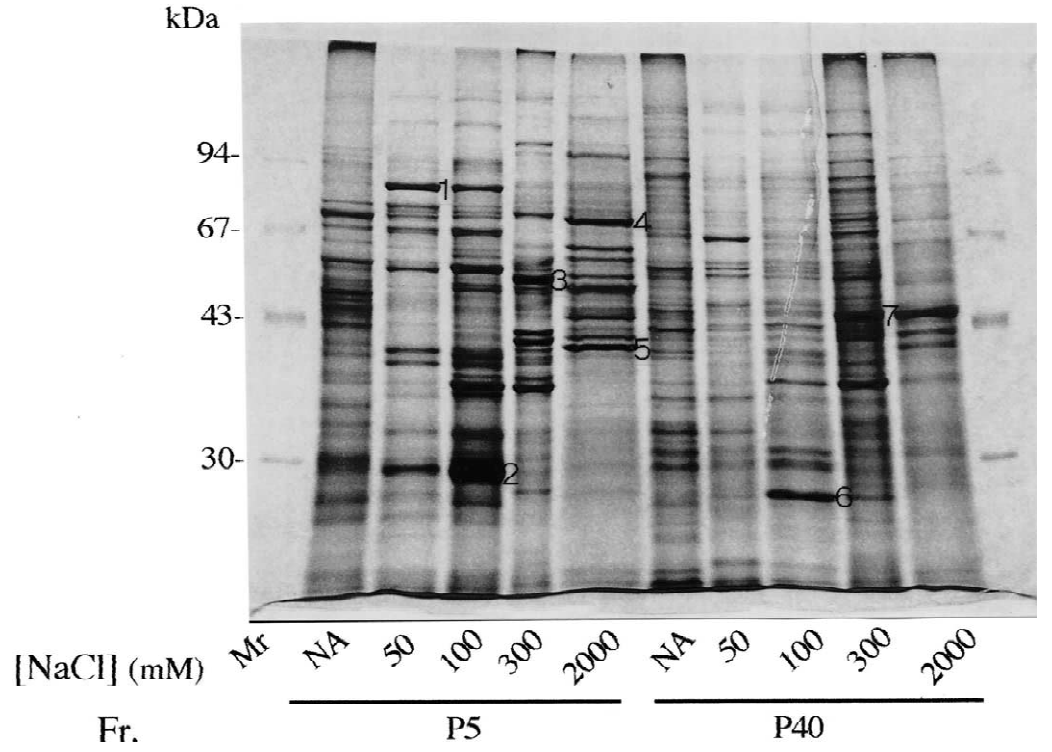

Fig. 1. Proteins eluted from heparin-gels with NaCl. Pooled P5 or P40 fractions equivalent to 2-l cultures were solubilized with 1% octyl glucoside–10 m*M* sodium phosphate (pH 7.4) and extracts were prepared as described in Section 2. The extracts (P5, 13.0 mg and P40, 16.5 mg proteins) were applied to a Heparin-Sepharose CL-6B column (0.5 ml bed volume) equilibrated with the solubilizing buffer. After washing the column with the same buffer, proteins were eluted successively with 10 column volumes of 0.05, 0.1, 0.3 and 2 *M* NaCl solution in the same buffer. The eluates were dialysed, concentrated and 10 μg proteins in each fraction were subjected to SDS–PAGE, followed by silver staining. NA represents non-adsorbed flow through fractions.

and 12 with higher concentrations of Mg^{2+} (Fig. 2a). Proteins in bands 15, 16, 17, 18, 27 and 28 were released from the gels with the low-Ca^{2+} buffers, whereas bands 19, 20 and 24 with the high-Ca^{2+} buffers (Fig. 2b). Proteins in bands 13, 21, 22, 23, 25 and 26 required 2 *M* NaCl for the elution.

3.3. Rechromatography

The above results showed that Ca^{2+} and Mg^{2+} can be used for isolating the specific MHBP. To establish the method, further analysis was made by rechromatography experiments; membrane proteins were eluted from the heparin-gels with $MgCl_2$ in the first step and with $CaCl_2$ in the second step.

Fig. 3 shows the results obtained with P5 fractions. Bands 1, 2, 4 and 7 proteins from the first gels were eluted with lower concentrations of $CaCl_2$ in the second gels. Relatively higher concentrations of $CaCl_2$ were required for the elution of band 3 protein. Binding of bands 5/8 and 6/9 proteins seemed to be strong, since higher concentrations of $MgCl_2$ in the first gels and 20 m*M* $CaCl_2$ or 2 *M* NaCl in the second gels were necessary for the elution.

The proteins solubilized from P40 fractions were analysed in the same procedure (Fig. 4). Band 1 protein seemed to have high affinity for heparin, but to lack ion specificity, since the protein was present in 5 and 20 m*M* $MgCl_2$ fractions in the first gels and could be eluted completely with 20 m*M* $CaCl_2$ in the second gels. Electrophoretic mobilities of bands 2, 3 and 4 seemed to be the same as those of bands 14, 15 and 16, respectively. However, the latter proteins

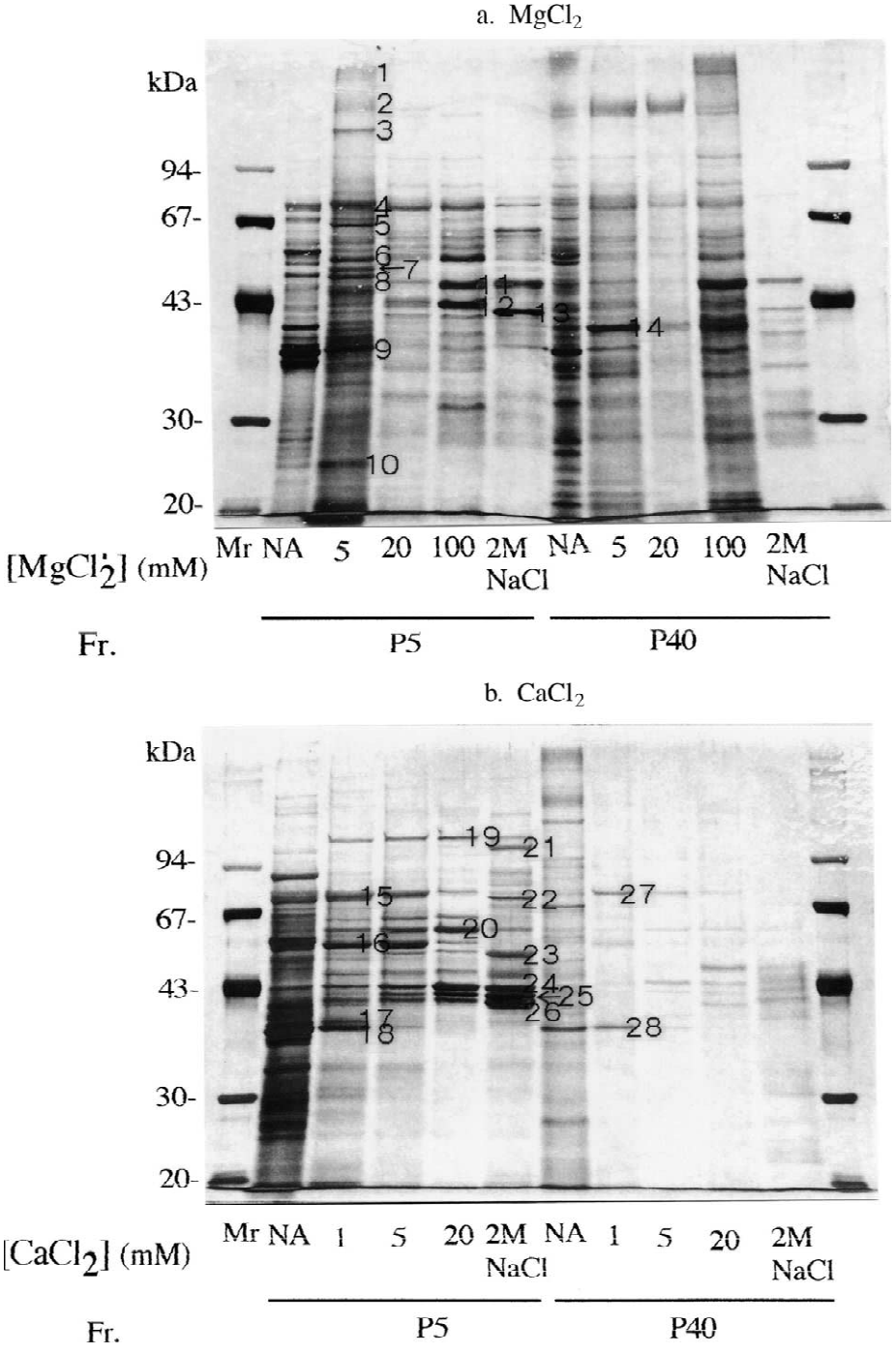

Fig. 2. Proteins eluted from heparin-gels with $CaCl_2$ or $MgCl_2$. The procedures were the same as in Fig. 1, except that the extracts were passed through a Sulfate-Cellulofine column before heparin-gel chromatography. The bed volume was 0.5 ml for both columns. The elution buffers consist of 1, 5, 20 m*M* $CaCl_2$ in 10 m*M* Tris–HCl (pH 7.4) or 5, 20, 100 m*M* $MgCl_2$ in 10 m*M* sodium phosphate (pH 7.4) containing 1% octyl glucoside and 0.15 *M* NaCl. The eluates were processed as in Fig. 1.

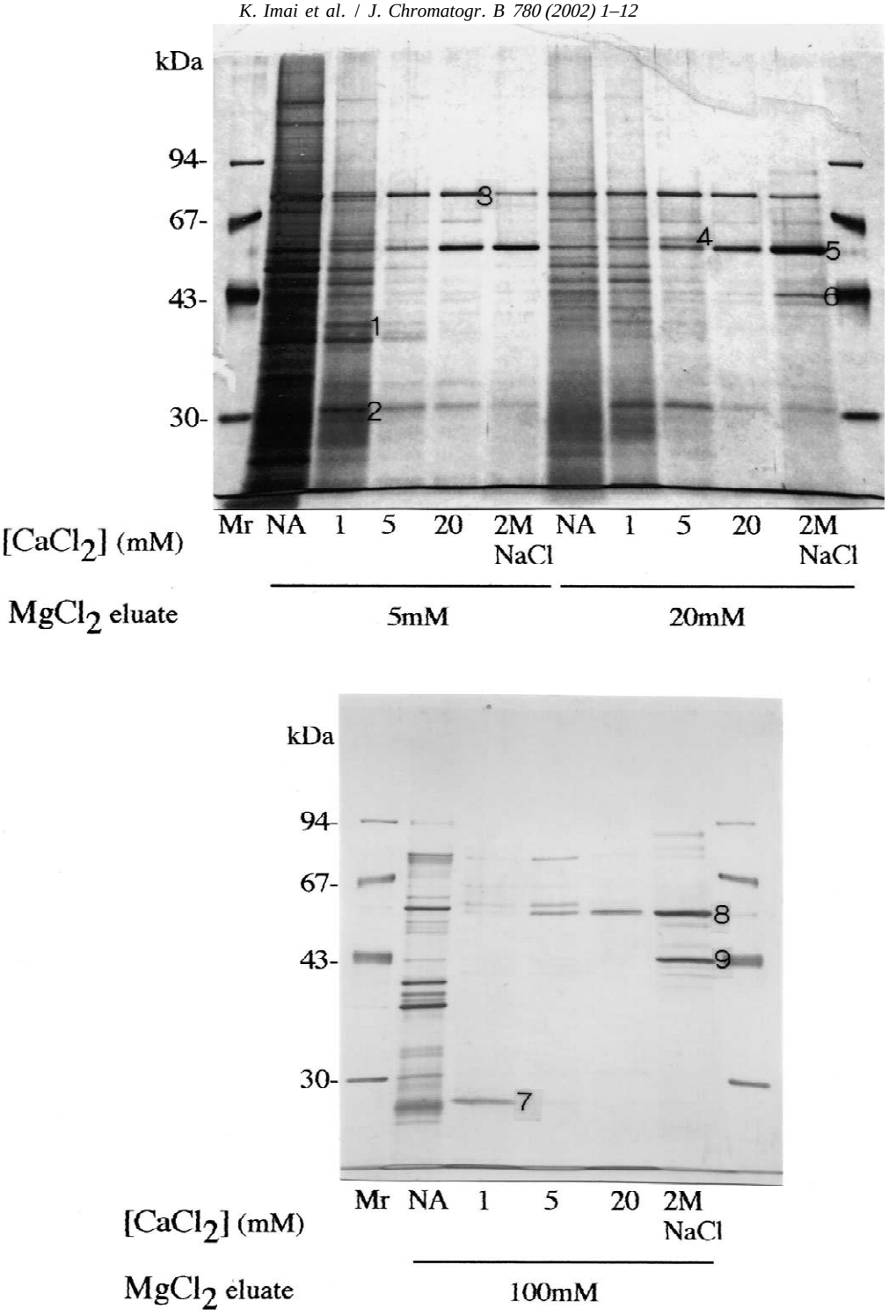

Fig. 3. Proteins in P5 fractions eluted from the second heparin-gels. P5 fractions were used as starting materials. Protein fractions of 5, 20 and 100 m*M* $MgCl_2$ from the first heparin-gels were prepared as described in Fig. 2. Dialysed and concentrated samples were applied to the second heparin-gels. Proteins were eluted with $CaCl_2$-containing buffer and eluates were processed as in Fig. 1.

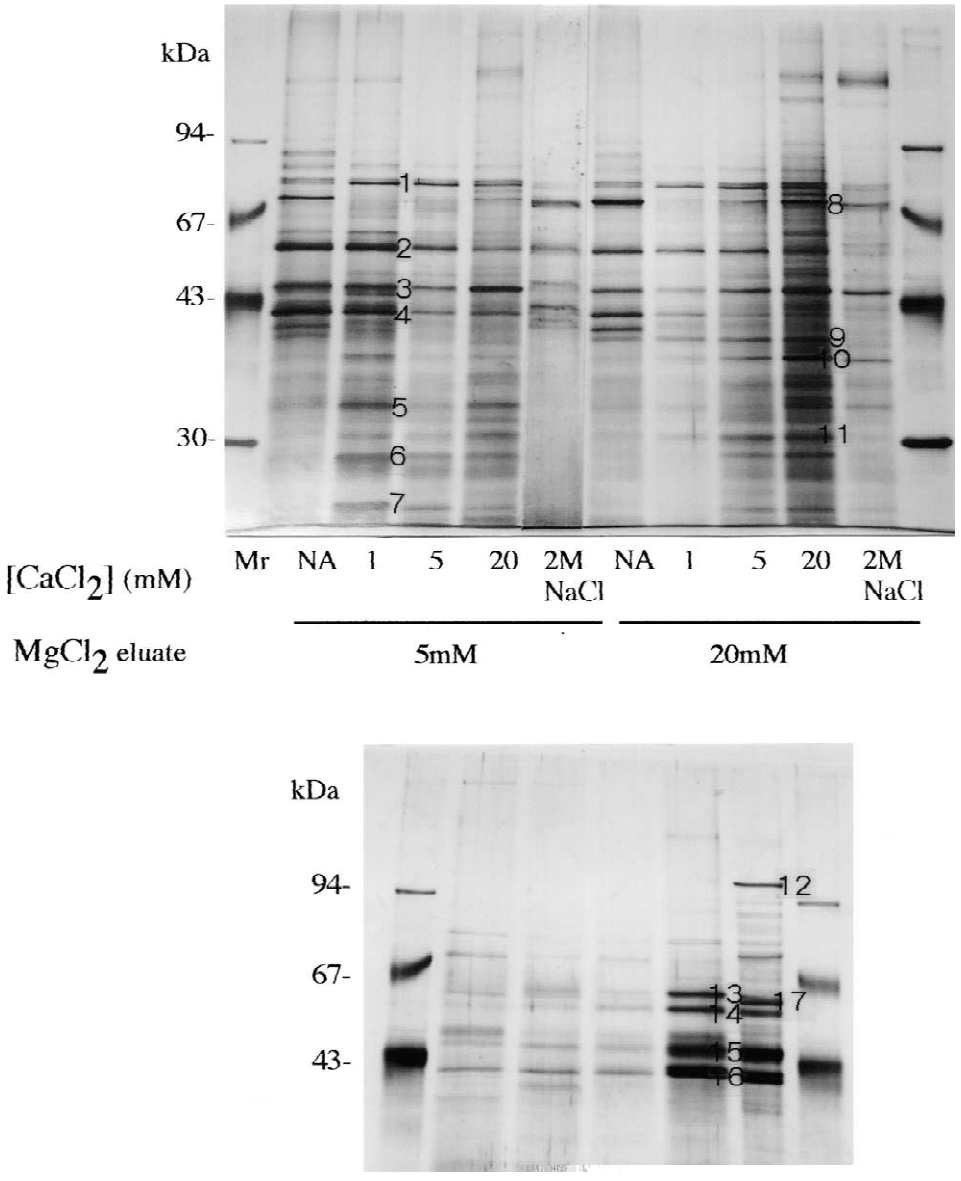

Fig. 4. Proteins in P40 fractions eluted from the second heparin-gels. Experimental procedures were the same as in Fig. 3, except that P40 fractions were used as starting materials.

Table 1
Sequences of N-terminal amino acids of proteins on bands

Band no.	M_r (kDa)	Sequence	Identified	Ref.
2	74	EGPDEDSSNR	Protein disulfide isomerase	[11]
5	34	GKVKVGVNGF	Glyceraldehyde 3-phosphate dehydrogenase	[15]
6	32	TAAKKNDKEA	Apurinic/apyrimidinic endonuclease	[13]
10	28	LHTKGALPLD	ERp29	[12]
12	81	PKPINVRVTT	Radixin; Villin 2	[19]; [18]
16	36	ATQGAAAAXX	hnRNP D	[20]
13	56	SDVLELTDDN	Microsomal protein P58	[14]
19	25	KQKVEGTEPT	Nucleolin	[16]

promote conformational change [22]. Though the endonuclease is mostly localized in nuclei, a substantial activity is found in microsomes or in cytoplasm [23,24]. Another nuclear protein of band 16 identified as heterogeneous nuclear ribonucleoprotein D has been reported to shuttle between the nucleus and cytoplasm [20].

Glyceraldehyde 3-phosphate dehydrogenase was identified via the 10 N-terminal amino acid sequence of the band 5 protein. The enzyme, originally described as a glycolytic enzyme, has recently been reported to exhibit diverse functions other than glycolytic activity [25]. Biological function of membrane association and heparin binding observed in the present study await further investigation.

Both radixin [19] and villin 2 [18] have exactly the same N-terminal amino acid sequence derived from the protein of band 12. Radixin was first found to be an 82 kDa barbed end-capping protein localized in the cell-to-cell adherence junction [26] and in addition radixin has been described as a member of the ezrin-radixin-moesin (ERM) family proteins, which link actin filaments to plasma membrane proteins [27].

A nucleolin 25 kDa fragment (band 19) was characterized via an internal sequence (amino acid residues 295–304) of 77 kDa nucleolin. Nucleolin is a nucleolar multifunctional protein which is essential for ribosome biogenesis [17]. Recent studies report intracellular redistribution of nucleolin upon cell activation [28] and infection [29]. Localization of nucleolin in cell surfaces has also been reported in various cell types [17,30]. The same amino acid sequence has also been found in the internal sequence (amino acid residues 226–235) of a protein similar to gastrin/cholecystokinin type B receptor, which consists of 641 amino acids [31]. The amino terminal domain of nucleolin is highly acidic and has been proposed to bind histone H1 [17]. Considering the difference in molecular mass between our result and the reported nucleolin, we assume that the 25 kDa protein is either a degradation product or a nucleolin-related protein lacking the acidic domain.

Sequencing of N-terminal amino acids with a blotted PVDF membrane is not applicable to proteins modified at the N-terminal, proteins present in extremely low amounts and for proteins with a very short half life. A large amount of cytoplasmic and nucleoplasmic soluble proteins are subjected to such N-terminal modification as N-acetylation or N-myristoylation [32]. However, it is not clear to what extent membrane proteins are modified. In the present study, six out of 20 proteins analysed seemed to be modified at the N-terminal residue. Modified proteins of bands 1 and 11 are interesting in that although they had a similar molecular size of 70 to 71 kDa, band 11 could be eluted with 1 m*M* $CaCl_2$, whereas band 1 protein required 2 *M* NaCl for its elution. To identify these modified proteins, analysis of internal sequence with partially digested samples will be necessary.

The simple methods described in this paper using divalent cations as eluents might allow the isolation of biologically important MHBP though the mechanisms are not completely elucidated. Mechanisms by which divalent cations eluted specific proteins might involve cation-exchange reactions among anionic groups in heparin and proteins. Solubilized proteins

may bind to heparins mainly electrostatically and could be dissociated by changing ionic strength. Various membrane functions requiring heparan sulfate have been reported to depend on divalent cations. In heparan sulfate-mediated cell aggregation, binding of heparan sulfate chains to a receptor occurs dependently on divalent cations [33]. Binding of cell adhesion molecule L-selectin to chondroitin sulfate and heparan sulfate requires divalent cations [34]. Fibroblast growth factor signaling requiring heparan sulfate was also shown to depend on divalent cations [35]. If divalent cations participate in the binding of proteins to proteoglycans, higher concentrations of mono- and divalent cations may cause dissociation of proteins. In addition to ion-exchanges, structural changes of proteins caused by divalent cations might contribute to the dissociation of specific proteins from the gels as well. Membrane proteins, integrins, have been reported to undergo drastic conformational changes by addition of divalent cations [36,37]. Specific dissociation of the proteins of interest from heparin-gels may be induced by alteration of the protein's affinity to heparin-gels, if the structural changes of the proteins operate in concert with ion-exchanges.

The present data suggest that certain membrane proteins bound to heparin can be differentially released by sequential elution with divalent cations. Recent proteomics owes its advance to a two-dimensional gel electrophoresis followed by sequencing. However, this approach is limited to only soluble proteins [38]. Systematic search for a detergent and conditions effective for solubilization and subsequent chromatography by the present methods may contribute to isolate and analyse novel membrane proteins which may depend on divalent cations for their functions.

5. Nomenclature

SDS–PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate
MHBP	membrane-bound heparin binding protein
TM solution	20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$

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