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Characterization of Hyaluronan-Binding Proteins on Guinea Pig Polymorphonuclear Leukocytes: Possible Involvement of Complement Receptor Type 3 (CR3, CD11b/CD18) in the Hyaluronan–Leukocyte Interaction

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Hyaluronan (HA), a high-molecular-weight glycosaminoglycan ubiquitously present in the extracellular matrices (ECMs) of animals, plays important roles in ECM organization and cell behavior through binding to hyaluronan-binding proteins (HABPs). We previously reported that HA has anti-inflammatory effects on guinea pig phagocytes, although the nature of guinea pig HABPs was unknown. In this study, we characterized guinea pig HABPs on peritoneal polymorphonuclear leukocytes (PMNs) and blood neutrophils by flow cytometry and affinity chromatography. It was found that PMNs express diverse HABPs with different molecular weights. These HABPs maximally bound with HA over a wide pH range (6–8), and recognized HAs as small as the pentadisaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. Furthermore, they could be divided into Mg^{2+}-dependent and Ca^{2+}/Mg^{2+}-independent groups. Interestingly, two proteins in the Mg^{2+}-dependent group were found to be the two subunits of complement receptor type 3 (CR3, CD11b/CD18). Unlike PMNs, blood neutrophils expressed several functionally inactive HABPs. Among these inactive HABPs, Mg^{2+}-dependent proteins including CR3 but not Ca^{2+}/Mg^{2+}-independent proteins were activated on phorbol ester-stimulation. These results show the existence of diverse HABPs on guinea pig neutrophils and the cell activation–dependent activation of HABPs. It is also suggested that the CR3-HA interaction is possibly involved in the regulation of neutrophil function.

Key words: complement receptor type 3, hyaluronan, hyaluronan receptor, neutrophils, polymorphonuclear leukocytes.

Abbreviations: BSA, bovine serum albumin; CR3, complement receptor type 3; DFP, diisopropyl fluorophosphate; ECM, extracellular matrix; F-HA30, fluorescein-conjugated HA30; FI, fluorescein isothiocyanate; fMLP, formyl-Met-Leu-Phe; HA, hyaluronan; HABP, HA-binding protein; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes; SOZ, serum-opsonized zymosan.

Hyaluronan (HA) is a major glycosaminoglycan ubiquitously present in essentially all vertebrate extracellular matrices (ECMs). It is composed of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine, and the molecular mass of HA in synovial fluid has been estimated to be more than several million Dalton (*1*–*3*). HA plays an important role not only in the construction of ECMs but also in the regulation of cell behavior such as cell aggregation, differentiation, proliferation and migration (*4*–*9*). It has thus been implicated that HA is essential for morphogenesis, wound healing, tissue regeneration, tumor invasion and metastasis (*10*–*14*). These diverse biological functions of HA are mediated by adhesive interactions with various types of HA-binding molecules.

A number of extracellular and cell-surface HA-binding molecules have been identified. Cartilage link protein and aggrecan are representative extracellular molecules that form highly hydrated huge complexes with HA to give structural integrity to ECMs (*15*–*17*). Tumor necrosis factor-α–stimulated gene-6 (TSG-6), a HA-binding molecule induced by inflammatory cytokines and secreted into the ECMs of inflamed tissues (*18*, *19*), binds HA through the Link module, which is a conservative sequence present in HA-binding molecules such as cartilage link protein and aggrecan (*20*, *21*). With respect to HA receptors expressed on various types of cells, CD44 (*22*–*24*), the receptor for hyaluronan-mediated motility (RHAMM) (*25*, *26*), the hyaluronan receptor for endocytosis (HARE) (*27*, *28*), CD38 (*29*), and stabilin (*30*) have been identified and molecularly characterized. It has been reported that stimulation of HA receptors produces certain intracellular signals that may regulate a variety of cellular functions (*31*, *32*). CD44 binds to HA through the Link module, and mediates intracellular signaling by associating with protein kinases and cytoskeletal proteins (*21*, *31*, *33*). RHAMM binds to HA through

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the BX_7B HA-binding motif (*33*), and promotes protein tyrosin kinase activation and focal adhesion turnover leading to reorganization of the cytoskeletal network (*31*, *33*, *34*). However, a large part of the molecular mechanism by which HA receptor–mediated events are regulated in neutrophils has not been established.

In addition, the physical and biological functions of HA are usually affected by various factors such as the molecular size and concentration of HA. In some inflammatory diseases such as rheumatoid arthritis and osteoarthritis, HAs in the synovial fluid decrease in concentration and molecular size (*1–3*). Such small HAs, derived from high-molecular-weight HA, tend to show biological activity distinct from that of native high-molecular-weight HA (*31*). It is well known that pro-inflammatory gene expression is stimulated by small, but not by large HAs (*35*, *36*). We have also reported that high-molecular-weight HAs inhibit the chemotaxis for formyl-Met-Leu-Phe (fMLP), and the CR3-mediated phagocytosis of serum-opsonized zymosan (SOZ) by guinea pig phagocytes in concentration- and molecular weight-dependent manners (*37*). However, both O_2^- generation and lysosomal enzyme release elicited by SOZ stimulation were not inhibited by high-molecular-weight HAs, while the binding reactions of fMLP and SOZ with the cell surface were not disturbed by high-molecular-weight HAs (*37*, *38*). These findings suggest that the inhibitory effects of high-molecular-weight HAs on phagocyte functions might be explained by the intracellular events induced *via* HA receptors rather than by the physicochemical properties of high-molecular-weight HAs by which ligand binding to the cell surface is inhibited (*37*, *38*), whereas the nature of HA receptors on guinea pig phagocytes has not been elucidated so far. To investigate the molecular mechanism by which HA regulates the functions of phagocytes, we have first attempted to isolate and characterize HABPs on guinea pig peritoneal PMNs and blood neutrophils.

MATERIALS AND METHODS

Cells—Guinea pig peritoneal PMNs were isolated from animals of the Hartley strain at 16 h after intraperitoneal injection of 7% casein, as described previously (*39*, *40*). Guinea pig blood neutrophils were isolated from anticoagulated blood, as described previously (*41*). When needed, blood neutrophils were activated by incubation with 30 ng/ml phorbol 12-myristate 13-acetate (PMA) for 10 min at 37°C, washed three times and then used as PMA-activated neutrophils.

Reagents—Highly purified HAs with molecular masses of 300 kDa (HA30), 40 kDa (HA4), and 2 kDa (HA0.2) were obtained from Denki Kagaku Kogyo (Tokyo, Japan). Anti–Z-1 monoclonal antibody (mAb) directed to the α subunit of guinea pig CR3 and B6E9 mAb directed to ovalbumin were previously established in this laboratory (*41*, *42*). The $F(ab')_2$ fragments of these mAbs were used in this study, and expressed as anti–Z-1 mAb and B6E9 mAb, respectively. The following materials were obtained from the indicated sources: BSA (fraction V), aprotinin, leupeptin, pepstatin, 5-aminofluorescein, fluorescein isothiocyanate and PMA from Sigma (St. Louis, MO); an LMW electrophoresis calibration kit, EHA-Sepharose 4B and CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology (Uppsala, Sweden); fibronectin from Seikagaku Kogyo (Tokyo, Japan); Nonidet P-40 from Nakarai Chemicals (Kyoto, Japan); diisopropyl fluorophosphate (DFP) from Kishida Chemicals (Osaka, Japan); and carrier-free $Na^{125}I$ from Movavek Biochemicals (Brea, CA).

Flow Cytometry Analysis—HA30 was coupled with 5-aminofluorescein according to the method of Ogamo *et al.* (*43*). The number of dye molecules bound per mol of HA30 was approximately 15. In the flow cytometry experiments, 2×10^6 peritoneal PMNs or blood neutrophils were incubated for 1 h with 10 μg/ml of fluorescein-conjugated HA30 (F-HA30) in 1 ml of Krebs-Ringer phosphate buffer, pH 7.2, containing 0.05% NaN_3 with or without 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$. After incubation, the mixture was centrifuged at 400 × *g* for 5 min. The sedimented cells were washed twice with reaction buffer and then analyzed with a Coulter EPICS Elite flow cytometer, as described previously (*41*, *42*). CR3 expression was also determined by flow cytometry using 5 μg/ml of fluorescein isothiocyanate (FI)–labeled anti–Z-1 mAb, as described previously (*41*, *42*).

Preparation of Adsorbents—HA30 was coupled with EHA-Sepharose 4B according to the method of Tengblad (*44*). The amount of HA30 attached to Sepharose was 9 mg per ml of wet gel. HA4-Sepharose (7 mg of HA4 per ml of wet gel) and HA0.2-Sepharose (1 mg of HA0.2 per ml of wet gel) were similarly prepared. Sepharose coupled with either $F(ab')_2$ of anti–Z-1 mAb (anti–Z-1-Sepharose), $F(ab')_2$ of B6E9 mAb (B6E9-Sepharose), or bovine serum albumin (BSA-Sepharose) was prepared using CNBr-activated Sepharose 4B, as described (*42*, *45*). Approximately 1–2 mg of the each protein bound per ml of wet gel.

Radioiodination and Solubilization of Plasma Membrane Proteins—Peritoneal PMNs or blood neutrophils (2×10^7 cells) were surface-labeled with $Na^{125}I$ (1 mCi) by the lactoperoxidase method (*42*, *46*), and then solubilized with 2 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.2% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin and 2 mM DFP [solubilization buffer] for 30 min on ice. After solubilization, residual insoluble materials were removed by centrifugation at 10,000 × *g* for 30 min and the supernatant obtained was used as the detergent-extract. For determination of the optimum pH for HA binding by HABPs, the detergent-extract was prepared with 5 mM phosphate buffer, of pH values ranging from 5.0 to 8.5, containing 0.2% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 2 mM DFP, 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$.

Affinity Chromatography and Immunoprecipitation Analysis—One ml of detergent-extract (equivalent to 2×10^6 cells), diluted with an appropriate buffer, was preadsorbed to 30 μl of BSA-Sepharose for 1 h at 20°C. The recovered supernatant was then reacted with 30 μl of HA30-Sepharose for 1 h at 20°C. In some experiments, the detergent-extract was sequentially adsorbed as follows: first with BSA-Sepharose, then with either anti–Z-1-Sepharose or B6E9-Sepharose, and finally with HA30-Sepharose. After washing each gel eight times with reaction buffer followed by twice with detergent-free reaction buffer, the materials bound to the gel were eluted by heating in a boiling water bath with 40 μl of Laemmli's sample buffer with or without 5% 2-mercaptoethanol (*42*, *47*). The materials eluted were analyzed by SDS-PAGE

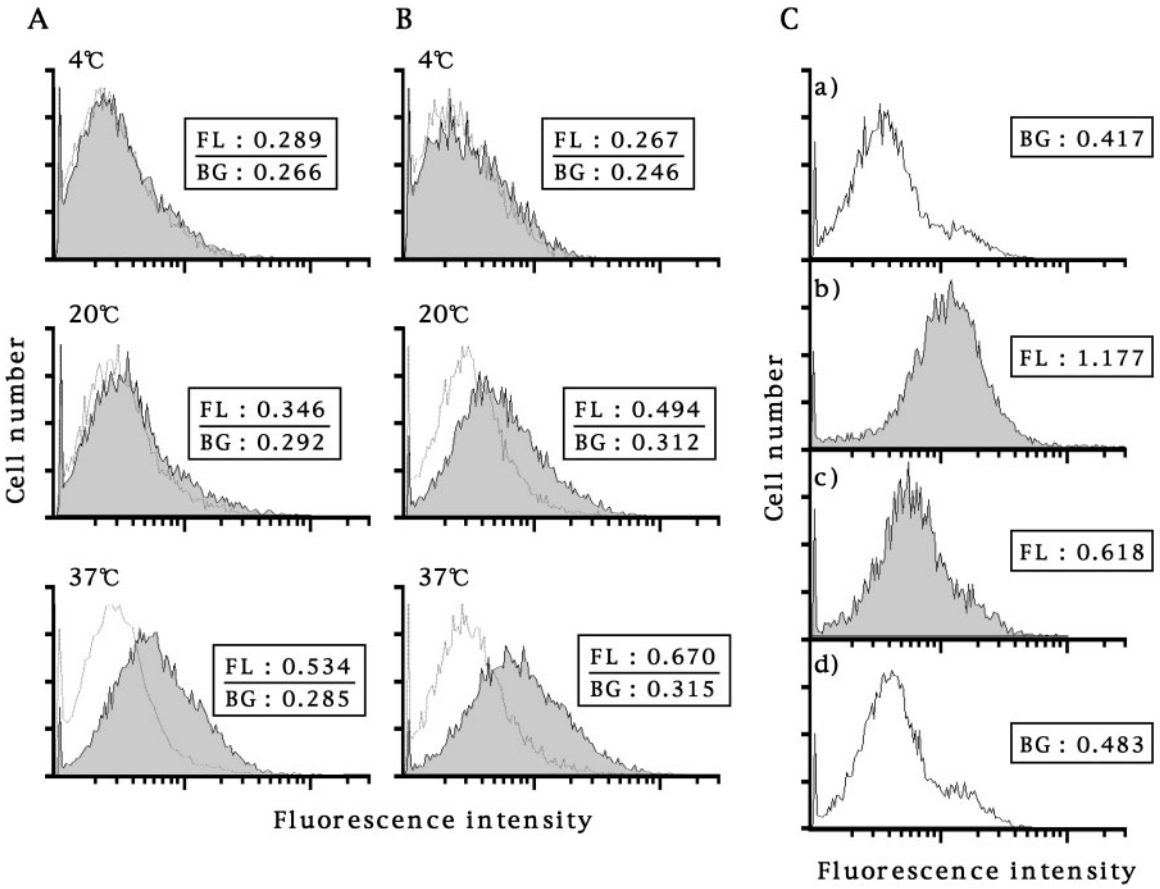

Fig. 1. **Flow cytometric analysis of the binding of F-HA30 to guinea pig peritoneal PMNs.** PMNs were incubated with F-HA30 in the absence (A) or presence (B) of both 0.6 mM Ca^{2+} and 1 mM Mg^{2+} for 1 h at 4°C, 20°C or 37°C. (C) PMNs in the buffer containing 0.6 mM Ca^{2+} and 1 mM Mg^{2+} were incubated for 1 h at 20°C as follows: (a) cells alone, (b) with 10 μg/ml of F-HA30, (c) with 10 μg/ml of F-HA30 and 1 mg/ml of non-labeled HA30, (d) with 1 mg/ml of non-labeled HA30. Cell-bound fluorescence was analyzed with a flow cytometer, and is presented as a shaded histogram with the logarithm of fluorescence intensity on the abscissa and the cell number on the ordinate. Controls involving unstained cells are presented as open histograms. One representative experiment of three with similar results is shown. The numbers in the squares indicate the mean values of the mean fluorescence intensity in each histogram obtained for duplicate determinations in one experiment (FL: fluorescein-stained cells, BG: control cells).

followed by autoradiography, as described previously (*40*, *42*). The standards used for estimation of the molecular masses of non-reduced proteins were rabbit IgG (150 kDa), $F(ab')_2$ of rabbit IgG (100 kDa), BSA (67 kDa), and Fab′ of rabbit IgG (50 kDa). For estimation of the molecular masses of reduced proteins, fibronectin (210 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), BSA (67 kDa), and ovalbumin (43 kDa) were used as standards. To quantify the radioactive protein bands on autoradiographs, densitometric analysis was performed with Image Master (Pharmacia LKB Biotechnology).

RESULTS

Binding of F-HA30 with Casein-Induced Peritoneal PMNs—The binding of F-HA30 with casein-induced peritoneal PMNs was analysed by flow cytometry. When the binding reactions were carried out at 4°C in the absence (Fig. 1A) or presence (Fig. 1B) of 0.6 mM Ca^{2+} and 1 mM Mg^{2+}, no significant binding was observed. In contrast, F-HA30 could bind to the cells at higher temperature, such as 20°C and 37°C, even in the absence of Ca^{2+} and Mg^{2+} (Fig. 1A). The amount of F-HA30 bound to the cells further increased when the reaction medium was supplemented with 0.6 mM Ca^{2+} and 1 mM Mg^{2+} (Fig. 1B). These binding reactions were inhibited by a 100-fold excess amount of non-labeled HA30 (Fig. 1C). The results showed that F-HA30 binds to HA-binding sites on the cell surface in a temperature-dependent manner. In addition, it was demonstrated that casein-induced peritoneal PMNs express at least two types of HA-binding sites: one type requires divalent cations, Ca^{2+} and/or Mg^{2+}, for their binding activity and the other does not.

Isolation of HABPs by Affinity Chromatography—To characterize HABPs expressed on the surface of casein-induced peritoneal PMNs, HABPs were isolated from the detergent-extract of ^{125}I-labeled cells by affinity chromatography using HA30-Sepharose. For this purpose, the detergent-extract was precleared with BSA-Sepharose and then reacted with HA30-Sepharose in the presence of 0.6 mM Ca^{2+} and 1 mM Mg^{2+}. The materials bound to HA30-Sepharose were eluted and analyzed by SDS-PAGE. Figure 2 shows autoradiographs of the materials eluted from the adsorbent. Under non-reducing condition, at least eight distinct radioactive bands are visible (lane –SH). Some bands are very close to each other. The sizes of the

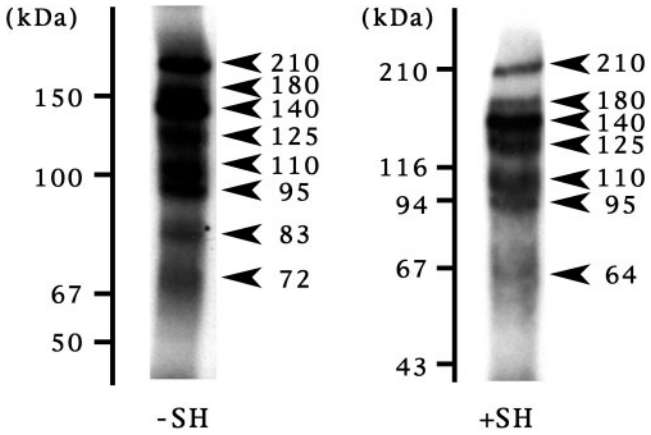

Fig. 2. **Isolation of HABPs from the detergent-extract of ^{125}I-labeled peritoneal PMNs by affinity chromatography.** A detergent-extract supplemented with 0.6 mM Ca^{2+} and 1 mM Mg^{2+} was preadsorbed with BSA-Sepharose and then reacted with HA30-Sepharose, as described under "MATERIALS AND METHODS." The materials bound to HA30-Sepharose were analyzed by SDS-PAGE under non-reducing (–SH) and reducing (+SH) conditions followed by autoradiography. Molecular weight standards are shown on the left of each panel.

bands were estimated to be 210 kDa, 180 kDa, 140 kDa, 125 kDa, 110 kDa, 95 kDa, 83 kDa and 72 kDa, respectively. The 72 kDa band was very diffuse. Reduction of the same sample with 2-mercaptoethanol resulted in disappearance of only the 83 kDa band (lane +SH), indicating that the proteins other than the 83 kDa one are single polypeptides. Thus, SDS-PAGE analysis of HABPs was henceforth performed under non-reducing conditions. Note that the BSA-Sepharose used as a control did not bind any radioactive material (data not shown), and that the amount of the 83 kDa protein detected with HA30-Sepharose varied from experiment to experiment and was sometimes undetectable, suggesting nonspecific binding of the 83 kDa protein.

Effects of HA Size on the Binding of HABPs to HA30-Sepharose—The binding of detergent-solubilized HABPs of PMNs to HA30-Sepharose was specific because an excess amount of soluble HA30 markedly reduced the amounts of all HABPs bound to the adsorbent (Fig. 3A, lane 4). In the preliminary experiments, monosaccharides such as D-glucuronic acid and *N*-acetyl-D-glucosamine did not show any inhibitory effect on the binding of these HABPs with HA30-Sepharose (data not shown), suggesting that HABPs do not recognize monosaccharides. Soluble HA4 and HA0.2 inhibited the binding of HABPs to the adsorbent whereas their inhibitory effects decreased with a decrease in the size of HA (Fig 3A, lanes 2 and 3). The densitometric analysis of the 210 kDa, 125 kDa, 140 kDa and 72 kDa protein bands showed that soluble HA0.2 most effectively inhibited the binding of the 140 kDa protein, and less effectively the binding of the 210 kDa and 125 kDa proteins (Fig. 3B). Other protein bands were difficult to subject to densitometric analysis because of overlapping with adjacent protein bands in very close proximity. Figure 3C shows the SDS-PAGE patterns of HABPs isolated with either HA0.2-, HA4-, or HA30-Sepharose. Essentially the same HABPs were detected with these adsorbents. These results suggest that the minimum size of HA recognized by HABPs is less than 2 kDa, which corresponds to the size of the pentadisaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. The estimated minimum HA size is approximately the same as that reported for the TSG-6 link module (*48*), suggesting that the size of the HA-binding site on guinea pig HABPs may be similar to that on link module-bearing HABPs.

Effects of pH and Ionic Strength on the Binding of HABPs to HA30-Sepharose—When the binding of detergent-solubilized HABPs to HA30-Sepharose was analyzed at various pHs, all the HABPs maximally bound to the adsorbent at pHs ranging from 6.0 to 8.0 (Fig. 4, A and B). At lower pHs than 6.0 and higher pHs than 8.0, the number of species of HABPs bound to the adsorbent decreased. The major HABPs detected at pH 5.5 were the 125 kDa and 110 kDa proteins, and a further decrease in pH resulted in disappearance of the 125 kDa protein. At pH 8.5, the major HABPs detected were the 110 kDa and 72 kDa proteins. The binding of these HABPs to HA30-Sepharose was also susceptible to changes in ionic strength and gradually disappeared with increased NaCl concentration (Fig. 4, C and D). The binding of the 95 kDa and 140 kDa proteins was more susceptible to increasing NaCl concentration than that of the 210 kDa and 125 kDa proteins, and was hardly detected even in the presence of NaCl at a concentration as low as 25 mM. With concentrations of NaCl higher than 100 mM, all HABPs became undetectable. These results suggest the involvement of ionic interactions in HA binding by the detergent-solubilized HABPs.

Effects of Ca^{2+} and Mg^{2+} on the Binding of HABPs to HA-Sepharose—HABPs of PMNs were further characterized by affinity chromatography in the presence or absence of Ca^{2+} and Mg^{2+}. When either or both 0.6 mM Ca^{2+} and 1 mM Mg^{2+} were included in the reaction buffer, essentially the same bands as those in Fig. 2 were seen (Fig. 5, lanes 2–4). Removal of Ca^{2+} by the addition of Mg^{2+} and EGTA did not affect the binding of HABPs (lane 1). Under the conditions where Ca^{2+} and Mg^{2+} were chelated with EDTA, the 72 kDa, 95 kDa and 140 kDa proteins became undetectable (lane 5), whereas binding of the 210 kDa, 180 kDa, 125 kDa and 110 kDa proteins to HA30-Sepharose was still observed (lane 5). These results demonstrate that PMNs express at least two types of HABPs on their plasma membrane, as observed with intact cells: one is a group of 72 kDa, 95 kDa and 140 kDa proteins that require Mg^{2+} for HA binding, and the other a Ca^{2+}/Mg^{2+}-independent group of 110 kDa, 125 kDa, 180 kDa and 210 kDa proteins.

Identification of the 140-kDa and 95-kDa Proteins as the Two Subunits of CR3—There was a striking resemblance between the molecular sizes of the Mg^{2+}-dependent 140 kDa and 95 kDa proteins and those of the α (140 kDa) and β (95 kDa) subunits of guinea pig CR3 (*42*). In addition, CR3 is known to require divalent cations and a warm temperature for optimum ligand binding (*49*, *50*). It is, therefore, possible that guinea pig CR3 may function as an HABP. To examine this possibility, we conducted the following two experiments. The first was an inhibition experiment involving an anti–Z-1 mAb that is capable of inhibiting zymosan phagocytosis of phagocytes through binding to the α subunit of CR3 without affecting ligand binding (*40*, *42*).

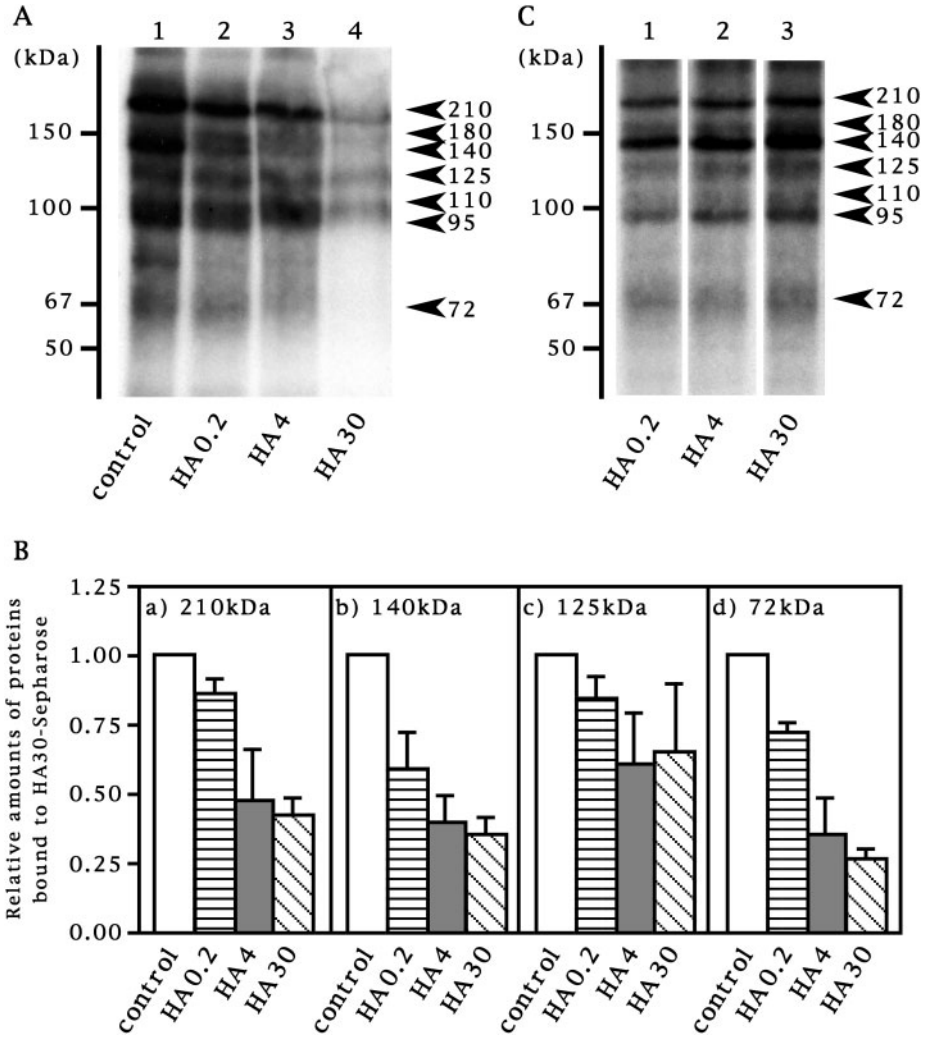

Fig. 3. **Effect of molecular size of HA on the binding of HABPs to HA30-Sepharose.** A detergent-extract of ^{125}I-labeled peritoneal PMNs was supplemented with 0.6 mM Ca^{2+} and 1 mM Mg^{2+} and then preadsorbed with BSA-Sepharose. (A) The recovered supernatant was reacted with HA30-Sepharose at 20°C in the absence of soluble HA (lane 1: control), or the presence of 1.5 mg/ml of soluble HA0.2 (lane 2), HA4 (lane 3), and HA30 (lane 4). The materials bound to each adsorbent were analyzed by SDS-PAGE under non-reducing conditions followed by autoradiography. One representative experiment of three with similar results is shown. (B) The radioactive proteins in the autoradiograph shown in (A) were quantified by densitometric analysis and expressed as relative amounts of proteins. The results are the means ± SD for three experiments. (C) The detergent-extract preadsorbed with BSA-Sepharose was reacted with Sepharose bearing either HA0.2 (lane 1), HA4 (lane 2), or HA30 (lane 3), and then analyzed as in (A). One representative experiment of three with similar results is shown.

In this experiment, the detergent-extract of ^{125}I-labeled PMNs was incubated with anti–Z-1 mAb (5 μg/ml) for 20 min and then reacted with HA30-Sepharose. The second experiment consisted of adsorption of CR3 in the detergent-extract of ^{125}I-labeled PMNs with anti–Z-1-Sepharose. As a control, B6E9-Sepharose was used in place of anti–Z-1-Sepharose. After adsorption, HABPs in the unadsorbed fraction were analyzed by affinity chromatography using HA30-Sepharose. These results are shown in Fig. 6A. An inhibitory effect of soluble anti–Z-1 mAb on the binding of both the 140 kDa and 95 kDa proteins with HA30-Sepharose was not observed (lane 4), and all HABPs bound with HA30-Sepharose, as in the control (lane 1). On the other hand, after adsorption of CR3 in the detergent-extract with anti–Z-1-Sepharose (lane 5), the 140 kDa and 95 kDa proteins became undetectable with HA30-Sepharose (lane 2), whereas other HABPs were seen (lane 2), as in the controls (lanes 1 and 3). Adsorption with B6E9-Sepharose did not cause any change in the electrophoretic pattern of HABPs (lane 3). These results suggest that CR3 may act as an HABP.

To further confirm the above results, divalent cation-dependent HABPs were partially purified by HA30-Sepharose chromatography using 5 mM EDTA-containing solubilization buffer as the elution solution. The eluate was dialyzed against an EDTA-free solubilization buffer and then diluted to 5 ml. One-milliliter aliquots (equivalent to 2×10^6 cells) were used for subsequent analysis. As shown in Fig. 6B, of the seven proteins bound to HA30-Sepharose (lane 1), the Mg^{2+}-dependent HABPs of 140 kDa, 95 kDa and 72 kDa were effectively eluted from the adsorbent (lane 2) and recovered in the eluate. When 1-ml aliquots of the eluate were re-incubated with HA30-Sepharose, it was demonstrated that the partially

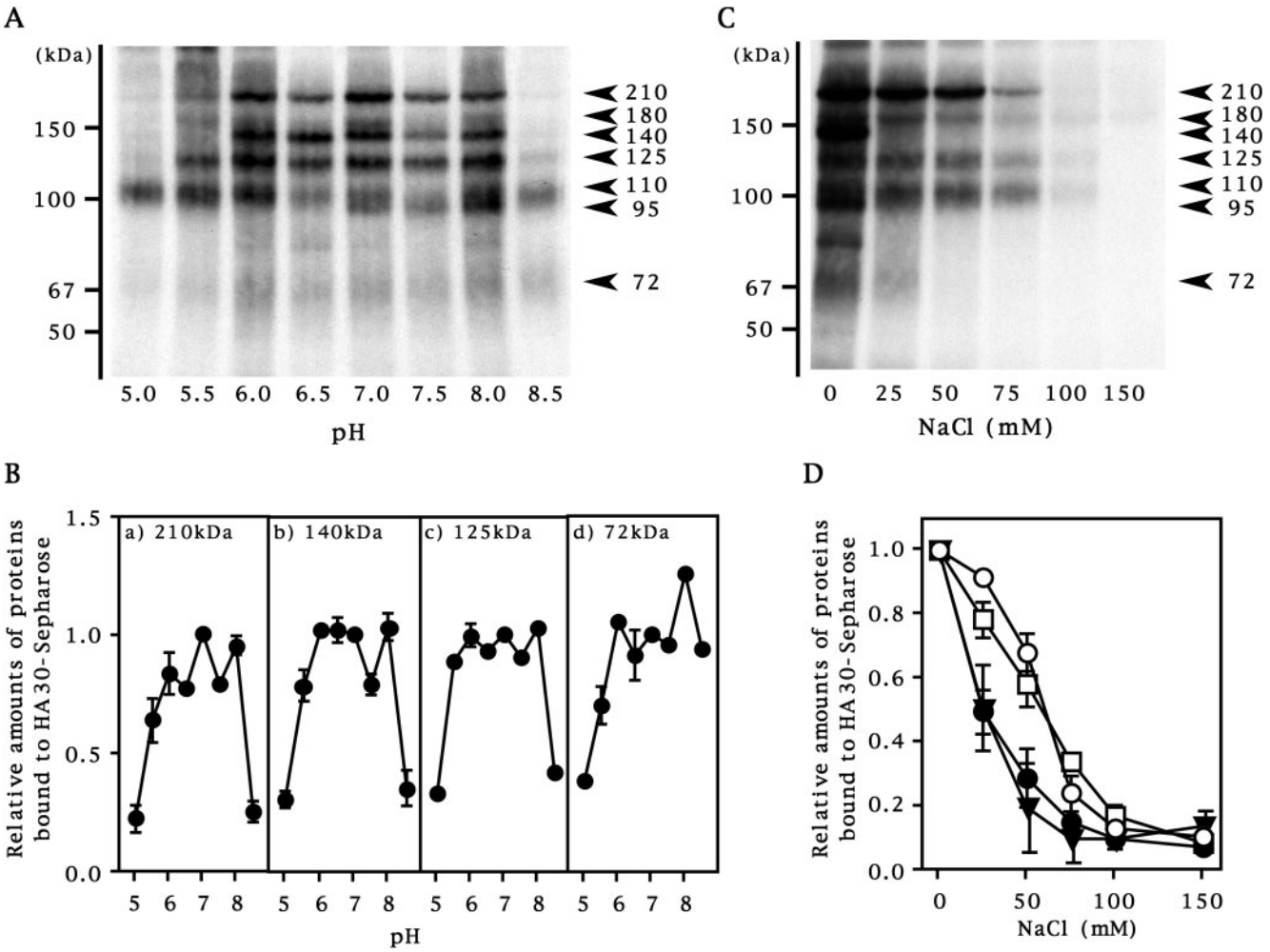

Fig. 4. **Effects of pH and ionic strength on the binding of HABPs to HA30-Sepharose.** A detergent-extract of ^{125}I-labeled peritoneal PMNs was prepared with a solubilization buffer comprising (A) 5 mM phosphate buffer of various pH values or (C) with various concentrations of NaCl, as described under "MATERIALS AND METHODS," and supplemented with 0.6 mM Ca^{2+} and 1 mM Mg^{2+}. After preadsorption with BSA-Sepharose, the supernatants recovered were reacted with HA30-Sepharose and then the materials bound to HA30-Sepharose were analyzed by SDS-PAGE followed by autoradiography. One representative experiment of three with similar results is shown. (B and D) The amounts of the 210 kDa (open circles), 140 kDa (solid circles), 125 kDa (open squares), and 72 kDa (inverted triangles) proteins in the autoradiographs (A and C), respectively, were determined by densitometric analysis and expressed as relative amounts of proteins. The results are the means ± SD for three experiments.

purified 140 kDa, 95 kDa and 72 kDa HABPs retained their original divalent cation–dependent HA-binding ability (lanes 3 and 4). Although the eluate still contained the 210 kDa and 125 kDa proteins (lane 4), the proportions of these two proteins in the partially purified HABP preparation were markedly decreased when compared with those in the control (lane 1). After adsorption of CR3 in the eluate with anti–Z-1-Sepharose (lane 6), the 140 kDa and 95 kDa proteins became undetectable with HA30-Sepharose (lane 5), whereas the amounts of the 210 kDa, 125 kDa and 72 kDa proteins detected with HA30-Sepharose were the same before and after adsorption with anti–Z-1-Sepharose (lanes 4 and 5).

These results indicate that the Mg^{2+}-dependent 140 kDa and 95 kDa proteins are the α and β subunits of CR3, respectively, and that both Ca^{2+}/Mg^{2+}-independent and Mg^{2+}-dependent HABPs can bind to HA30-Sepharose independently of each other. It was also demonstrated that CR3 does not associate with other HABP molecules in the solubilization buffer used, since soluble anti–Z-1 mAb did not affect the binding of HABPs including CR3 to HA30-Sepharose whereas anti–Z-1-Sepharose could adsorb CR3 alone. It was thus suggested that CR3 itself may possess HA-binding ability. Similarly, it was suggested that the 72 kDa protein may not be associated with other HABPs and that it can bind to HA by itself.

Characterization of HABPs on Blood Neutrophils—To compare the characteristics of HABPs expressed on blood neutrophils with those of HABPs on peritoneal PMNs, we then analyzed the binding of F-HA30 with blood neutrophils by flow cytometry. However, unlike for the peritoneal PMNs, the binding of F-HA30 with resting neutrophils was scarcely observed even when the binding reaction was carried out at 37°C in the presence of Ca^{2+} and Mg^{2+} (Fig. 7a). In contrast, after stimulation with PMA, the cells became active and could bind F-HA30 on their surface (Fig. 7b), suggesting that a certain cell activation process is required for the acquisition of HA-binding ability. Since it

is known that PMA promotes up-regulated expression and activation of CR3 on human neutrophils (*51*), we then analyzed CR3 expression on both resting and PMA-activated cells by flow cytometry using FI–anti–Z-1 mAb. As shown in Fig. 7c, a substantial amount of CR3 was detected on the surface of resting cells, as previously observed (*41*), and PMA-stimulation caused a further increase in CR3 expression on the cell surface, *i.e.*, to approximately three-fold that in resting cells (Fig. 7d).

If CR3 were the predominant HABP in blood neutrophils, its up-regulation and activation, which are promoted by PMA, might explain at least a part of the mechanism for the cell activation–dependent HABP activation. To examine this possibility, we then isolated HABPs expressed on blood neutrophils by affinity chromatography using HA30-Sepharose. As shown in Fig. 8A, despite the lack of HA-binding activity in unactivated intact cells, five proteins could be isolated from resting cells. Three of the five proteins isolated were Mg^{2+}-dependent, and their molecular sizes were 140 kDa, 95 kDa and 58 kDa, respectively (lanes 1–3). The other two proteins, 210 kDa and 125 kDa, were Ca^{2+}/Mg^{2+}-independent. The binding of the 210 kDa protein was rather inhibited by Ca^{2+} (lanes 1–3). The 180 kDa, 110 kDa and 72 kDa HABPs that were found on peritoneal PMNs (Fig. 2) were not isolated from resting blood neutrophils. Furthermore, two major proteins of 140 kDa and 95 kDa were identified as the two subunits of CR3 by adsorption with anti–Z-1-Sepharose (lanes 4–7).

In the case of PMA-activated cells, as shown in Fig. 8B, only three proteins of 140 kDa, 95 kDa and 58 kDa were isolated (lanes 2 and 3). These proteins exhibited Mg^{2+}-dependency in HA binding (lanes 1–3). The two proteins of 140 kDa and 95 kDa disappeared after adsorption with anti–Z-1-Sepharose, showing again that these are the two subunits of CR3 and that CR3 does not associated

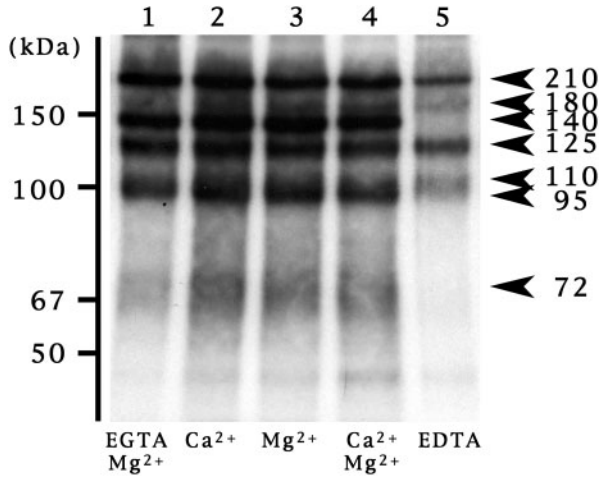

Fig. 5. **Effect of divalent cations on the binding of HABPs to HA30-Sepharose.** A detergent extract of ^{125}I-labeled peritoneal PMNs was diluted with solubilization buffer containing either 5 mM EGTA and 1 mM Mg^{2+} (lane 1), 0.6 mM Ca^{2+} (lane 2), 1 mM Mg^{2+} (lane 3), 0.6 mM Ca^{2+} and 1 mM Mg^{2+} (lane 4), or 5 mM EDTA (lane 5). Each mixture (equivalent to 2×10^6 cells) was preadsorbed with BSA-Sepharose and then reacted with HA30-Sepharose. The materials bound to HA30-Sepharose were analyzed as described in the legend to Fig. 3. One representative experiment of three with similar results is shown.

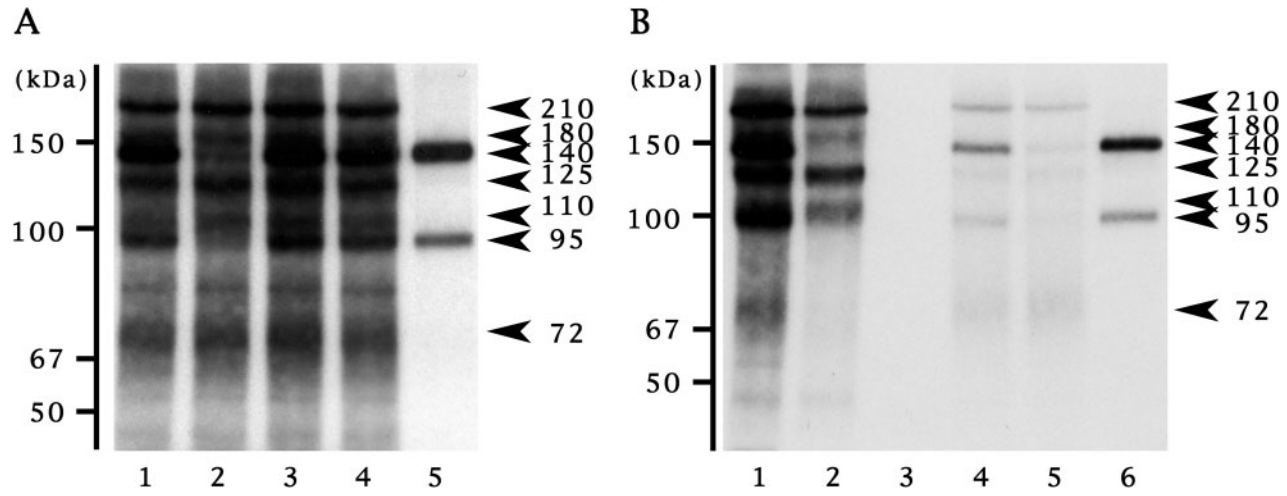

Fig. 6. **Identification of the 140 kDa and 95 kDa proteins as the α and β subunits of CR3.** A detergent extract (equivalent to 2×10^6 cells) of ^{125}I-labeled peritoneal PMNs was supplemented with 0.6 mM Ca^{2+} and 1 mM Mg^{2+}, and then the mixture was precleared with BSA-Sepharose. (A) For the inhibition experiment, the precleared mixture was further incubated with 5 μg/ml of soluble anti–Z-1 mAb for 20 min and then reacted with HA30-Sepharose (lane 4). For the CR3-adsorption experiment, the precleared mixture was adsorbed with either B6E9-Sepharose or anti–Z-1-Sepharose, and then reacted with HA30-Sepharose (lane 2: HA30-Sepharose after anti–Z-1-Sepharose, lane 3: HA30-Sepharose after B6E9-Sepharose, lane 5: anti–Z-1-Sepharose). As a control, the precleared mixture was reacted with HA30-Sepharose (lane 1). The materials bound to each adsorbent were analyzed as described in the legend to Fig. 3. One representative experiment of three with similar results is shown. (B) As in (A), the detergent extract precleared with B6E9-Sepharose was reacted with HA30-Sepharose in the presence of 0.6 mM Ca^{2+} and 1 mM Mg^{2+} (lane 1). HABPs bound to HA30-Sepharose were eluted with solubilization buffer containing 5 mM EDTA, and the eluate was dialysed against EDTA-free solubilization buffer. HABPs thus purified were again reacted with HA30-Sepharose in the presence of either 5 mM EDTA (lane 3) or both 0.6 mM Ca^{2+} and 1 mM Mg^{2+} (lane 4). The purified HABPs were also reacted with HA30-Sepharose in the presence of both 0.6 mM Ca^{2+} and 1 mM Mg^{2+} after adsorption with anti–Z-1-Sepharose (lane 5). The materials bound to each adsorbent were analyzed as described in the legend to Fig. 3. HABPs that remained on HA30-Sepharose after EDTA-elution (lane 2) and CR3 adsorbed with anti–Z-1-Sepharose (lane 6) are also shown. One representative experiment of three with similar results is shown.

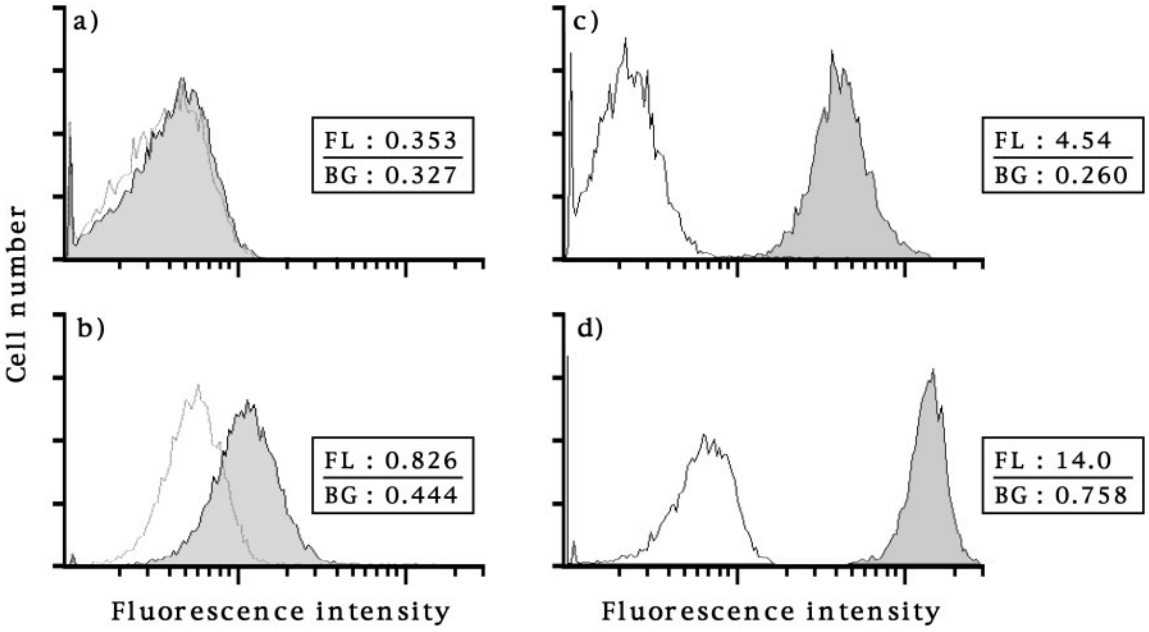

Fig. 7. **Flow cytometric analysis of the expression of HABPs and CR3 on guinea pig blood neutrophils.** Resting blood neutrophils (a and c) and PMA-activated blood neutrophils (b and d) were incubated with F-HA30 in the presence of 0.6 mM Ca^{2+} and 1 mM Mg^{2+} for 1 h at 37°C (a and b), or with FI–anti–Z-1 mAb for 15 min at room temperature (c and d). The cell-bound fluorescence was analyzed with a flow cytometer, and is presented as a shaded histogram with the logarithm of fluorescence intensity on the abscissa and the cell number on the ordinate. Controls involving unstained cells are presented as open histograms. One representative experiment of three with similar results is shown. The numbers in the squares indicate the mean values of the mean fluorescence intensity in each histogram obtained for duplicate determinations in one experiment (FL: fluorescein-stained cells, BG: control cells).

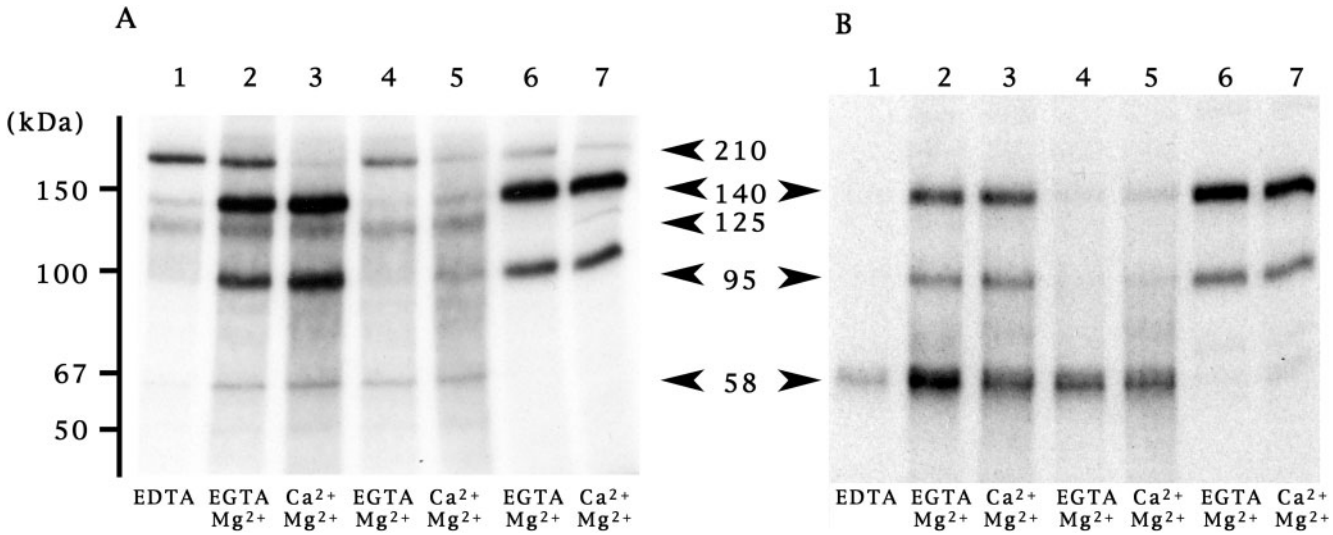

Fig. 8. **Characterization of HABPs on blood neutrophils by affinity chromatography.** A detergent extract of ^{125}I-labeled blood neutrophils (A) or PMA-activated neutrophils (B) was supplemented with either 5 mM EDTA (lane 1), 5 mM EGTA and 1 mM Mg^{2+} (lanes 2 and 4), or 0.6 mM Ca^{2+} and 1 mM Mg^{2+} (lanes 3 and 5), and then preadsorbed with B6E9-Sepharose (lanes 1–3) or anti–Z-1-Sepharose (lanes 4 and 5). The preadsorbed supernatants were then reacted with HA30-Sepharose. The materials bound to HA30-Sepharose (lanes 1–5) and anti–Z-1-Sepharose (lanes 6 and 7) were analyzed as described in the legend to Fig. 3. One representative experiment of three with similar results is shown.

with other membrane proteins under the experimental conditions used (lanes 4–7).

These results suggest that unactivated blood neutrophils express not only CR3 but also small amounts of the 210 kDa, 125 kDa and 58 kDa proteins on their surface. However, these proteins were functionally inactive on unactivated intact cells and required a certain activation process for the acquisition of their HA-binding activity. It was shown that both solubilization of the cells with a non-ionic detergent and stimulation of the cells with PMA were effective for the activation of functionally inactive HABPs. The effectiveness of a non-ionic detergent for the activation

of both Mg^{2+}-dependent and Ca^{2+}/Mg^{2+}-independent HABPs on resting cells, and the experimental conditions used for affinity chromatography suggest that the binding of either or both non-ionic detergent molecules and Mg^{2+} in the assay buffer to HABPs might cause certain structural changes in pre-existing HABP molecules, thereby activating previously inactive HABP molecules. It was also suggested that Ca^{2+} may also be involved in the regulation of the activity of the 210 kDa protein. In the case of PMA-activation, it is likely that PMA selectively activated CR3 and the 58-kDa protein, probably through phosphorylation of intracellular proteins, since PMA is known to trigger the intracellular signaling cascade by activating Ca^{2+}- and phospholipid-dependent protein kinase C. Indeed, it has been reported that PMA promotes the phosphorylation of CD18 in guinea pig and human leukocytes (*42*, *52*). In contrast, the HABPs of 210-kDa and 125-kDa could not be detected with HA30-Sepharose after stimulation with PMA, suggesting that PMA-treatment might selectively inactivate the 210-kDa and 125-kDa HABPs although other possibilities cannot be excluded.

DISCUSSION

In the present study, we have demonstrated the diversity of HABP molecules expressed on guinea pig peritoneal PMNs and blood neutrophils. Both types of cells express several kinds of HABP molecules with different molecular weights on their surface (Figs. 2 and 8). All the HABPs exhibited similar maximal HA-binding capacities over a wide pH range (from 6 to 8) and require an ionic interaction with HA (Fig. 4). These HABPs could be divided into two groups; one is Mg^{2+}-dependent and the other Ca^{2+}/Mg^{2+}-independent (Fig. 5). Since both divalent cation–dependent and –independent HA-binding reactions were also observed with intact cells (Fig. 1), it is likely that the HABPs identified in this study are involved in HA binding at the intact cell surface as well.

It has been implied that there might be a significant reduction in the pH of inflamed tissue and the pH gradient generated might affect neutrophil function. Indeed, Simkin and Bassett reported that lactic acid promotes local acidosis in the chronically inflamed synovium and the pH of the synovial fluid decreases from 7.3 in normal fluid to 6.8 in inflamed fluid (*54*). Even under inflamed conditions, neutrophils must accomplish their functions such as migration and phagocytosis in order to protect the body. In the present study, all HABPs exhibited maximal HA-binding ability over a wide pH range (Fig. 4): therefore, the binding characteristics of HABPs seem to be suitable for mediating a stable interaction between HA and PMNs not only under healthy conditions but also under inflamed conditions. In other words, our data suggest that all the HABPs found in this study have the potential to allow continuous regulation of PMN functions through binding to surrounding HA molecules in the ECM during inflammation. Similar optimum pH profiles have been reported for link protein and aggrecan (*55*). Both ECM components are essential for stabilization of the structural integrity of cartilage under both healthy and inflamed conditions by binding to HA molecules to form highly hydrated huge complexes with load-bearing properties.

Unlike HABPs on the intact cell surface (Fig. 1), the detergent-solubilized HABPs did not bind to HA under isotonic conditions (Fig. 4). Macklem and Sim also showed that detergent-solubilized human complement receptor type 1 and type 2 could bind to ligand-coupled Sepharose (iC3b-Sepharose) at low ionic strength and could be eluted from the affinity resin with the addition of 10–50 mM NaCl (*53*). Therefore, the enhanced susceptibility to ionic strength observed for the detergent-solubilized HABPs is not only the case for guinea pig HABPs. As a possible explanation for the different susceptibility to ionic strength observed between intact cell surface HABPs and detergent-solubilized HABPs, it is likely that homo- and heterotypic interactions between the HABP molecules or with other molecules may occur in the intact cell membrane, which stabilize the active conformation and the multimerization of the HABP molecules, thereby enhancing the avidity of the HABP molecules to bind HA molecules. On the other hand, solubilization with a non-ionic detergent may reduce the chance for such molecular interactions to occur and the HA-binding activity of each HABP molecule may become more susceptible to change in ionic strength than those in the intact cell membrane. Although other possibilities cannot be ruled out, the changes in ionic susceptibility of HABPs may be important for understanding the function–structure relationship of HABP molecules.

The identity of most HABPs found in this study is unknown. However, at least, the Mg^{2+}-dependent 95 kDa and 140 kDa proteins were found to be the two subunits of CR3 (CD11b/CD18) (Figs. 6 and 8). In addition, we presented several lines of indirect evidence suggesting that guinea pig CR3 may possess HA-binding activity, as follows: (i) CR3 could be isolated by HA-Sepharose chromatography, (ii) anti–Z-1 mAb-Sepharose immunoprecipitated CR3 alone, and (iii) HA binding of CR3 was not inhibited by soluble anti–Z-1 mAb. These data demonstrated that the CR3 molecule does not associate with other HABP molecules but binds to HA-Sepharose.

CR3, a member of the β_2-integrin family, is a heterodimeric molecule consisted of α-chain (CD11b) noncovalently associated with β_2-chain (CD18), and promotes a variety of leukocyte adhesive reactions. CR3 can bind to protein and polysaccharide ligands such as iC3b (*56*, *57*), fibrinogen (*58*), factor X (*59*), ICAM-1 (*60*), β-glucan (*61*), bacterial lipopolysaccharide (*50*), and heparin (*61*). The ligand-binding sites for protein ligands and heparin are located at an inserted region of ~200 amino acids termed the I domain in the α subunit of CR3 (*62*, *63*), while the binding site for β-glucan, the so-called lectin domain, is located outside of the I domain (*64*, *65*). Several studies have shown that the I domain has a unique cation coordination site termed the metal ion–dependent adhesion site (MIDAS), which is induced following cell activation, and coordinates Mg^{2+} and Mn^{2+} (*66*, *67*). Recent studies have shown the existence of an I domain–like structure in the β subunit of CR3 and its involvement in ligand binding (*68*). Collectively, these studies suggest that several separate regions of the CR3 molecule are concerned with ligand binding. Indeed, Li and Zhang have argued that three separate regions in the CR3 molecule (the I domain of the α chain, the I domain of the β chain and the β-propeller region of the α chain) reside proximally

in space and contribute to the formation of a ligand-binding site (*69*).

Most studies on the structural and functional characteristics of CR3 have been performed with human and murine systems. In the case of guinea pig CR3, the N-terminus amino acid sequence is identical with that of human CD11b (*70*), and our recent studies have shown that there is approximately 75.2% homology between the primary structure of guinea pig CD11b and that of human CD11b (Nochi *et al.*, unpublished data). It has thus been implied that guinea pig CR3 may have similar functions to those of human CR3. However, we have frequently obtained results that seem to reflect certain structural and functional differences in the CR3 molecule between the two species. For example, the detergent-solubilized human CR3 binds to iC3b and β-glucan whereas the guinea pig CR3 does not (*41*, *61*, *71*), and several mAbs to human CR3 do not cross-react with guinea pig CR3 (*41*). Thus, the details of the molecular characteristics of guinea pig CR3 and its ligand specificity are unknown. We propose here that HA may be an adhesive ligand for guinea pig CR3. Although our results are inconsistent with those reported by Diamond *et al.*, who showed heparin-binding ability of human CR3 but not HA-binding ability (*62*). The discrepancy may also be explained by the structural difference in CR3 between the two species, as mentioned above.

With respect to the localization of the HA-binding site on the CR3 molecule, the fact that CR3-mediated zymosan phagocytosis (*40*, *42*) and HA binding (the present study) exhibited different susceptibilities to soluble anti–Z-1 mAb suggests that the HA-binding site on the CR3 molecule may be distinct from that required for zymosan phagocytosis. Furthermore, our previous finding that high-molecular-weight HA inhibits CR3-mediated SOZ phagocytosis without affecting the binding of SOZ to the cell surface (*37*) suggests the HA-binding site may also be distinct from the SOZ-binding site. In addition, the observed Mg^{2+}-dependency of the CR3-HA interaction suggests that the existence of a MIDAS motif-like structure in guinea pig CR3 may be essential for HA binding as well. There have been many studies on the HA-binding sites of a variety of HA-binding molecules, and two structures required for HA binding have been identified; one is the BX_7B motif, where B is a basic residue and X is any amino acid that is not negatively charged (*33*), and the other a common structure termed the Link-module that is shared by most HA-binding molecules (*21*). It should be noted that, in our recent study, at least one BX_7B motif-like amino acid sequence was found in the α subunit of guinea pig CR3 but not in human CD11b (Nochi, et al., unpublished data). It is, therefore, possible that guinea pig CR3 might use the BX_7B motif-like structure as well for HA binding, although further studies on the molecular characteristics of guinea pig CR3 are needed to clarify this.

The following facts suggest that the expression of the HA-binding activities of diverse HABPs on guinea pig neutrophils are separately and/or sequentially modulated in a manner depending on the stage of cell activation: firstly, resting blood neutrophils constitutively expressed Mg^{2+}-dependent and Ca^{2+}/Mg^{2+}-independent HABPs (Fig. 8), whereas these HABPs were functionally inactive on intact cells (Fig. 7); secondly, PMA-stimulation of resting neutrophils only activated Mg^{2+}-dependent HABPs and up-regulated CR3 expression (Figs. 7 and 8); thirdly, the number of HABP species expressed on resting blood neutrophils was smaller than that expressed on casein-induced peritoneal PMNs (Figs. 2 and 8); and finally, casein-induced peritoneal PMNs that were activated *in vivo* expressed both Mg^{2+}-dependent and Ca^{2+}/Mg^{2+}-independent HABPs in a functionally active state (Figs. 1 and 5). In such processes, as mentioned previously, a conformational change and up-regulated surface expression of HABPs are possibly involved in and contribute to the regulation of neutrophil functions. These results suggesting cell activation-dependent HABP activation are consistent with those reported for other adhesion receptors such as β_2-integrin and CD44 (*51*, *72*). Both adhesion receptors pre-existing on the plasma membrane of resting cells are functionally inactive, and the stimulation of cells with various stimulants such as chemotactic factors and pro-inflammatory cytokines promotes their increased surface expression and induces structural changes in these receptor molecules to make them functional.

For further understanding of the roles of HABPs including CR3 in the regulation of neutrophil functions during inflammation, further studies on the molecular structure and function of each HABP are currently in progress.

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