Neutrophil Recognition Requires a Ca\textsuperscript{2+}-induced Conformational Change in the Lectin Domain of GMP-140*  

(Received for publication, May 14, 1991)

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GMP-140, a receptor for myeloid cells that is expressed on surfaces of thrombin-activated platelets and endothelial cells, is a member of the selectin family of adhesion molecules that regulate leukocyte interactions with the blood vessel wall. Each selectin contains an N-terminal domain homologous to Ca\textsuperscript{2+}-dependent lectins and mediates cell-cell contact by binding to oligosaccharide ligands in a Ca\textsuperscript{2+}-dependent manner. The mechanisms by which Ca\textsuperscript{2+} promotes selectin-dependent cellular interactions have not been defined. We demonstrate that purified GMP-140 contains two high affinity binding sites for Ca\textsuperscript{2+} as measured by equilibrium dialysis (K_d = 22 ± 2 µM). Occupancy of these sites by Ca\textsuperscript{2+} alters the conformation of the protein as detected by a reduction in intrinsic fluorescence emission intensity (K_d = 4.8 ± 0.2 µM). This Ca\textsuperscript{2+}-dependent conformational change exposes an epitope spanning residues 19–34 of the lectin domain that is recognized by a monoclonal antibody capable of blocking neutrophil adhesion to GMP-140 (half-maximal antibody binding at ~20 µM Ca\textsuperscript{2+}). Furthermore, a synthetic peptide encoding this epitope, CQNRTV-DLVAIQKNE, inhibits neutrophil binding to GMP-140. Mg\textsuperscript{2+} also alters the conformation of the protein, but not in a manner that will support leukocyte recognition in the absence of Ca\textsuperscript{2+}. There is a strong correlation between the Ca\textsuperscript{2+} levels required for neutrophil adhesion to GMP-140, for occupancy of the two Ca\textsuperscript{2+}-binding sites, for the fluorescence-detected conformational change, and for exposure of the antibody epitope in the lectin domain. We conclude that binding of Ca\textsuperscript{2+} to high affinity sites on GMP-140 modulates the conformation of the lectin domain in a manner that is essential for leukocyte recognition.

Leukocytes must bind to the vascular endothelium before their migration into tissues in response to inflammatory stimuli. Several classes of receptors that regulate these adhesive interactions have been identified (1), of which the most recently described are the selectins (reviewed in Ref. 2). The three known selectins are LAM-1 (peripheral lymph node homing receptor, LECAM-1), a molecule present on leukocytes that participates in neutrophil adhesion to cytokine-activated endothelium and homing of lymphocytes to high endothelial venules of peripheral lymph nodes (3–5); ELAM-1, a cytokine-inducible endothelial cell receptor for neutrophils (6), monocytes (7), and memory T cells (8, 9); and granule membrane protein-140 (GMP-140, PADGEM, CD62), a receptor for myeloid cells that is rapidly mobilized from secretory granule membranes to surfaces of platelets and endothelial cells activated with agonists such as thrombin (reviewed in Ref. 10). Each of the selectins contains an N-terminal lectin-like domain, followed by an epidermal growth factor (EGF)-like module, a series of consensus repeats related to those in complement-binding proteins, a transmembrane domain, and a cytoplasmic tail (3–6, 11).

The presence of a lectin-like motif suggested that each selectin might promote cell-cell contact by interacting with carbohydrate moieties on opposing cells. This hypothesis has been confirmed by recent studies demonstrating that all of the selectins bind to sialylated oligosaccharides (12). Most of the new information concerns the carbohydrate structures on myeloid cells recognized by ELAM-1 and GMP-140, both of which bind to sialylated, fucosylated lactosaminoglycans, including the tetrasaccharide sialyl Lewis x (13–20).

The N-terminal domains of the selectins are homologous to a family of "C-type" lectins that require Ca\textsuperscript{2+} to bind carbohydrate (21). Consistent with this structural feature, the selectins all require Ca\textsuperscript{2+} to mediate cellular interactions (2). The mechanisms responsible for Ca\textsuperscript{2+}-dependent modulation of cell recognition by selectins are not known. In the case of GMP-140, half-maximal neutrophil adhesion to the purified protein is supported by ~20 µM Ca\textsuperscript{2+} (22). Mg\textsuperscript{2+} at a concentration of 1 mM does not support neutrophil binding to GMP-140, but allows lower concentrations of Ca\textsuperscript{2+} to mediate adhesion (half-maximal at ~2 µM) (22). The latter observation suggests that there are two or more binding sites for divalent cations that participate in neutrophil recognition, at least one of which is specific for Ca\textsuperscript{2+}.

In this study, we demonstrate that Ca\textsuperscript{2+} binds to two high affinity sites on GMP-140. Occupancy of these sites alters the conformation of the molecule, at least in part by modulating regions of the lectin domain essential for neutrophil recognition. Mg\textsuperscript{2+} also alters the conformation of GMP-140, apparently by binding to a site(s) distinct from those filled by Ca\textsuperscript{2+}.

* The abbreviations used are: GMP-140, granule membrane protein-140; EGF, epidermal growth factor; KLH, keyhole limpet hemocyanin; TBS, Tris-buffered saline; EGTJA, [ethylenebis(oxyethyl)enitrilo]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.

1-piperazineethanesulfonic acid.
The anti-GMP-140 monoclonal antibodies G1, G2, G3, S12, and W40 were prepared and characterized as described (22-24); the antibodies were all of the IgG1 subclass and were used as purified murine immunoglobulin. Antibodies G1, G2, and G3 do not cross-react with ELAM-1 as determined by immunoperoxidase (22) or Western blot analysis of COS cells transfected with ELAM-1 cDNA. Furthermore, G1 and G3 have been shown not to cross-react with LAM-1, because they bind to platelets but not to leukocytes as assessed by flow cytometry.

Two synthetic peptides designed from the GMP-140 sequence (11) were prepared by Peninsula Laboratories (Belmont, CA). The first spanned residues 19-34 of the lectin domain (CNQRFTY-DLVAGQKNNE) and the second spanned residues 761-777 of the cytoplasmic tail (KDDGKCPLNPHSHLCTGY). The peptides were purified by high performance liquid chromatography, and their identities were confirmed by amino acid analysis. Stock solutions of peptides were prepared in sterile deionized water; the pH was adjusted to 7.4 by addition of 1 M Hepes (final concentration of 50 mM). Peptides were coupled to keyhole limpet hemocyanin (KLH) (5 mg of peptide to 25 mg of KLH, 90% coupling efficiency) by Peninsula Laboratories. The 19-34 peptide was coupled to KLH via its N-terminal cysteine using N-maleimidobenzoic acid N-hydroxysuccinimide ester. The 762-777 peptide was coupled through primary amines using 1-ethyl-3-(3-dimethylaminopropyl)carboimidiomide. Six additional peptides spanning all but the most hydrophobic regions of the lectin domain were synthesized and purified by Dr. Kenneth Jackson in the Molecular Biology Resource Facility of the St. Francis Medical Research Institute, University of Oklahoma Health Sciences Center. The sequences of these peptides are as follows: residues 6-18 (STKAYSWNSIRKY); residues 31-43 (KNKEDYLKVKL), residues 54-72 (RRKKNWTWVTWKKALTN), residues 73-89 (AENWADNPNNKRNNE), residues 91-106 (VEYIKSP-SAPGKWDEH), and residues 110-116 (LKKKHAL).

Details of these peptides will be described elsewhere.2 Preparation of GMP-140 for Divalent Cation Binding Studies—GMP-140 was purified from human platelet lysates as previously described (19). For experiments requiring metal-free conditions, GMP-140 was extensively dialyzed at 4 °C for 36 h in polypropylene containers (Nalgene) against three changes of a 1000-fold volume ratio of 10 mM Tris-HCl pH 7.5, 0.1 M NaCl, and 0.1% (v/v) Lubrol PX that had previously been percolated through a Chelex 100 column (25 x 40 cm). A 50-ml sample of the Chelex-treated buffer was lyophilized and then redissolved in 5 ml of the initial Chelex-treated buffer; measurements of the concentrated sample using an atomic absorption spectrophotometer (Varian-Techtron model 1200) showed that the unconcentrated Chelex-treated buffer contained less than 1 μM Ca2+ and Mg2+. Measurements with the atomic absorption spectrophotometer also showed that a stock 1 M CaCl2 solution contained less than 1 μM Mg2+ and a stock 1 M MgCl2 solution contained less than 1 μM Ca2+. Prior to use, proteins were exposed only to solutions which had been Chelex-treated. Analytical sedimentation velocity analysis indicated that purified GMP-140 is monomeric in buffers containing Lubrol PX at or above its critical micelle concentration of 0.0058% (100 μM). Therefore, 0.02% Lubrol PX was included in all buffers to ensure that GMP-140 was studied in its monomeric form.

Equilibrium Dialysis—CaCl2 binding studies were performed in 8-place dialyzing modules (EMD 103 dialysis membrane, M cutoff = 13,000-15,000, 0.25-ml cell, Hoefer Scientific Instruments). An aliquot of purified GMP-140 (0.6 mg/ml) was added to 100 μl of Chelex-treated buffer containing 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.02% (v/v) Lubrol PX; the sample was placed in the chamber on one side of the dialysis membrane; on the other side of the chamber was placed an equal volume of buffer containing 2 mM CaCl2 and various levels of unlabeled CaCl2 in the presence or absence of 1 mM MgCl2. After incubation for 24 h at 37 °C with vigorous agitation, aliquots of 40-μl sample were removed from each side of the membrane and transferred to 10 ml of Aquasol II. Radioactivity was quantitated in an LS 5000 CE liquid scintillation system (Beckman). Radioactivity remaining in the cells and membranes at the end of dialysis was less than 1% of that added. Analysis of protein samples by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis after equilibrium dialysis indicated that GMP-140 did not undergo proteolytic degradation during these experiments.

Fluorescence Measurements—All fluorescence spectra were obtained in 1 x 1-cm quartz cuvettes in an SLM-8000C fluorescence spectrophotometer (SLM-Aminco, Urbana, IL) equipped with a stirrer and computerized control. Excitation and emission slits were 10 nm. The terminal cysteine of the 762-777 peptide was maintained at 25 °C, and shutters were kept closed except during scans to avoid photodegradation of the sample. Samples were excited at 280 nm, and the band-pass was 4 nm on both excitation and emission light paths. Emission scans were recorded from 305-400 nm at 1-nm intervals using 5-ns integration times. GMP-140 (0.2 μM) in Chelex-treated 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.02% (v/v) Lubrol PX was titrated with CaCl2, MgCl2, or MnCl2 in the same buffer. To measure changes in intrinsic fluorescence emission intensity as a function of divalent cation concentration, tryptophans were excited at 285 nm and emission intensity was measured at an excitation wavelength of 340 nm. Measurements were made 30 min after each addition of titrant to ensure that the sample was at equilibrium. In all experiments, the background signals of protein-free samples were subtracted, and all data were corrected for dilution due to the addition of titrant.

Monoclonal Antibody Epitope Mapping with Synthetic Peptides—Binding of monoclonal antibodies to immobilized GMP-140 was measured by ELISA as described for the divalent cation titration experiments, except that 50 μl of GMP-140 at a concentration of 2.5 μg/ml was added to each microtiter well, all buffers were TBS or TBS/bovine serum albumin buffer containing 1 mM CaCl2, and the monoclonal antibodies were directly biotinylated (26), followed by detection with the peroxidase-streptavidin complex of the Vectorstain ABC kit. To determine whether any of the lectin-domain peptides inhibited antibody binding, monoclonal antibodies were incubated for 1 h at each of the peptide concentrations prior to addition to the wells. To determine whether antibodies bound directly to selected peptides, the lectin 19-34/KLH and C-terminal 761-777/KLH peptide-protein conjugates (0.5 mg/ml, 50 μl/well) were coated on microtiter wells at 4 °C overnight. Antibody binding was then measured by ELISA as described above for binding to immobilized GMP-140.

Inhibition of Neutrophil Adhesion to GMP-140 by Synthetic Peptides—Neutrophil adhesion to GMP-140 immobilized on microtiter wells was measured with a myeloperoxidase assay as previously described (22,25). To determine the effect of synthetic peptides derived from the GMP-140 sequence on adhesion, 50-μl aliquots of peptide dissolved in Hank’s balanced salt solution containing 1.26 mM Ca2+, 0.8 mM Mg2+, and 1% human serum albumin were preincubated with...
RESULTS

Ca2+ Binding to GMP-140—To determine if Ca2+ interacts directly with GMP-140, we measured the binding of 45Ca2+ to the purified protein by equilibrium dialysis. Fig. 1 demonstrates that Ca2+ bound to two indistinguishable high affinity sites on GMP-140 (Kd = 22 ± 2 µM). Addition of 1 mM Mg2+ had only a minor effect on either the affinity (Kd = 17 µM) or stoichiometry of 45Ca2+ binding to GMP-140 (Fig. 1). This indicates that Mg2+ does not bind tightly to either of the two high affinity Ca2+-binding sites. The stoichiometry and affinity of Mg2+ binding to GMP-140 was not directly determined using radioactive Mg2+.

Changes in Fluorescence Emission Induced by Divalent Cations—The binding of divalent cations to GMP-140 might support leukocyte recognition by inducing functionally relevant conformational changes in the protein. We therefore examined whether divalent cations altered the conformation of GMP-140 by monitoring its intrinsic fluorescence emission, since a metal ion-dependent conformational change may alter the immediate environments of one or more tryptophans and thereby change their fluorescence properties. Addition of 1 mM Ca2+ to a sample of divalent cation-free GMP-140 caused a blue-shifted decrease in emission intensity (Fig. 2A). No additional change in fluorescence was observed if 2.5 mM Mg2+ was then added to the sample (Fig. 2A). The observed change in emission intensity was completely reversible by addition of excess EDTA. The fluorescence change did not result from a divalent cation-induced protein aggregation, because sedimentation velocity studies showed that GMP-140 solubilized in 0.02% Lubrol PX retains a monomeric configuration in the presence of either 1 mM Ca2+ or EDTA. Thus, the binding of Ca2+ to GMP-140 elicits a fluorescence-detected conformational change.

To evaluate the divalent cation specificity of this structural change, 2.5 mM Mg2+ was added to a sample of Chelex-treated GMP-140 and this decreased the emission intensity of the protein (Fig. 2B). The subsequent addition of 0.1 mM Ca2+ caused a further decrease in emission intensity. These spectral changes were completely reversible with excess EDTA. Both Ca2+ and Mg2+ caused a greater decrease in emission intensity between 336 and 400 nm than between 305 and 335 nm, suggesting that their association with GMP-140 had a larger effect on the environment of relatively exposed tryptophans than of buried tryptophans. These results indicate that both Ca2+ and Mg2+ alter the conformation of GMP-140, but the conformational shifts are not equivalent.

The changes in fluorescence emission intensity of GMP-140 as a function of divalent cation concentration are shown in Fig. 3. Ca2+ elicited a 12% reduction in intrinsic fluorescence intensity (Fig. 3A), and double-reciprocal analysis of these data (Fig. 3B) suggests that Ca2+ interacted with a single class of binding sites with an apparent Kd of 4.8 ± 0.2 µM. Mg2+ caused a 7% reduction in emission intensity (Fig. 3A), and double-reciprocal analysis (not shown) suggests a single class of binding sites with an apparent Kd of 55 µM.

When Ca2+ was titrated into a sample of GMP-140 that already contained 0.1 mM Mg2+ and hence a reduced intrinsic fluorescence, a further decrease in emission intensity was observed (Fig. 3A). The final emission intensity was identical to that produced by Ca2+ alone. The apparent Kd for Ca2+ binding was only slightly lower (2.6 ± 0.2 µM) in the presence of 0.1 mM Mg2+ than in the absence of Mg2+ (Fig. 3B). However, the decrease in fluorescence intensity was close to maximal at Ca2+ levels of only 10 µM in the presence of Mg2+, whereas the same decrease in fluorescence intensity required Ca2+ concentrations of greater than 100 µM in the absence of Mg2+. The observed changes in intrinsic fluorescence emission intensity were specific for Ca2+ and Mg2+, because a titration with Mn2+ did not alter GMP-140 fluorescence intensity (Fig. 3A). Furthermore, titration of Ca2+ into a sample of GMP-140 that contained 0.1 mM Mn2+ caused a reduction in emission intensity at each Ca2+ concentration that was identical to that produced by Ca2+ titration in the absence of Mn2+ (not shown).

Mapping of an Antibody Epitope to a Functionally Important

![Fig. 1. Ca2+ binding to GMP-140. Equilibrium dialysis experiments were performed with 45Ca2+ in the presence or absence of 1 mM Mg2+, as described under "Experimental Procedures." The data are plotted as fractions moles of Ca2+ bound per mol of GMP-140 versus the concentration of Ca2+ free in solution. Data from two separate experiments are shown for the "Ca2+" binding study performed in the presence of Mg2+. Each point is the average of duplicate measurements. The inset is a Scatchard plot of the data generated by linear least squares regression analysis.](image-url)
Region of the Lectin Domain—The above studies indicate that binding of divalent cations induces conformational changes in GMP-140 that may be important for leukocyte recognition. However, the data do not localize the observed conformational changes to a particular domain of the protein. A candidate site for conformational alteration is the lectin domain. We previously described monoclonal antibodies to GMP-140 that block (G1, G2, and G3) or do not block (S12 and W40) binding to neutrophils (22, 24). Since GMP-140 mediates cell contact at least in part by interaction with oligosaccharides (18–20, 27, 28), the blocking antibodies might identify epitopes in the lectin domain, the predicted site for oligosaccharide recognition. To test this possibility, we synthesized a series of seven peptides spanning the lectin domain of GMP-140, as outlined under “Experimental Procedures,” and used an ELISA to test whether the peptides inhibited antibody binding to immobilized GMP-140. Only one of these peptides, consisting of residues 19–34 of the mature protein (CQNRYTDLVAIQRKNE), affected antibody binding. This peptide blocked binding of G3, but not S12, to GMP-140 in a dose-dependent manner (Fig. 4A). G3, but not S12, also bound directly to the 19–34 peptide of the lectin domain when it was conjugated to a carrier protein and coated on plastic. Neither G3 nor S12 bound to an immobilized control peptide conjugate, prepared from a synthetic peptide encoding residues 761–777 of the cytoplasmic domain of GMP-140 (Fig. 4B). The 19–34 peptide of the lectin domain also partially blocked binding of G1 and G2 to GMP-140, but these two antibodies did not bind directly to the 19–34 peptide conjugate (not shown). These data indicate that the 19–34 peptide of the lectin domain contains most or all of the epitope recognized by the blocking antibody G3 and part of the epitopes identified by G1 and G2.

The G3 antibody might inhibit neutrophil adhesion to GMP-140 either by binding to a site directly involved in leukocyte recognition or by indirectly altering another functionally important region of the molecule. Fig. 5 demonstrates that the lectin 19–34 peptide containing the G3 epitope inhibited neutrophil adhesion to immobilized GMP-140 in a dose-dependent fashion, whereas the control peptide containing...
sequence from the cytoplasmic domain had no effect. This result suggests that the region encompassed by residues 19–34 of the lectin domain participates directly in interactions with leukocytes.

Ca\(^{2+}\)-dependent Exposure of an Antibody Epitope in the Lectin Domain—If the binding of Ca\(^{2+}\) to GMP-140 alters the conformation of the lectin domain in a manner that is essential for leukocyte recognition, it might also modulate the epitopes for certain blocking antibodies such as G3 that identify functionally important sites in the lectin domain. To examine this possibility, we measured the binding of monoclonal antibodies to immobilized GMP-140 as a function of divalent cation concentration. Fig. 6 demonstrates that the interaction of G3 with GMP-140 was Ca\(^{2+}\)-dependent, with half-maximal binding at ~20 μM levels of the metal ion. Binding of the other blocking antibodies, G1 and G2, required similar levels of Ca\(^{2+}\). At Ca\(^{2+}\) levels of 1 μM or less, binding of these three antibodies to immobilized GMP-140 was no greater than that to microtiter wells coated only with albumin. In contrast, the nonblocking antibodies S12 (Fig. 6) and W40 (not shown) interacted equally well with GMP-140 in the presence or absence of Ca\(^{2+}\). Unlike Ca\(^{2+}\), Mg\(^{2+}\) at concentrations up to 1 mM did not support binding of G1, G2, or G3 to GMP-140. Furthermore, Mg\(^{2+}\) at a concentration of 1 mM did not allow lower concentrations of Ca\(^{2+}\) to support antibody binding (not shown).

DISCUSSION

Our results show that GMP-140 contains two high affinity binding sites for Ca\(^{2+}\). The Scatchard plot (Fig. 1) indicates that the dissociation constants for Ca\(^{2+}\) binding to these two sites are the same, or nearly so, and the binding to each site occurs independently. The binding of Ca\(^{2+}\) to GMP-140 alters the conformation of the protein, as shown by the Ca\(^{2+}\)-dependent changes in intrinsic fluorescence emission intensity. The linearity of the double-reciprocal plot (Fig. 3B) suggests that the Ca\(^{2+}\)-induced conformational change is mediated by Ca\(^{2+}\) binding to two independent sites with equivalent (or nearly so) affinities for Ca\(^{2+}\). It is also possible that the occupation of only one of the sites by Ca\(^{2+}\) elicits a change in intrinsic emission intensity and that the binding of Ca\(^{2+}\) to the second site is spectroscopically undetectable. Whatever the case, at least part of this conformational change occurs in a functionally important region of the lectin domain recognized by a monoclonal antibody, G3, that blocks neutrophil recognition by GMP-140.

Previous studies have demonstrated that Ca\(^{2+}\) is required for adhesive interactions of GMP-140 with leukocytes (22, 24, 29, 30) and that adhesion is mediated at least in part by lectin-like recognition of oligosaccharide ligands (18–20, 27, 28). There is a strong correlation between the Ca\(^{2+}\) levels required for neutrophil adhesion to GMP-140 (half-maximal binding at ~20 μM) (22), for occupancy of the two Ca\(^{2+}\)-binding sites (Kd = 22 μM), for the fluorescence-detected conformational change in the protein (Kd = 4.8 μM), and for recognition of a functionally significant epitope in the lectin domain by the G3 antibody (half-maximal binding at ~20 μM). Together, these data strongly suggest that binding of Ca\(^{2+}\) to GMP-140 mediates a conformational change in the lectin domain that is essential for leukocyte recognition.

Mg\(^{2+}\) also interacts with GMP-140 and induces a conformational alteration that is detected by a reduction in intrinsic fluorescence emission intensity. Because the binding of Mg\(^{2+}\) was not measured by equilibrium dialysis, the stoichiometry and affinity of this interaction could not be determined directly. However, the Kd for the fluorescence emission change induced by Mg\(^{2+}\) (55 μM) is 10 times higher than the Kd for fluorescence change induced by Ca\(^{2+}\) (4.8 μM). This observation suggests that the binding site(s) on GMP-140 for Mg\(^{2+}\) is of lower affinity than those for Ca\(^{2+}\).

The presence of Mg\(^{2+}\) alone is not sufficient to support neutrophil adhesion to GMP-140 (22). However, addition of Mg\(^{2+}\) allows lower concentrations of Ca\(^{2+}\) to mediate neutrophil recognition (22) and to produce the maximal conformational change detected by reduction in fluorescence emission intensity (Fig. 3A). It is not clear how Mg\(^{2+}\) potentiates the Ca\(^{2+}\)-mediated structural and functional changes in the protein. Mg\(^{2+}\) appears to bind to sites different from those occupied by Ca\(^{2+}\), because addition of 1 mM Mg\(^{2+}\) has little effect on the stoichiometry or affinity of binding of 4Ca\(^{2+}\) to GMP-140. Although Mg\(^{2+}\) is not required for interactions of GMP-140 with leukocytes in vitro, it may support other important functions not measured in our assays.

Although we have not yet located the actual binding sites for divalent cations on GMP-140, the most likely areas are in the lectin and EGF domains. In the case of the lectin domain, previous studies have examined the effects of Ca\(^{2+}\) on the structure and function of another well characterized member of the C-type lectin family, the asialoglycoprotein receptor. A monomeric proteolytic fragment of the chicken receptor consisting only of the carbohydrate recognition domain contained two low affinity Ca\(^{2+}\)-binding sites (Kd ~ 1–2 mM) as estimated by mathematical modeling (31). The Kd for binding correlated with the Ca\(^{2+}\) requirements for carbohydrate recognition and for conformational changes detected by protease sensitivity and circular dichroism studies. Mg\(^{2+}\) levels up to 15 mM did not alter the conformation of the chicken receptor as measured by changes in protease sensitivity. Ca\(^{2+}\) also modulated the conformation of the rabbit receptor as detected by reductions in intrinsic fluorescence emission intensity (32). Filtration studies with 4Ca\(^{2+}\) indicated that the rabbit molecule contained two Ca\(^{2+}\)-binding sites (Kd ~ 0.35 mM) and possibly a third very low affinity site which could be inhibited by 100 mM Mg\(^{2+}\). Since the receptor molecules examined in these studies consisted exclusively or predominantly of a C-type lectin domain, the sites of divalent cation binding could be assigned to this domain. By analogy, the binding sites for Ca\(^{2+}\), and possibly also for Mg\(^{2+}\), may be in the lectin domain of GMP-140. This positioning of divalent cation-binding sites would be compatible with the induction of local conformational changes in the lectin domain that are required for recognition of carbohydrate ligands on leukocytes. However, if the binding sites for Ca\(^{2+}\) on GMP-140 are in the lectin

![Fig. 6. Ca\(^{2+}\) dependence of monoclonal antibody binding to GMP-140.](image-url)

GMP-140 was immobilized on microtiter wells in the indicated concentration of Ca\(^{2+}\). The binding of monoclonal antibodies to GMP-140 was then measured by ELISA as described under “Experimental Procedures,” using buffers containing Ca\(^{2+}\) concentrations identical to those used for immobilization of the protein. Each point represents the mean ± S.D. of triplicate determinations from three independent experiments.
domain, they are of significantly higher affinity than those of the corresponding motif in the asialglycoprotein receptor. Like the other selectins, GMP-140 contains an EGF-like motif located next to the N-terminal lectin domain (11). The EGF domain may be required for selectin-mediated recognition of target cells, since certain functionally important epitopes in the lectin domains of the homing receptor and ELAM-1 require an adjacent EGF domain for expression (14, 33). Furthermore, leukocyte adhesion is blocked by a monoclonal antibody to an epitope created by an amino acid substitution in the EGF domain of the homing receptor (34). Some EGF domains in other proteins have been demonstrated to contain high affinity Ca\(^2+\)-binding sites; occupancy of these sites by Ca\(^2+\) can alter the conformation of the domain as well as the function of the protein (35-40). By analogy, one of the high affinity Ca\(^2+\)-binding sites on GMP-140 may be in the EGF domain. By altering the structure of the EGF domain, Ca\(^2+\) might modulate the conformation of the lectin domain through domain-domain interactions. Alternatively, binding of Ca\(^2+\) might support direct interactions of the EGF domain with a protein component of the ligand on target cells.

Some EGF domains contain the consensus sequence CX\(CD\)NXXXXF/YXXCX, where the Asp or Asn residue is hydroxylated (41). It was initially proposed that the hydroxylated residue was required for Ca\(^2+\) binding (36-38, 42), although it has since been demonstrated that Ca\(^2+\) binds to EGF domains lacking this modification (40). The EGF domain in GMP-140 (11) contains a similar consensus sequence, except that a Glu residue is present instead of the Asp or Asn. Although it is theoretically possible that a Glu in this position might undergo hydroxylation like Asp or Asn, no such modification has been detected in amino acid hydrolysates of purified GMP-140.\(^4\)

Cell-cell contact mediated by LAM-1 and ELAM-1 requires Ca\(^2+\) (7, 43), suggesting that these selectins, like GMP-140, contain binding sites for Ca\(^2+\) and perhaps Mg\(^2+\) that modulate their structure and function. Binding of divalent cations is also critical in supporting the adhesive properties of other two classes of cell surface receptors, cadherins (44) and integrins (45). Additional study of the mechanisms by which divalent cations interact with selectins should provide insight into the structural basis for cellular communication mediated by these proteins.

Acknowledgments—We thank Charles Esmon for many helpful discussions, Ginger Hampton for excellent technical assistance, and Naomi Esmon and James Morrissey for critical reading of the manuscript.

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\(^4\) Ca\(^2+\) Modulation of GMP-140 Structure