Characteristics of Cation Binding to the I Domains of LFA-1 and MAC-1

THE LFA-1 I DOMAIN CONTAINS A Ca\(^{2+}\)-BINDING SITE*

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The crystal structures of the I domains of integrins MAC-1 (α\(_{M}β_2\)) and LFA-1 (α\(_{L}β_2\)) show that a single conserved cation-binding site is present in each protein. Purified recombinant I domains have intrinsic ligand binding activity, and in several systems this interaction has been demonstrated to be cation-dependent. It has been proposed that the I domain cation-binding site represents a general metal ion-dependent adhesion motif utilized for binding protein ligands. Here we show that the purified recombinant I domain of LFA-1 (α\(_{L}I\)) binds cations, but with significantly different characteristics compared with the I domain of MAC-1 (α\(_{M}I\)). Both α\(_{L}I\) and α\(_{M}I\) bind \(5\text{mM} \text{Mn}^{2+}\) in a conformation-dependent manner, and in general, cations with charge and size characteristics similar to \(\text{Mn}^{2+}\) most effectively inhibit \(54\text{Mn}^{2+}\) binding. Surprisingly, however, physiological levels of \(\text{Ca}^{2+}\) (1–2 mM) inhibited \(54\text{Mn}^{2+}\) binding to purified α\(_{L}I\), but not to α\(_{M}I\). Using \(\text{Ca}^{2+}\) and \(\text{Mn}^{2+}\) in direct binding studies, the dissociation constants (\(K_D\)) for the interactions between these cations and α\(_{L}I\) were estimated to be 5–6 × 10^{-7} and 1–2 × 10^{-5} M, respectively. Together with the available structural information, the data suggest differential affinities for \(\text{Mn}^{2+}\) and \(\text{Ca}^{2+}\) binding to the I domain of LFA-1, but not to MAC-1, and to the I domain of NIF, a hookworm-derived MAC-1 inhibitor. Our results indicate that like α\(_{M}I\), α\(_{L}I\) may be related to the \(\text{Ca}^{2+}\) binding activity of the LFA-1 I domain.

LFA-1 (α\(_{L}β_2\)) and MAC-1 (α\(_{M}β_2\)) are closely related leukocyte integrins that are essential for normal immune system functions (1, 2). Both integrins bind to several distinct ligands, but share in the ability to bind ICAM-1, a widely expressed cell surface protein (3–5). Recent studies suggest that in the β\(_2\) and other non-RGD binding integrins, additional and perhaps multiple subdomains within both the α and β subunits may contribute to form the complete ligand-binding domain. One of these subdomains is the I domain (also known as the A domain), a region of approximately 200 amino acids found in a variety of proteins as well as the α subunit ectodomain of all β\(_2\) integrins and VLA-1 (α\(_{1}β_1\)), VLA-2 (α\(_{2}β_1\)), and α\(_{5}β_7\) (6, 7).

Several lines of evidence suggest that the I domain in the context of the complete integrin may play a significant and direct role in ligand binding. The activities of both integrin-neutralizing and integrin-activating monoclonal antibodies, and NIF, a hookworm-derived MAC-1 inhibitor have been mapped to the I domain region (8–14). Significantly, purified recombinant forms of the I domains derived from LFA-1, MAC-1, and VLA-2 have intrinsic ligand binding activity (12, 15–19) and in several systems it has been shown that this protein-protein interaction is cation-dependent (12, 19). Determination of the I domain crystal structures has provided a structural basis for conceptualizing the role of cations in α\(_{M}I\) and α\(_{L}I\) interaction with ligands (19–21). The structures show that α\(_{M}I\) and α\(_{L}I\) domains contain a single metal cation-binding site, and that residues involved in coordinating the metal ion in each protein are completely conserved (21). Lee and co-workers (19) proposed that this novel cation-binding site represents a general metal ion-dependent adhesion (MIDAS)\(^1\) motif for binding protein ligands. Interestingly, crystallization of α\(_{M}I\) in the presence of different cations has been shown to result in significant changes in metal coordination and protein structure (19), suggesting that differential effects of cation binding on integrin function may be possible.

Divalent cations have multiple effects on integrin-mediated cell adhesion including enhancement, suppression, and modification of ligand binding activity. Mg\(^{2+}\) and \(\text{Mn}^{2+}\) induce conformational alterations of several integrins, including LFA-1 and MAC-1, concomitant with activation of integrin-mediated adhesion to ligands (22–24). In contrast, \(\text{Ca}^{2+}\) has been shown to inhibit LFA-1, but not MAC-1, mediated adhesion to ligands (24–28). We speculated that the differential effects of \(\text{Ca}^{2+}\) and \(\text{Mn}^{2+}\) on LFA-1 and MAC-1 function might be related to differences in the divalent cation binding properties of their I domains. In the work described herein, we compared the cation binding properties of purified recombinant α\(_{L}I\) and α\(_{M}I\) domains. Our results indicate that like α\(_{M}I\), α\(_{L}I\) preferentially binds \(\text{Mn}^{2+}\) over most other cations. However, \(\text{Mn}^{2+}\) interaction with α\(_{L}I\) was inhibitable to some degree using a variety of cations, and these studies revealed a pattern of binding selectivity that was clearly distinct from α\(_{M}I\). Interestingly, \(\text{Ca}^{2+}\) inhibited \(\text{Mn}^{2+}\) binding to α\(_{L}I\), but had little effect on \(\text{Mn}^{2+}\) binding to α\(_{M}I\), underscoring a fundamental difference in the cation-binding properties of these two I domains. Furthermore, experiments confirmed that α\(_{L}I\) binds both Mn\(^{2+}\) and Ca\(^{2+}\) and the results are consistent with the notion that the α\(_{L}I\) contains a single mixed-type Mn\(^{2+}\)/Ca\(^{2+}\)-binding site. It is possible that the activation state of LFA-1 is regulated in part by the interaction of the I domain with cations present in the extracellular environment. Ca\(^{2+}\) antagonism of LFA-1, but not MAC-1, mediated cell adhesion may be

\(^1\) The abbreviations used are: MIDAS, metal ion-dependent adhesion; MBF, maltoose-binding protein; BSA, bovine serum albumin; ICP-AES, inductively coupled plasma-atomic emission spectrometry; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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a consequence of the Ca$^{2+}$ binding activity of the I domain of LFA-1 that the Mn$^{2+}$ and Ca$^{2+}$ complexes of αI may represent, respectively, high- and low-affinity ligand binding states.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Purified maltose-binding protein (MBP2) was obtained from New England Biolabs (Beverly, MA). Purified bovine serum albumin (BSA) was obtained from Pierce (Rockford, IL). Concentrations of proteins were determined using the Bio-Rad Protein Assay Reagent (Hercules, CA) with BSA as standard.

All buffer solutions were prepared in single-use plastic containers. Water was obtained by a Milli-Q water system (Millipore Corporation, Bedford, MA). Reagents used for the preparation of buffers were of the highest quality available. The concentration of Ca$^{2+}$ and Mn$^{2+}$ in the water and overlay buffer (described below) was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) using a Jarell Ash Atomcomp 975 (Thermo Jarell Ash Corp., Franklin, MA) instrument. The lower limit of detection for Ca$^{2+}$ and Mn$^{2+}$ in water and overlay buffer by ICP-AES was 0.50 and 0.55 μM, respectively, and 2.5 and 0.91 μM, respectively. The measured Ca$^{2+}$ and Mn$^{2+}$ concentrations in water and buffer were below the limit of detection by ICP-AES.

**Expression with Purification of I Domain Polyptides—** αI was expressed as a fusion protein with glutathione S-transferase (GST), purified from the soluble fraction of the cleared Escherichia coli lysate by affinity chromatography and then cleaved with thrombin to separate the I domain from the GST moiety. The αI domain was amplified by polymerase chain reaction, using as template plasmid pMON24304 (obtained from B. Harding, Monsanto Co., St. Louis, MO) that contains a 2.45-kilobase cDNA fragment that includes the I domain region of LFA-1. A cDNA encoding the full-length αI was digested with BamHI and EcoRI, denatured into pMAI-c (New England Biolabs), and the accuracy of the DNA sequence was verified. The resulting coding sequence joins the MBP to CD11a at amino acid Asn$^{118}$ and Leu$^{244}$ corresponding to the same residues contained in the free αI protein described above. An overnight culture of E. coli strain DH5α containing the expression plasmid was subcultured 1:10 into 2 liters of LB broth with ampicillin and grown at 37 °C for 1 h. Expression was induced by the presence of the 0.2 mM isopropyl-β-D-galactoside (IPTG) for 2 h. Cells were centrifuged and stored frozen at −80°C overnight. After thawing, cells were resuspended in 60 ml of MBP column buffer (20 mM Tris pH 7.4, 200 mM NaCl, 5 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride), frozen in a dry ice/ethanol bath, thawed, and sonicated on ice for 30-s intervals until lysis was achieved. The sample was centrifuged, and the clarified lysate was loaded onto a column of glutathione-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). Binding to filters was determined to be dose-dependent and equilibrated with MBP column buffer. The column was washed extensively with MBP column buffer and the protein eluted into fractions with MBP column buffer containing 10 mM maltose. Protein identity and purity was confirmed by amino-terminal peptide sequencing and SDS-PAGE analysis, respectively.

**Expression Binding Assays—** $^{54}$Mn$^{2+}$ and $^{45}$Ca$^{2+}$ binding to purified proteins was measured by procedures based on those previously described by Michishita et al. (29). Equimolar amounts of purified recombinant proteins were bound to nitrocellulose (PHTB, 0.1 μM pore size, Schleicher and Schuell, Keene, NH) using the Mini-Fold II Slot Blot System (Schleicher and Schuell). Filters were washed twice for 10 min with 10 mM imidazole, pH 6.8, 60 mM EDTA, 0.05% Tween 20, and then incubated with $^{54}$MnCl$_2$ (5 μM) containing 10 μM EDTA, followed by four washes for 30 s with overlay buffer lacking EDTA. The filter was then incubated for 10 min in overlay buffer containing 0.5 μM CaCl$_2$ and 5 μM $^{45}$CaCl$_2$ (40 Ci/g, NEN Life Science Products, Boston, MA) or $^{54}$MnCl$_2$ (10–75 Ci/g, NEN Life Science Products) in the presence or absence of cold competitor cations diluted from freshly prepared stock solutions. Filters were washed with 20 mM Tris, pH 7.4, and scanned. The bound radioactivity on the filter was measured either by autoradiography or by PhosphorImage analysis using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager system. The efficiency of protein binding to nitrocellulose was assessed by staining the filter with naphthol blue black as described previously (30), and scanning using an LKB Ultrascan XL densitometer (LKB, Uppsala, Sweden). Binding to filters was determined to be dose-dependent and saturable with a Hill coefficient of 1.0. The dissociation constant (Kd) was calculated by curve fitting to a single-site binding model using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

**RESULTS**

**Expression of Recombinant Proteins—** The I domain coding regions were derived by polymerase chain reaction amplification using CD11a and CD11b cDNA clones as templates as described under “Experimental Procedures.” The sequence of
the MAC-1 I domain (α₃M) was identical to that reported previously (31). However, the sequence of the LFA-1 I domain (α₁I), as determined from two independent clones from the cDNA library, varied from that reported by Larson et al. (32) at a single nucleotide that results in substitution of tryptophan 189 by arginine. This substitution has also been identified by other researchers (21) and may represent a natural allelic variation. The position of this residue lies on the surface of the protein, far from the MIDAS motif, and thus is unlikely to affect the metal binding characteristics.

α₁I was expressed as a fusion protein with GST, purified from the soluble fraction of the cleared E. coli lysate by affinity chromatography, and then cleaved with thrombin to separate the I domain from the GST moiety. In contrast, α₃I expressed in E. coli as a fusion protein with GST, using conditions similar to those utilized for the expression of α₃M, formed intracellular inclusion bodies. Modification of the expression conditions, as described under “Experimental Procedures,” yielded small amounts of soluble GST-α₃I that was cleavable with thrombin.

In order to generate larger amounts of soluble protein, α₃I was expressed as a fusion protein with MBP. In this expression context, the protein was found almost exclusively in the soluble fraction and at high concentration. Unfortunately, cleavage of the MBP-α₃I fusion protein with thrombin resulted in I domain aggregation and precipitation. Due to the apparent ability of the MBP moiety to enhance the solubility of the LFA-1 I domain and the high yields of recovered protein (e.g. up to 30 mg/liter of cell culture) the majority of experiments utilized α₃I expressed as a fusion protein with MBP. However, to verify that structural differences imposed by the nature of the MBP-α₃I fusion protein construct did not contribute to α₃I cation binding properties, some experiments were conducted with α₃I cleaved and purified from the GST-α₃I fusion protein. For the sake of clarity, we subsequently use the term α₃If to refer to MBP-α₃I fusion protein and the term α₃Ii to refer to the purified I domain of LFA-1 isolated from the thrombin cleavage of the GST-α₃I fusion protein. The accuracy of protease cleavage of the fusion proteins for the release of α₃M and α₃I was confirmed by identification of the correct amino terminus by amino acid peptide sequencing. The molecular masses of α₃M and α₃I, determined by MALDI mass spectrometry, were within 0.05 and 0.01%, respectively, of their expected values. Purity of each protein was greater than 95% as determined by SDS-PAGE analysis (data not shown).

The I Domains of LFA-1 and MAC-1 Bind Metal Cations—To demonstrate that the LFA-1 and MAC-1 I domains contain qualitatively similar cation-binding sites, α₃M and α₃If were immobilized on nitrocellulose filters and incubated with ⁵⁴Mn²⁺ as described under “Experimental Procedures.” The autoradiogram shown in Fig. 4A shows that both α₃If and α₃Ii, but not BSA, bind ⁵⁴Mn²⁺ and that unlabeled Mn²⁺ competitively inhibits binding in a dose-dependent fashion. Quantitative PhosphorImager analysis of the nitrocellulose filters confirmed that ⁵⁴Mn²⁺ binding to control proteins BSA or MBP was less than 10% of ⁵⁴Mn²⁺ binding to either of the purified I domains (Fig. 1, B and C). Moreover, boiling of α₃Ii prior to immobilization on nitrocellulose reduced ⁵⁴Mn²⁺ binding to background levels. These results showed that the purified I domains of each integrin contain a conformationally sensitive Mn²⁺-binding site.

To assess possible qualitative variation in the cation binding activity of the I domain of each integrin, we screened various divalent and trivalent metal ions for the capacity to inhibit ⁵⁴Mn²⁺ binding to α₃M and α₃If. The results in Fig. 2 and Fig. 3A demonstrate that 500 μM unlabeled Mn²⁺ reduced ⁵⁴Mn²⁺ binding to α₃M and α₃If by approximately 90%. In these experiments, the concentration of ⁵⁴Mn²⁺ was approximately 0.2 μM, and hence the observed residual binding is likely to be nonspecific. In agreement with the earlier data of Michishita et al. (29), Mg²⁺, Ni²⁺, Co²⁺, Zn²⁺, and Cd²⁺ all were excellent competitors of Mn²⁺ binding to α₃M, as were two previously untested divalent cations, Fe²⁺ and Cu²⁺ (Fig. 2). Relative to
effect on $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{M}}$I, but did inhibit $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{I}}$I by greater than 70%. Together the data suggest that ionic size may be a more important attribute than charge in determining cation binding specificity to $\alpha_{\text{M}}$I, although the specific coordination geometry preferred by the cations may also play a roll. The data further suggest that the LFA-1 I domain may be complexed with Ca$^{2+}$ at physiological concentrations.

The $I$ Domain of LFA-1, but Not MAC-1, Contains a Binding Site for Calcium—Ca$^{2+}$ inhibits LFA-1, but not MAC-1, mediated cell adhesion (24–28, 33), an effect that conceivably may be related to differences in the divalent cation binding properties of the I domains of these integrins. Since the results presented above suggested that Ca$^{2+}$ binds to $\alpha_{\text{M}}$I but not $\alpha_{\text{I}}$I, we examined in further detail the binding of this cation to these purified polypeptides. The effect of increasing concentrations of unlabeled Mn$^{2+}$ and Ca$^{2+}$ on $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{M}}$I and $\alpha_{\text{I}}$I is presented in Fig. 3A. In agreement with the results of Michishita et al. (29), unlabeled Mn$^{2+}$ and Ca$^{2+}$ inhibited $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{M}}$I with estimated IC$_{50}$ values (inhibitory concentration resulting in 50% control binding) of 1–2 µM and greater than 10,000 µM, respectively. In contrast to these results, unlabeled Mn$^{2+}$ and Ca$^{2+}$ were both potent inhibitors of $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{I}}$I inhibiting $^{54}\text{Mn}^{2+}$ binding with IC$_{50}$ values of 10–20 and 50–100 µM, respectively. Furthermore, 1 mM Ca$^{2+}$ reduced $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{I}}$I to a level equivalent to that observed with the control proteins BSA and MBP (data not shown). These results showed clearly that Ca$^{2+}$ inhibited $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{I}}$I but not $\alpha_{\text{M}}$I, and indicate that the I domain of LFA-1, but not MAC-1, has a Ca$^{2+}$-binding site.

In the experiments described above, the cation binding comparison was made between $\alpha_{\text{M}}$I (i.e. the MBP-$\alpha_{\text{M}}$I fusion protein) and $\alpha_{\text{I}}$I, the I domain of $\alpha_{\text{M}}$ prepared by cleavage of the GST-$\alpha_{\text{M}}$I fusion protein. To rule out the possibility that the differences in the cation binding activities of the two I domains was related to structural differences imposed by the nature of the protein constructs, $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{I}}$I, the I domain of LFA-1 prepared by thrombin cleavage of the GST-$\alpha_{\text{I}}$I fusion protein, was determined in the presence of increasing concentrations of unlabeled Mn$^{2+}$ and Ca$^{2+}$. In agreement with the results shown in the previous experiment, Fig. 3B shows that $^{54}\text{Mn}^{2+}$ binds to $\alpha_{\text{I}}$I, and that both unlabeled Mn$^{2+}$ and Ca$^{2+}$ inhibit $^{54}\text{Mn}^{2+}$ binding (IC$_{50}$ = 39 ± 19 and 278 ± 108 µM, respectively). Furthermore, the results suggest that the cation binding activities of $\alpha_{\text{I}}$I and $\alpha_{\text{M}}$I are similar, and that the Ca$^{2+}$-binding site is associated with the I domain peptide.

The hypothesis that the LFA-1, but not the MAC-1 I domain, has a Ca$^{2+}$-binding site was further confirmed by direct binding studies utilizing $^{45}\text{Ca}^{2+}$. In the first experiment, $\alpha_{\text{M}}$I, MBP, and $\alpha_{\text{I}}$I were immobilized on nitrocellulose paper and then incubated with 0.2 µM $^{45}\text{Ca}^{2+}$. Fig. 4A shows that $^{45}\text{Ca}^{2+}$ binds directly to $\alpha_{\text{M}}$I but not to MBP or $\alpha_{\text{I}}$I, results consistent with those in Fig. 3, and that together confirm that the $^{45}\text{Ca}^{2+}$-binding activity is contained within the LFA-1 I domain rather than the associated MBP moiety. To further confirm these results, the experiment was repeated utilizing $\alpha_{\text{I}}$I, which lacks all MBP sequence. $\alpha_{\text{I}}$I was immobilized on nitrocellulose paper and incubated with 45Ca$^{2+}$ in the absence or presence (>1000-fold excess) of unlabeled Ca$^{2+}$. The results shown in Fig. 4B demonstrate that $^{45}\text{Ca}^{2+}$ binds to $\alpha_{\text{I}}$I, but not to $\alpha_{\text{M}}$I, and that excess unlabeled Ca$^{2+}$ completely inhibited $^{45}\text{Ca}^{2+}$ binding to $\alpha_{\text{I}}$I. Together, the results of these experiments strongly suggest that the LFA-1 I domain contains a Ca$^{2+}$-binding site. Furthermore, these results suggest that Ca$^{2+}$ binding to the I domain of LFA-1, but not MAC-1, may occur at cation concentrations found in plasma (34).

**Determination of the Dissociation Constant (K$_{D}$) for Mn$^{2+}$**

The hypothesis that the LFA-1, but not the MAC-1 I domain, has a Ca$^{2+}$-binding site was further confirmed by direct binding studies utilizing $^{45}\text{Ca}^{2+}$. In the first experiment, $\alpha_{\text{M}}$I, MBP, and $\alpha_{\text{I}}$I were immobilized on nitrocellulose paper and then incubated with 0.2 µM $^{45}\text{Ca}^{2+}$. Fig. 4A shows that $^{45}\text{Ca}^{2+}$ binds directly to $\alpha_{\text{M}}$I but not to MBP or $\alpha_{\text{I}}$I, results consistent with those in Fig. 3, and that together confirm that the $^{45}\text{Ca}^{2+}$-binding activity is contained within the LFA-1 I domain rather than the associated MBP moiety. To further confirm these results, the experiment was repeated utilizing $\alpha_{\text{I}}$I, which lacks all MBP sequence. $\alpha_{\text{I}}$I was immobilized on nitrocellulose paper and incubated with 45Ca$^{2+}$ in the absence or presence (>1000-fold excess) of unlabeled Ca$^{2+}$. The results shown in Fig. 4B demonstrate that $^{45}\text{Ca}^{2+}$ binds to $\alpha_{\text{I}}$I, but not to $\alpha_{\text{M}}$I, and that excess unlabeled Ca$^{2+}$ completely inhibited $^{45}\text{Ca}^{2+}$ binding to $\alpha_{\text{I}}$I. Together, the results of these experiments strongly suggest that the LFA-1 I domain contains a Ca$^{2+}$-binding site. Furthermore, these results suggest that Ca$^{2+}$ binding to the I domain of LFA-1, but not MAC-1, may occur at cation concentrations found in plasma (34).

**FIG. 2.** Various metal cations inhibit $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{M}}$I (A) and $\alpha_{\text{I}}$I (B). $^{54}\text{Mn}^{2+}$ (0.2 µM) binding to $\alpha_{\text{M}}$I and $\alpha_{\text{I}}$I was determined in the presence of excess cold competitor cation (500 µM) to examine the cation binding specificity. Binding is expressed as the percentage of binding observed in the absence of inhibitor. Bound $^{54}\text{Mn}^{2+}$ was quantified by PhosphorImager analysis as described under “Experimental Procedures.” The results represent the mean of values derived from three independent experiments.

| Cation Competitor | Control Binding |
|-------------------|------------------|
| Mn$^{2+}$ | 1.00 |
| Zn$^{2+}$ | 0.75 |
| Co$^{2+}$ | 0.50 |
| Cd$^{2+}$ | 0.75 |
| Mg$^{2+}$ | 0.50 |
| Ni$^{2+}$ | 0.25 |
| Fe$^{2+}$ | 0.50 |
| Au$^{3+}$ | 0.25 |
| Sr$^{2+}$ | 0.25 |

**FIG. 2.** Various metal cations inhibit $^{54}\text{Mn}^{2+}$ binding to $\alpha_{\text{M}}$I (A) and $\alpha_{\text{I}}$I (B). $^{54}\text{Mn}^{2+}$ (0.2 µM) binding to $\alpha_{\text{M}}$I and $\alpha_{\text{I}}$I was determined in the presence of excess cold competitor cation (500 µM) to examine the cation binding specificity. Binding is expressed as the percentage of binding observed in the absence of inhibitor. Bound $^{54}\text{Mn}^{2+}$ was quantified by PhosphorImager analysis as described under “Experimental Procedures.” The results represent the mean of values derived from three independent experiments.
and Ca$^{2+}$ Interaction with αI$\beta$IIa—The affinity constant for Ca$^{2+}$ and Mn$^{2+}$ binding to αI$\beta$IIa were determined by hot saturation binding studies and analysis of the equilibrium binding data as described below. Initial experiments showed that Ca$^{2+}$ and Mn$^{2+}$ binding to αI$\beta$IIa immobilized on nitrocellulose was reversible, reached equilibrium in less than 1 min, and that bound 54Mn$^{2+}$ and Mn$^{2+}$ dissociated rapidly when filters were incubated in overlay buffer containing cold competitor cation (data not shown). Cation dissociation from αI$\beta$IIa was stopped completely by immersing and washing nitrocellulose filters in 50% ethanol as described above (data not shown). Quantitation of Ca$^{2+}$ or Mn$^{2+}$ bound to the filters was performed by autoradiography and PhosphorImaging, and the data was analyzed using the EOBDA/LIGAND software (Biosoft, Milltown NJ). Ca$^{2+}$ and Mn$^{2+}$ binding to αI$\beta$IIa is shown in Fig. 5. Binding to αI$\beta$IIa was dose dependent and saturable (Fig. 5, A and B, insets), and the Scatchard plot of the binding data was curvilinear. LIGAND resolved the binding isotherm into two components corresponding to high and low affinity binding sites. The estimate for the $K_D$ of the high affinity Ca$^{2+}$- and Mn$^{2+}$-binding sites was $5.6 \pm 0.7 \times 10^{-5}$ and $1.4 \pm 0.2 \times 10^{-5}$ M, respectively. The estimate of the $K_D$ values for the low affinity Ca$^{2+}$- and Mn$^{2+}$-binding sites was $1.5 \times 10^{-5}$ M. The maximal binding capacity of the high affinity site for Mn$^{2+}$ and Ca$^{2+}$ was 0.4 and 0.8 mol of cation bound/mol of αI$\beta$IIa, respectively. The slope of the Hill plot of the binding data for both Ca$^{2+}$ and Mn$^{2+}$ was close to unity, indicating the apparent absence of cooperativity between the high and low affinity sites (data not shown).

We questioned whether the low affinity metal-binding site might represent Ca$^{2+}$ and Mn$^{2+}$ binding to the MBP component of the fusion protein. Therefore, the affinity of each of these cations for purified MBP, lacking any I domain sequences, was determined as described above. Scatchard analysis of the binding data showed a single low affinity site for Ca$^{2+}$ and Mn$^{2+}$ in MBP with a $K_D$ corresponding to the low affinity site identified in αI$\beta$IIa (data not shown). We conclude that the low affinity metal-binding site in αI$\beta$IIa is due to Ca$^{2+}$ and Mn$^{2+}$ binding to the MBP component, whereas the higher affinity metal-binding site is associated with the I domain of the LFA-1 integrin.

**DISCUSSION**

The results of this study clearly show that the purified I domains of LFA-1 and MAC-1 bind cations with distinct selectivity. Binding of 54Mn$^{2+}$ to αI$\beta$IIa was inhibitable by a variety of divalent and trivalent cations with a range of ionic radii. In contrast, binding to αM$\beta$IIa appeared to be more specific for smaller divalent cations. Notably, Ca$^{2+}$ inhibited 54Mn$^{2+}$ binding to αI$\beta$IIa at concentrations well below those found in physiological environments (34), whereas Ca$^{2+}$ failed to inhibit 54Mn$^{2+}$ binding to αM$\beta$IIa even when present at approximately 10$^{-5}$ M excess. These results demonstrate a fundamental biochemical difference between these two closely related integrins, a finding with potential functional significance in the modulation of cell-ligand interactions.

In initial experiments, the comparison of the binding activities of the I domains of αI$\beta$IIa and αM$\beta$IIa were made between the intact MBP-αI fusion protein (αI$\beta$IIa) and αM$\beta$IIa, the I domain of αM$\beta$IIa that was cleaved from the GST-αM$\beta$IIa fusion protein. It seemed possible that the cation binding characteristics of αI$\beta$IIa were unique to the MBP-αI fusion protein and not reflective of the inherent cation binding activity of the LFA-1 I domain. To test this notion, we purified the isolated I domain of LFA-1 (αI$\beta$IIa) and then compared the cation binding characteristics of...
both forms of the LFA-1 I domain. The results of these experiments showed that: 1) $^{54}\text{Mn}^{2+}$ binds to both $\alpha_{L}I$ and $\alpha_{M}I$ in a saturable manner; 2) binding is inhibitable with both unlabeled Ca$^{2+}$ and Mn$^{2+}$; and 3) both $\alpha_{L}I$ and $\alpha_{M}I$, but not $\alpha_{M}I$, bind $^{45}\text{Ca}^{2+}$. Furthermore, in these experiments, the concentration of $^{54}\text{Mn}^{2+}$ was approximately 0.2 $\mu$M, a Mn$^{2+}$ concentration approximately 5000-fold below the $K_D$ of the MBP low affinity cation-binding site in $\alpha_{L}I$. Under these conditions $^{54}\text{Mn}^{2+}$ binding is restricted to the LFA-1 I domain component of $\alpha_{L}I$, a conclusion supported by the observation that Ca$^{2+}$ binds to immobilized $\alpha_{L}I$ and $\alpha_{M}I$, but not to MBP, a consequence of the relatively disparate cation binding affinities of the I domain and MBP components of $\alpha_{L}I$. Collectively, the results suggest that $\alpha_{L}I$ and $\alpha_{M}I$ have similar cation binding characteristics and appear to be interchangeable in these experiments.

Fig. 5. Determination of the dissociation constant ($K_D$) for Mn$^{2+}$ and Ca$^{2+}$ interaction with $\alpha_{L}I$. $^{54}\text{Mn}^{2+}$ (A) or $^{45}\text{Ca}^{2+}$ (B) binding to $\alpha_{L}I$ was determined by PhosphorImager analysis. The binding data was replotted (inset) by the method of Scatchard (43) and analyzed using the program LIGAND as described under “Experimental Procedures.” The values reported are the results of a single experiment; this experiment was repeated three times with similar results.

The binding data suggest that Ca$^{2+}$ and Mn$^{2+}$ bind to a single site within the I domain of LFA-1. $^{45}\text{Ca}^{2+}$ and $^{54}\text{Mn}^{2+}$ binding was inhibited by either unlabeled cation, demonstrating that these two cations bind to the LFA-1 I domain in a mutually competitive manner. Ca$^{2+}$ and Mn$^{2+}$ bind to the I domain of LFA-1 with similar affinity and stoichiometry with 1 $\mu$mol of cation bound/mol of I domain indicative of a Ca$^{2+}$/Mn$^{2+}$ mixed type binding site. We speculate that the Ca$^{2+}$/Mn$^{2+}$-binding site in $\alpha_{L}I$ may represent an equivalent Ca$^{2+}$/Mg$^{2+}$-binding site since, as we show here, Mg$^{2+}$ is an effective inhibitor of Mn$^{2+}$ binding to $\alpha_{L}I$. Collectively, these results are consistent with the available structural information showing that the I domains of LFA-1 and MAC-1 contain a single Mn$^{2+}$/Mg$^{2+}$-binding site. Comparable structural data describing an I domain Ca$^{2+}$-binding site has not been presented; the information herein is the first demonstration that the I domain of LFA-1 binds Ca$^{2+}$ as well as Mn$^{2+}$ and that both cations bind to a single site in a competitive manner. It should be noted that these metal binding studies were conducted using solid-phase binding techniques and it is possible that the physical interaction of the proteins with the nitrocellulose membrane could affect the metal binding characteristics. Consequently, confirmation of the reported binding constants using solution phase equilibrium binding dialysis would be useful.

Considerable evidence supports the idea that cation-binding to integrin I domain MIDAS motifs plays a role in modulating the interaction of intact integrin with ligand. Mg$^{2+}$ and Mn$^{2+}$ induce conformational changes in integrins and both cations stimulate LFA-1-mediated cell adhesion to ICAM-1 (22,24,26,27). In contrast, Ca$^{2+}$ inhibits Mn$^{2+}$-induced activation of LFA-1, and Mg$^{2+}$ stimulation of LFA-1 mediated cell adhesion to ICAM-1 requires prior chelation of Ca$^{2+}$ by EGTA treatment (24, 26, 27). That the effects of divalent cations on integrin-mediated cell adhesion may be related to cation binding and cation-induced changes in I domain conformation is suggested by several observations. First, the recombinant forms of the I domains derived from a variety of integrins have intrinsic ligand binding activity. Second, ligand binding activity is cation-dependent and supported by Mn$^{2+}$ and Mg$^{2+}$, results that reflect the activity of these cations on the behavior of the intact integrin (12, 16–19). Third, activation-specific conformational changes (neoepitopes) have been mapped to the I domain of MAC-1 (17, 35). Finally, the structural data reported by Lee et al. (20) showed $\alpha_{M}I$ crystals grown in Mg$^{2+}$ display large differences in conformation and dramatic alteration of the surface of the protein implicated in ligand binding compared with crystals grown in Mn$^{2+}$. These investigators proposed that the Mg$^{2+}$ and Mn$^{2+}$ structures represent conformations of the I domain that exist in the active and inactive states of the integrin, respectively (20). In addition, it was speculated that Ca$^{2+}$ binding to the integrin I domain might stabilize the inactive form of the integrin (20). Together, these observations suggest that cation binding to the I domain modifies integrin interaction with ligand and raises the question whether cation-I domain complexes might exert activating or inactivating ligand-binding effects that are dependent upon the cation type.

Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ are present in the extracellular environment and all are available to compete for binding to the I domain. The normal concentration of Ca$^{2+}$ and Mg$^{2+}$ in serum is approximately 1 mM (34), and it has been estimated that the concentration of Mn$^{2+}$ may range from 1 to 50 $\mu$M depending upon the particular tissue environment (36). Assuming that the cation binding properties of the isolated recombinant I domain and the native I domain within the context of the holoprotein are similar, the high concentration of cation relative to the $K_D$ for cation binding to the I domain predicts that the metal-binding site in the I domain of LFA-1 will be occupied by cation. Furthermore, based on the evidence discussed above supporting the idea that cation-binding to integrin I domain MIDAS motifs play a role in modifying the integrin interaction with ligand, it is conceivable that I domain of low affinity LFA-1 on circulating leukocytes may be complexed with calcium, and that activation to the high affinity form may be accompanied by the replacement of Ca$^{2+}$ with Mg$^{2+}$ or Mn$^{2+}$.

It seems possible that integrin activation could be driven in...
some environments by dynamic alteration in the ratio of cation concentration, for example, at sites of vascular or tissue injury or bone resorption (22, 36–39). However, a more likely scenario is that cation binding to the I domain of LFA-1 is only one of multiple factors that together coordinate and regulate LFA-1 activation. Additional components of the activation process likely include conformational changes conferred by inside-out signaling mechanisms and ligand binding (40–42). Consequently, structural changes in the integrin ectodomain mediated by inside-out signaling processes, ligand binding, and the association of Mg$^{2+}$ or Mn$^{2+}$ cation binding with the I domain, may combine to produce the activated form of LFA-1.

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