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J Immunol 1998; 161:4016-4022; ;
http://www.jimmunol.org/content/161/8/4016

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Affinity and Kinetic Analysis of the Molecular Interaction of ICAM-1 and Leukocyte Function-Associated Antigen-1

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LFA-1 is a member of the β₂ integrin family, and interacts with ICAM-1, a member of the Ig superfamily containing five Ig-like domains. Interaction of LFA-1 with ICAM-1 is important in a number of cellular events, including Ag-specific T cell activation and leukocyte transendothelial migration, which are known to be typically transient and highly regulated. In this study, we have used surface plasmon resonance technology to study the ICAM-1/LFA-1 interaction at the molecular level. A soluble form of LFA-1 (sLFA-1), normally expressed as two noncovalently associated membrane-bound subunits, has been produced, and its interaction with ICAM-1 has been examined. The kinetic analysis of a monomeric sLFA-1 binding to the first two domains of ICAM-1 expressed as a chimeric IgG fusion protein (D1D2-IgG) revealed that sLFA-1 was bound to the D1D2-IgG chimera with a $K_d$ of 500 nM and dissociated with a $k_{diss}$ value of 0.1 s$^{-1}$. Monomeric membrane-bound LFA-1 purified from plasma membranes showed a similar kinetic to sLFA-1. These results suggest that the monovalent interaction between ICAM-1 and LFA-1 has a primarily high affinity and a slow dissociation rate constant as compared with other adhesion molecules, suggesting a potential mechanism for firm adhesion. The Journal of Immunology, 1998, 161: 4016–4022.

The CD11/CD18 (β₂ integrins) family consists of three heterodimeric surface-membrane glycoproteins, each with a distinct α subunit (CD11 a, b, c) noncovalently associated with a common β subunit (CD18) (1, 2). The members of this family are LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150, 95 (CD11c/CD18) (3). As other integrins, association of the CD11 and CD18 subunits is required for normal surface-membrane expression and function of these receptors (4, 5). LFA-1 is expressed on all leukocytes and mediates adhesion to a variety of cell types that express one or more of the LFA-1 ligand’s ICAM-1 (CD54) (6–8), ICAM-2 (CD102) (9, 10), and ICAM-3 (CD50) (11–14).

ICAM-1 is a counter-receptor for the leukocyte integrins LFA-1 and Mac-1 and promotes a wide range of cellular interactions important in inflammation (15–18). ICAM-1 is a membrane protein with five Ig superfamily extracellular domains, a hydrophobic transmembrane domain, and a short cytoplasmic domain (19, 20). The LFA-1 binding site is located in domain 1 of ICAM-1, although domain 2 is known to play a role in maintaining the conformation of domain 1 (21). ICAM-1/LFA-1 interaction includes adhesion of leukocytes to the endothelium, followed by their extravasation at sites of inflammation, costimulatory signaling for T cell activation, and adherence of killer T cells to target cells (3). LFA-1 is maintained in an inactive form on resting leukocytes and becomes activated following signaling through other cell surface receptors such as the TCR/CD3 complex (22).

Several groups have reported that the ligand binding site in LFA-1 is located in the I domain. For example, Champe et al. (23) have shown that a number of mAbs that block LFA-1 binding to ICAM-1 map to the I domain of LFA-1. Other reports showed that some point mutations in the I domain significantly reduced LFA-1 binding to ICAM-1 (24, 25). In addition, I domain-IgG chimeras, which are bivalent molecules, specifically bind to ICAM-1 (26). On the other hand, Bajt et al. (27) have reported that the Iβ subunit is essential for the ligand-binding function of LFA-1. Since the I domain as well as domains V and VI of CD11a (28) have been implicated in the ligand-binding function, it is likely that multiple sites in LFA-1 cooperate in the recognition of ligands. While a purified LFA-1 from leukocytes has been reported to bind to purified ICAM-1 and ICAM-1-expressing cells (22, 29), LFA-1 protein micelles may exhibit a higher avidity due to their multivalency. Therefore, we have produced a recombinant soluble form of LFA-1 (sLFA-1), a truncated form of LFA-1 lacking the transmembrane and cytoplasmic domains, in mammalian cells to facilitate the study of the ICAM-1/LFA-1 interaction (30).

In the present study, we have characterized the ICAM-1/LFA-1 interaction at the molecular level, using a novel technique based on surface plasmon resonance. Our results show that a soluble form of monomeric LFA-1 binds the first two domains of ICAM-1 (D1-D2) expressed as a chimeric IgG fusion protein (D1D2-IgG) with a $K_d$ value of 500 nM and a $k_{diss}$ value of 0.1 s$^{-1}$.

Materials and Methods
Ab and reagents

Reagents were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Daigo’s T media and ITES (2 μg/ml insulin, 2 μg/ml transferrin, 122 ng/ml ethanolamine, and 9.14 ng/ml sodium selenite) were purchased from Wako (Osaka, Japan). DMEM/F-12 media, FCS, and G418 were obtained from Life Technologies (Grand Island, NY). The hybridoma lines producing anti-human CD11a mAb (TS1/22, TS2/4) or anti-human CD18

2 Abbreviations used in this paper: sLFA-1, soluble LFA-1; D1D2, domains 1–2 of ICAM-1; D1D5, domains 1–5 of ICAM-1; mLFA-1, membrane-bound LFA-1; RU, resonance unit; sICAM-1, soluble ICAM-1; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1.
mAb (TS1/18) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Purified MEM-83 (anti-CD11a) and MEM-48 (anti-CD18) were purchased from Sanbio BV (Uden, Netherlands). All anti-ICAM-1 mAbs used in this study were generated and characterized by the authors (30). The mAbs 3D6 and 4E3 directed against epitopes within domain 1 of ICAM-1 have been shown to inhibit the ICAM-1/LFA-1 interaction in a previous study (30).

Production of chimeric forms of ICAM-1 (D1D2-IgG, D1D5-IgG)

Two chimeric soluble forms of ICAM-1, termed D1D2-IgG and D1D5-IgG, were prepared as previously described (30). Briefly, chimeric ICAM-1 was prepared by fusing either the first two Ig domains of ICAM-1 (D1D2: 1–185) or the five Ig domains of ICAM-1 (D1D5: 1–453) to the Fc portion (hinge, C₂₂ and C₃₃ domains) of human IgG1 (31) using conventional cDNA techniques. CHO-K1 cells were transfected with the vector pRS/CMV containing the chimeric ICAM-1 cDNA using calcium-phosphate methods. Chimeric fusion proteins were purified from culture supernatants using protein A-Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden).

Purification and analysis of sLFA-1

sLFA-1 was purified from the culture supernatants of a stable line of sLFA-1-transfected CHO-K1 cells, as previously described (30). Fractions containing sLFA-1 were concentrated using Centriplus 100 microconcentrators (Amicon, Beverly, MA) and dialyzed against HBS(−) buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM MgCl₂). Purified sLFA-1 was analyzed by SDS-PAGE and Coomassie blue staining. Protein concentrations were estimated by the AccQ-Tag amino acid composition analysis of acid-hydrolyzed protein samples according to the manufacturer’s instructions (Waters, Milford, MA) and by ELISA, as previously described (30).

Production of chimeric forms of ICAM-1

Membrane-bound LFA-1 (mLFA-1) was purified from LFA-1-transfected CHO-K1 cell lysates using immunoaffinity chromatography, as described by Rustin et al. (29) with modifications. The TS2/4 column (5 ml at 5 ml/min) was prepared by covalently attaching TS2/4 to N-hydroxysuccinimide (NHS)-activated HitTrap (Pharmacia), according to the manufacturer’s instructions. After neutralization of the fractions from the TS2/4 column, samples were precleared with HitTrap-protein G (Pharmacia) and dialyzed against HBS buffer, following the addition of 0.05% Tween-20. The protein concentration was estimated by BCA protein assay (Pierce, Rockford, IL) and confirmed by AccQ-Tag amino acid composition analysis. The reactivity of mLFA-1 with mAbs was demonstrated by ELISA (30).

For the BIAcore analysis, mLFA-1 was fractionated by gel filtration on a Superose 6 HR10/30 FPLC column (Pharmacia) in HBS buffer. Fractions (0.5 ml) were collected at a flow rate of 0.1 ml/min. The column elution profile of the fractionated mLFA-1 was compared with calibration standards (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa) (Pharmacia) to determine the m.w.

Expression of membrane-bound LFA-1

Human CD11a (32) and CD18 (33) cDNA were cloned into the expression vector pSV2neo (ATCC, Manassas, VA). Purified MEM-83 (anti-CD11a) and MEM-48 (anti-CD18) were purchased from Sanbio BV (Uden, Netherlands). All anti-ICAM-1 mAbs used in this study were generated and characterized by the authors (30). The mAbs 3D6 and 4E3 directed against epitopes within domain 1 of ICAM-1 have been shown to inhibit the ICAM-1/LFA-1 interaction in a previous study (30).

BIAcore analysis

The interaction of LFA-1 with immobilized ICAM-1 was studied on a BIAcore 2000 biosensor (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were performed at 25°C. All of the protein injections were dialyzed against HBS buffer and diluted with HBS buffer. To immobilize D1D2-IgG to a CM5 sensor chip (Pharmacia Biosensor AB), polyclonal goat anti-human IgG (γ-chain) Ab (Zymed, San Francisco, CA) was coupled to the sensor chip (about 11,000 RU) using the amine-coupling kit (Pharmacia Biosensor AB), as described (34), except that the Ab was injected at 50 μg/ml in 10 mM Na acetate pH 4.5. After injection of D1D2-IgG at 50 μg/ml for immobilization via the goat-anti-human Ig Ab, LFA-1 was injected at a flow rate of 20 μl/min. The sensor surface was regenerated at the end of each experiment with 10 mM HCl.

Analysis of the binding data in BIAcore

The analysis of kinetic data for LFA-1 binding to captured D1D2-IgG was performed using standard kinetic equations described by Karlsson et al. (34). The portion of the sensorgram that corresponds to the dissociation of sLFA-1 from immobilized D1D2-IgG was analyzed to obtain the dissociation rate constant (kₗ). Nonlinear curve fitting was conducted with the BIA evaluation 2.0 program (Pharmacia Biosensor AB). The association rate constant (kₕ) was determined by nonlinear curve fitting to the association phase data using the model of one site. kₗ was calculated from the ratio kₕ/kₗarr. The advantage that all of the immobilized chimeric ICAM-1 is present in the same orientation on the sensor surface. When sLFA-1 was injected over the sensor surface with D1D2-IgG, a large response was observed for sLFA-1 (Fig. 1A). In contrast, sLFA-1 induced little or no response when it was injected over a control sensor surface on which D1D2-IgG was not captured. Saturation of immobilized D1D2-IgG with anti-ICAM-1 mAb, 3D6, which blocks sLFA-1 binding (30), resulted in a decrease in the response to the baseline level, suggesting that the observed interaction between sLFA-1 and D1D2-IgG on a BIAcore biosensor. Specific binding of sLFA-1 to D1D2-IgG was demonstrated by a solid-phase binding assay (30). D1D2-IgG was indirectly immobilized on the sensor surface through the covalently coupled goat anti-human IgG Ab, which binds the IgG portion of D1D2-IgG. This has the advantage that all of the immobilized chimeric ICAM-1 is present in the same orientation on the sensor surface. When sLFA-1 was injected over the sensor surface with D1D2-IgG, a large response was observed for sLFA-1 (Fig. 1A). In contrast, sLFA-1 induced little or no response when it was injected over a control sensor surface on which D1D2-IgG was not captured. Saturation of immobilized D1D2-IgG with anti-ICAM-1 mAb, 3D6, which blocks sLFA-1 binding (30), resulted in a decrease in the response to the baseline level, suggesting that the observed interaction between sLFA-1 and D1D2-IgG on a BIAcore biosensor is specific. Sensorgrams obtained in a typical experiment are overlaid in Figure 1B. When sLFA-1 (250–500 nM) was injected over the sensor surface with D1D2-IgG (300 RU), it increased the response in a dose-dependent manner. Interaction of sLFA-1 with immobilized D1D2-IgG might be multiphasic in that the plots of both the ln(RUo/RU) versus time and the ln(abs(DRU/dt)) versus time do not give linear plots.

Analysis of the binding of monomeric sLFA-1

The results in Figure 1B suggest that there are at least two types of binding activities in the sLFA-1 preparation: one dissociates fast and the other dissociates slowly. The kinetic analysis of the binding data indicated that association and dissociation of sLFA-1 to immobilized D1D2-IgG were biphasic. It seemed likely that the slow dissociation was due to the binding of aggregated sLFA-1. We, therefore, performed the gel filtration of the sLFA-1 preparation to separate the monomeric sLFA-1 and aggregated sLFA-1, and then distinguished each response on a BIAcore biosensor. sLFA-1 was fractionated into two peaks on Superose 6 (Fig. 2A). The main peak (Fig. 2A, peak 2) corresponded to monomeric sLFA-1, since it eluted from the column in the size range expected...
for the monomeric form of sLFA-1 (258 kDa). The expected m.w. of the shoulder peak (Fig. 2A, peak 1) was consistent with the molecular size of the dimerized sLFA-1 (516 kDa).

We analyzed the binding activity of each fraction to D1D2-IgG on a BIAcore biosensor. sLFA-1 fractionated on Superose 6 were injected over the D1D2-IgG surface (Fig. 2C) and simultaneously injected over a control sensor surface with only goat anti-human IgG Ab to distinguish specific reactions from nonspecific ones. When sLFA-1 from the major peak (fr.10, peak 2) was injected over the D1D2-IgG-immobilized surface (Fig. 2C), the component of both the slow association and slow dissociation was reduced significantly in comparison with the unfractionated sLFA-1 (Fig. 2B). The association rate constant ($k_{\text{ass}}$) and the dissociation rate constant ($k_{\text{diss}}$) for the reaction of the monomeric sLFA-1 were $2 \times 10^7$ M$^{-1}$ s$^{-1}$ and $1 \times 10^{-1}$ s$^{-1}$, respectively. The equilibrium dissociation constant ($K_d$) for the monomeric sLFA-1 was calculated to be 500 nM. Furthermore, the sLFA-1 in the minor peak (fr.8, peak 1) was bound with a high avidity, as expected for a multimeric interaction (Fig. 2C). No binding activity was detected from the other peak, which eluted from the column later than the main peak, indicating that these were lower m.w. contaminants. These results clearly indicated that the binding of sLFA-1 to ICAM-1 is monophasic and that the multiphasic interaction of sLFA-1 with D1D2-IgG was due to the presence of approximately 10% multimeric sLFA-1 in the sLFA-1 preparation.

Interaction of mLFA-1 with chimeric ICAM-1
To exclude the possibility that the truncation of the cytoplasmic and transmembrane domain of LFA-1 affects the receptor-ligand interaction, we repeated the BIAcore analysis using mLFA-1, a full-length heterodimeric receptor. We purified mLFA-1 from CHO-K1 cells transfected with CD11a/CD18 by TS2/4 affinity chromatography. The interaction of the immunoaffinity-purified mLFA-1 with the ICAM-1 chimera was analyzed using a BIAcore biosensor. D1D2-IgG was immobilized on the sensor chip via the goat anti-human IgG Ab. When immobilized D1D2-IgG was saturated with 3D6, the response was reduced to almost the level seen in the control flowcell, whereas the irrelevant Ab had no effect (Fig. 3A). Various concentrations of mLFA-1 (100–200 nM) were injected over the surface, while regenerating the surface at the end of each experiment. The overlay plot for the mLFA-1 interaction with chimeric ICAM-1 at different concentrations of mLFA-1 is shown in Figure 3B. The plot of the dissociation phase from 300 to 600 s was calculated using the BIAcore software. Dissociation is expressed as the natural log (ln) of the drop in resonance units (RUo/RU). Association (0 to 300 s) is expressed as the natural log (ln) of the absolute value of the rate of change of resonance units (abs(dRU/dt)) (RUo, resonance units at the indicated time; abs, absolute value). When the ln(RUo/RU) versus time and the ln(abs(dRU/dt)) versus time were plotted, these graphs do not give linear plots. This result indicates that the binding of mLFA-1 to the immobilized chimeric ICAM-1 might be multiphasic during both the association and dissociation phases. We, therefore, analyzed binding of monomeric mLFA-1 fractionated by gel-filtration chromatography.

Gel-filtration chromatography on a Superose 6 column was performed to determine the apparent molecular size of mLFA-1 using calibration proteins from 158 to 669 kDa. Although monomeric
mLFA-1, which has a molecular size of 275 kDa, is expected to elute between ferritin (440 kDa) and catalase (232 kDa). mLFA-1 was detected in the broad range by ELISA, which could identify the intact heterodimers (Fig. 4A), while the purity of the mLFA-1 was greater than 95%, as determined by Coomassie blue staining of SDS-PAGE, indicating that mLFA-1 is heterogeneous in terms of molecular size. To determine whether the multimerization of mLFA-1 was critical for its binding characteristics to ICAM-1, we analyzed the binding activity of each fraction containing mLFA-1 on a BIAcore biosensor. Fractions containing mLFA-1 ranging from 250 to 300 kDa apparent molecular mass, which correspond to monomeric mLFA-1, were concentrated fivefold in Centricon 30 microconcentrators (Amicon), and immediately tested for their ability to bind to immobilized D1D2-IGC. As shown in Figure 4B, the monomer-enriched fractions (fr.26, 27) showed a fast dissociation as compared with the higher molecular size fractions (fr.20, 22). The result was quite similar to that obtained by sLFA-1.

Discussion

In the present study, we have shown that a monomeric soluble form of human LFA-1 binds to chimeric soluble human ICAM-1 with a $K_d$ of 500 nM and that this interaction has a fast dissociation rate constant ($k_{diss}$ $2 \times 10^5$ M$^{-1}$ s$^{-1}$). To our knowledge, this is the first affinity and kinetic analysis conducted in a cell-free system of the interaction between ICAM-1 and LFA-1. Recent studies have shown that multimeric forms of ICAM-1 bind to LFA-1 more efficiently than monomeric ICAM-1. It was demonstrated that the binding activity of the dimer-enriched recombinant soluble ICAM-1 (sICAM-1) to purified LFA-1 was four times more potent as monomeric ICAM-1 (35). Dimerization of sICAM-1 using non-blocking mAbs directed against domain 4 or domain 5 of ICAM-1 increased the affinity by two orders of magnitude relative to monomeric sICAM-1 (36). These studies have concluded that while monomeric sICAM-1 binds immobilized LFA-1 with an affinity in the 100 nM range, dimerization of sICAM-1 results in an increase in the affinity for LFA-1 by several orders of magnitude. For affinity and kinetic analysis, it is critical that the interaction is monovalent, because increasing the binding valency leads to dramatic increases in the strength and stability of an interaction (37). In the present study, monovalency was ensured by using a monomeric form of sLFA-1 purified by size-exclusion chromatography (Fig. 2A). The monomeric peak of sLFA-1 was used for affinity and kinetic measurements, suggesting that this study has estimated the true affinity.

Divalent cations such as Mg$^{2+}$ regulate ligand interactions through selective binding to several sites on integrins and are
thought to directly associate with the ligand binding site and control access to a cryptic binding site through altering the conformation of the integrin (38, 39). Although Mg\(^{2+}\) is directly involved in the affinity of LFA-1 for its ligand, Ca\(^{2+}\) correlates with avidity regulation of LFA-1 by clustering LFA-1 molecules at the cell surface of T cells, thereby facilitating LFA-1-ligand interaction.

FIGURE 3. Binding of mLFA-1 to immobilized D1D2-IgG chimeric protein and competition by anti-ICAM-1 mAb. A, mLFA-1 (500 nM) was injected for 2 min (indicated with bars) over the D1D2-IgG surface with (curve a) or without (curve b) pretreatment with anti-ICAM-1 mAb, 3D6. Pretreatment with 3D6 injected at 500 μg/ml for 5 min (indicated with a bar) inhibited the reaction to the basal level. B, Binding of mLFA-1, obtained at mLFA-1 concentrations of 200, 180, 160, 140, 120, and 100 nM, to D1D2-IgG immobilized on the sensor chip via goat anti-human IgG Ab. mLFA-1 was injected for 5 min through a flowcell with D1D2-IgG immobilized. Bound materials were eluted with 10 mM HCl for 1 min.

FIGURE 4. A, Analysis of the immunoaffinity-purified mLFA-1. mLFA-1 was fractionated by gel filtration on a Superose 6 HR10/30 FPLC column. The amount of mLFA-1 in fractions was determined by ELISA. Fractions (0.5 ml) were collected at a flow rate of 0.1 ml/min. The column elution positions of the m.w. standards were determined for comparison. B, Binding of fractionated mLFA-1 to immobilized D1D5-IgG. Fractions 20, 22, 25, 26, and 27 were tested for their ability to bind to D1D5-IgG. mLFA-1 was injected for 2 min through a flowcell with D1D5-IgG immobilized. The buffer flow rate was 10 μl/min. D1D5-IgG was captured with goat anti-human IgG covalently linked to the sensor chip.
Recent studies have provided affinity and kinetic data on the interactions of CD2 with its ligands CD48 and CD58 with an affinity in the 100 μM range. CD80 has been shown to bind CD28 with a low affinity ($K_d$ = 4 μM) and very fast kinetics ($k_{\text{diss}}$ = 1.6 s$^{-1}$) (46). These kinetic studies of cell-cell recognition molecules have revealed that rapid binding kinetics may be a general feature of the molecular interactions mediating cell-cell recognition. Nicholson et al. (47) have shown that CD62L (L-selectin) binds immobilized GlyCAM-1 with a very low affinity ($K_d$ = 108 μM) and a very fast dissociation rate constant ($\geq 10^3$ s$^{-1}$). The extremely fast $k_{\text{diss}}$ of CD62L/GlyCAM-1 interaction may have an influence on the duration of leukocyte tethers and the velocity of leukocyte rolling. The affinity measured in the present study for monomeric LFA-1 binding to ICAM-1 is much higher than that measured for these adhesive interactions (Table I). Monomeric interaction of LFA-1 in high affinity state with ICAM-1 has an affinity with 500 nM, while the affinity measured for LFA-1 binding to ICAM-1 on unstimulated T cells is very low, about 100 μM (42). As compared with CD62L, LFA-1 has high affinity and forms long-lived bonds with ICAM-1, suggesting a potential mechanism for firm adhesion. Stimulation of leukocytes with physiologic stimuli or PMA induces clustering of LFA-1 as well as conformational changes of LFA-1 itself into high affinity state. Despite a slow dissociation rate constant of LFA-1, monomeric interaction of LFA-1 would not be sufficient for firm adhesion. Clustering of high affinity LFA-1 would induce cooperative interaction of each molecule, increase in the avidity, and thus induce firm adhesion.

In conclusion, in the first real-time analysis of affinity and kinetic study of the interaction between ICAM-1 and well-defined LFA-1, we have shown that LFA-1 binds to ICAM-1 with a $K_d$ of 500 nM and $k_{\text{diss}}$ of 0.1 s$^{-1}$. Thus, LFA-1 has high affinity and forms long-lived bonds with ICAM-1, suggesting a potential mechanism for firm adhesion.

**References**


