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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 β_2 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} 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 (β_2 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 in 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 appears to play an essential 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 β 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[2]), 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

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[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.

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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, C_H2, and C_H3 domains) of human IgG1 (31) using conventional rDNA techniques. CHO-K1 cells were transfected with the vector pRc/CMV (Invitrogen, San Diego, CA) containing 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).

Production 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 $MgCl_2$). 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).

Before BIAcore analysis, sLFA-1 (1.2 μg/ml) was further fractionated by gel filtration on a Superose 6 PC3.2/30 SMART column (Pharmacia) in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and 0.05% Tween-20). The detection of eluted components was monitored by absorbance at 280 nm. The column elution positions of the fractionated sLFA-1 were 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 *Eco*RI site of pBluescript II SK (Stratagene, La Jolla, CA), as previously described (30). For the expression of the LFA-1 heterodimer, CD11a and CD18 cDNA fragments were subcloned into the expression vector containing the SV40 early promoter (30). These expression vectors and pSV2neo (ATCC) were then cotransfected into CHO-K1 cells using the calcium-phosphate method, and G418-resistant clones were selected. The expression of LFA-1 on the cell surface was determined by the flow-cytometric analysis on a FACScan (Becton Dickinson, Mountain View, CA) using TS1/22 and TS1/18. LFA-1 stably expressed on the surface of CHO-K1 cells has the same properties as naturally occurring LFA-1 on human leukocytes, e.g., SKW-3 and JY, in terms of both the binding activity to ICAM-1-expressing cells and the reactivity with mAbs directed against LFA-1. A stable line of LFA-1-expressing CHO-K1 cell was cultured in DMEM/F-12 media supplemented with 10% heat-inactivated FCS.

Purification of membrane-bound LFA-1

Membrane-bound LFA-1 (mLFA-1) was purified from LFA-1-transfected CHO-K1 cell lysates using immunoaffinity chromatography, as described by Dustin et al. (29) with modifications. The TS2/4 column (5 ml at 5 mg/ml) was prepared by covalently attaching TS2/4 to *N*-hydroxysuccinimide (NHS)-activated HiTrap (Pharmacia), according to the manufacturer's instructions. After neutralization of the fractions from the TS2/4 column, samples were precleared with HiTrap-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 positions of the fractionated LFA-1 were compared with calibration standards, as already described. LFA-1 was quantitated by ELISA using MEM48 (anti-CD18) and TS2/4 (anti-CD11a).

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 proteins for injection 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 IgG 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 (k_{diss}). Nonlinear curve fitting was conducted with the BIA evaluation 2.0 program (Pharmacia Biosensor AB). The association rate constant (k_{ass}) was determined by nonlinear curve fitting to the association phase data using the model of one site. K_d was calculated from the ratio k_{diss}/k_{ass}.

Results

Binding of sLFA-1 to immobilized chimeric ICAM-1

The interaction between LFA-1 and ICAM-1 was studied using a soluble form of human LFA-1 (sLFA-1) and a chimeric molecule consisting of the amino-terminal two Ig domains of human ICAM-1 (D1D2) fused to the Fc portion (hinge, C_H2, and C_H3 domains) of human IgG1 (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. 1*A*). 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 1*B*. 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 1*B* 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. 2*A*). The main peak (Fig. 2*A*, peak 2) corresponded to monomeric sLFA-1, since it eluted from the column in the size range expected

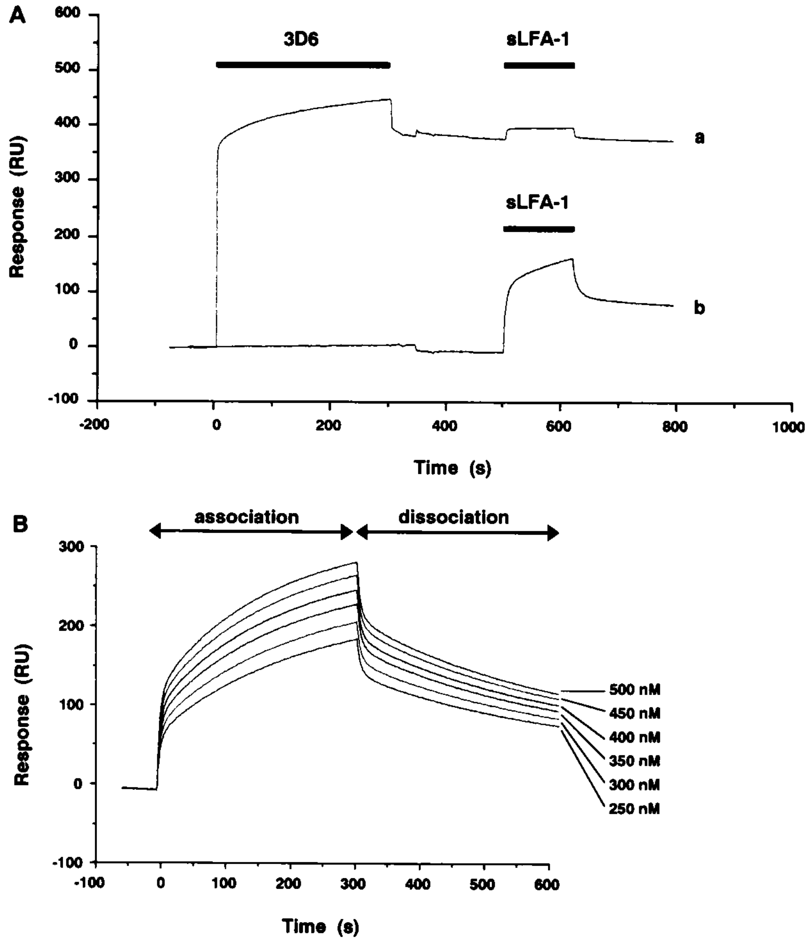

FIGURE 1. Binding of sLFA-1 to immobilized D1D2-IgG chimeric protein and competition by anti-ICAM-1 mAb. *A*, sLFA-1 (500 nM) was injected for 2 min (indicated with bars) over the D1D2-IgG surface with (*curve a*) or without (*curve b*) pretreatment with anti-ICAM-1 mAb, 3D6. Pretreatment with 3D6 injected at 500 μg/ml for 5 min (indicated with a bar) inhibited the reaction to the basal level. *B*, sLFA-1 was injected at 250, 300, 350, 400, 450, or 500 nM for 5 min after immobilization of D1D2-IgG captured with goat anti-human IgG Ab covalently coupled to a sensor chip. Bound proteins were dissociated at the end of each experiment with 10 mM HCl for 1 min.

for the monomeric form of sLFA-1 (258 kDa). The expected m.w. of the shoulder peak (Fig. 2*A*, 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. 2*C*) 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. 2*C*), the component of both the slow association and slow dissociation was reduced significantly in comparison with the unfractionated sLFA-1 (Fig. 2*B*). The association rate constant (k_{ass}) and the dissociation rate constant (k_{diss}) for the reaction of the monomeric sLFA-1 were 2×10^5 M^{-1} s^{-1} and 1×10^{-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. 2*C*). 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. 3*A*). 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 3*B*. 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 beginning of dissociation; RU, 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

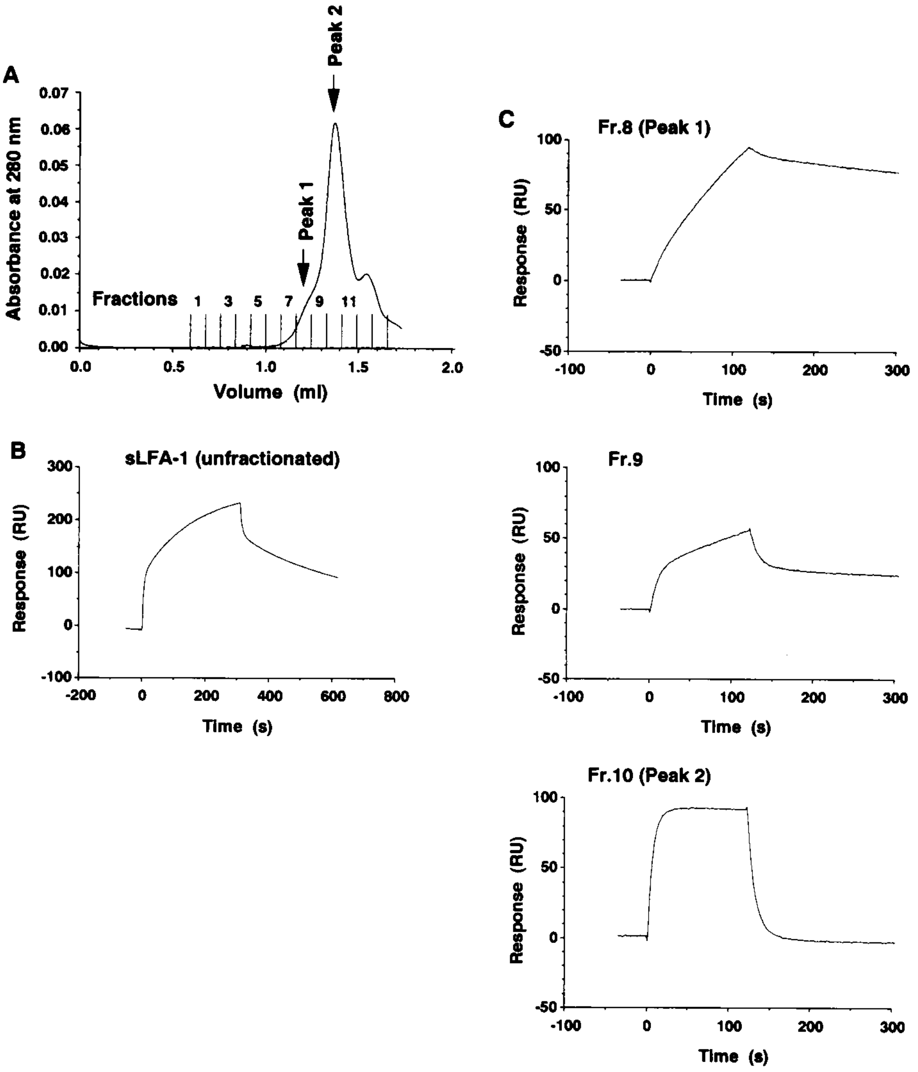

FIGURE 2. *A*, Analysis of multimeric aggregates in sLFA-1 preparation. sLFA-1 was loaded onto a Superose 6 PC3.2/30 SMART column (Pharmacia) in a 10-μl sample, and 80-μl fractions were collected at 40 μl/min. Peaks: 1, dimeric sLFA-1 (516 kDa); 2, monomer of sLFA-1 (258 kDa). *B*, Binding of unfractionated sLFA-1 to immobilized D1D2-IgG. *C*, Binding characteristics of sLFA-1 fractionated on a Superose 6 PC3.2/30 SMART column. Fractions corresponding to the monomeric sLFA-1 peak (fr.10), the multimeric sLFA-1 peak (fr.8), and the mixture (fr.9) were analyzed by their binding to immobilized D1D2-IgG on BIAcore. The surface was regenerated by injection of 10 mM HCl for 1 min.

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. 4*A*), 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 chimeric ICAM-1 on a BIAcore biosensor. As shown in Figure 4*B*, 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} 1×10^{-1} s^{-1}) and a moderately fast association rate constant (k_{ass} 2×10^5 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. 2*A*). 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

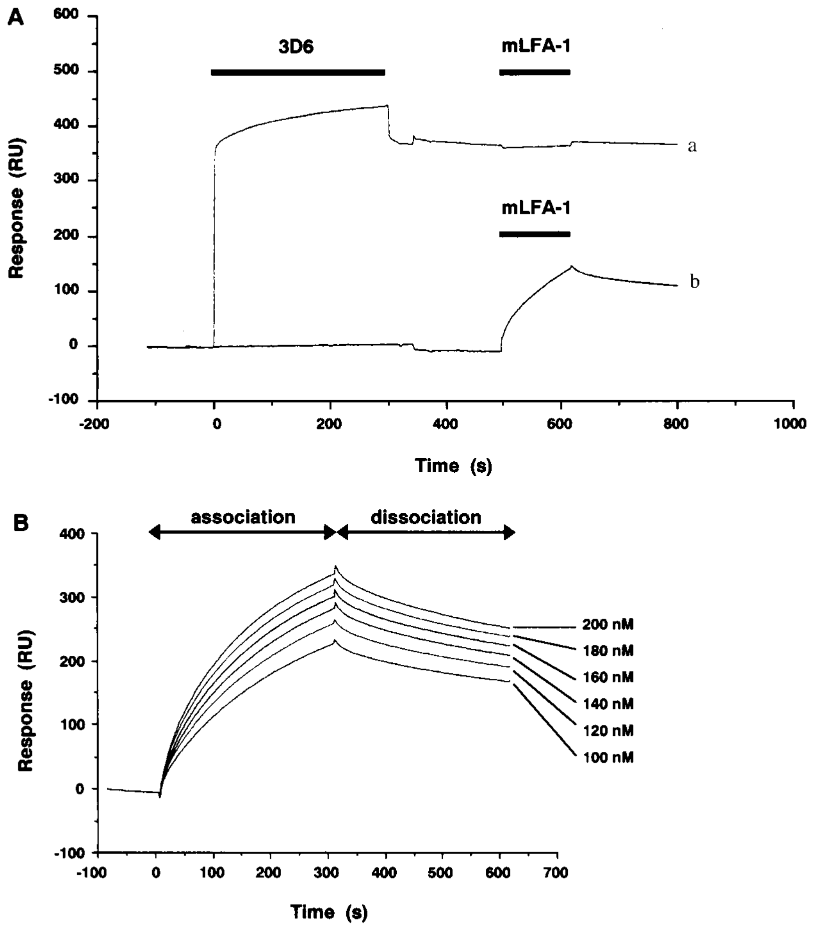

FIGURE 3. Binding of mLFA-1 to immobilized D1D2-IgG chimeric protein and competition by anti-ICAM-1 mAb. *A*, mLFA-1 (500 nM) was injected for 2 min (indicated with bars) over the D1D2-IgG surface with (*curve a*) or without (*curve b*) pretreatment with anti-ICAM-1 mAb, 3D6. Pretreatment with 3D6 injected 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.

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

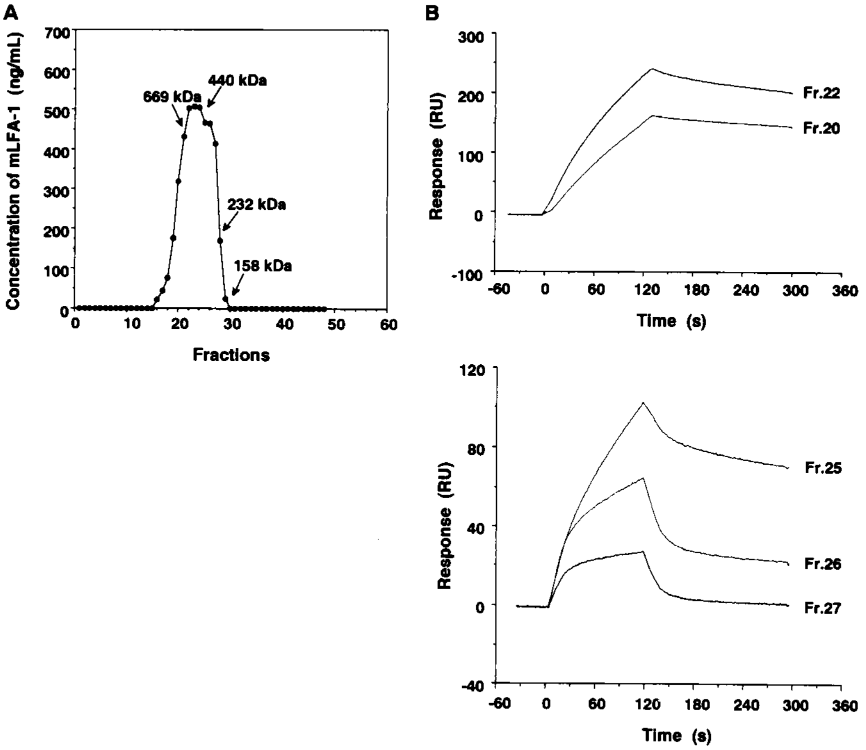

FIGURE 4. *A*, Analysis of the immunoaffinity-purified mLFA-1. mLFA-1 was fractionated by gel filtration on a Superose 6 HR10/30 FPLC column. The amount of mLFA-1 in fractions was determined by ELISA. Fractions (0.5 ml) were collected at a flow rate of 0.1 ml/min. The column elution positions of the 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.

(40). Other reports showed that Ca^{2+} inhibits Mg^{2+}-induced T cell adhesion by inhibiting the expression of the Mg^{2+}-induced 24 epitope on LFA-1 (41). Addition of 1 mM Ca^{2+} inhibited the binding of LFA-1 to immobilized chimeric ICAM-1 by 20% in our BIAcore analysis (data not shown). We, therefore, performed affinity and kinetic measurements in the absence of Ca^{2+}, but in the presence of Mg^{2+} to induce high affinity state of LFA-1.

In the BIAcore analysis, sLFA-1 (250–500 nM) was bound to the immobilized D1D2-IgG (300 RU), but little or no binding was observed when ICAM-1 was not present on the sensor surface. Binding specificity was clearly demonstrated by the fact that binding of sLFA-1 to immobilized D1D2-IgG was completely inhibited when ICAM-1 on the sensor surface was pretreated with 3D6 mAb that binds domain 1 of ICAM-1 and blocks LFA-1 binding (Fig. 1*A*). In the association phase, a fast association was observed at the very beginning of the reaction, followed by a slow association phase, and the reaction did not reach the equilibrium state during the injection period (Fig. 1*B*). Since we showed that the sLFA-1 preparation contained approximately 10% of the dimeric sLFA-1 (Fig. 2*A*), the slow association in the reaction might be due to the binding of sLFA-1 to D1D2-IgG as a dimer with two binding sites to replace the sLFA-1 monomer binding to the surface. It appears likely that the dimeric forms of sLFA-1 slowly diffuse due to their higher molecular size. The binding activity of the monomeric sLFA-1 was not likely to be detected in conventional binding assays as a result of its k_{diss} value.

When we used the mAb affinity-purified mLFA-1, the multiphasic association and dissociation steps were observed during the biosensor kinetic analysis of the interaction between mLFA-1 and the chimeric ICAM-1. However, mLFA-1 fractionated in lower molecular size fractions have kinetics similar to that of the monomeric sLFA-1 (Fig. 4*B*). The monomer-enriched fractions of mLFA-1 dissociated from the immobilized D1D5-IgG faster than the multimeric mLFA-1. We confirmed that similar results were obtained with the D1D2-IgG-immobilized surface (data not shown) and that D1D5 (D1D5-IgG) has the same potency as D1D2 (D1D2-IgG) with binding to sLFA-1, as previously described (30). We also tried to analyze the binding of D1D5 to mLFA-1 immobilized on the sensor surface indirectly using TS2/4 or MEM48. Binding of monomeric D1D5 fractionated by size-exclusion chromatography to immobilized mLFA-1 revealed rapid binding kinetics. However, we could not analyze the interaction kinetically, because the mLFA-1 baseline gradually decreased during the experiment as a result of dissociation of mLFA-1 from the sensor surface (data not shown). The interaction between the sICAM-1 and immobilized mLFA-1 has been studied in a number of laboratories. Dissociation constants ranging from 100 nM (35) to 130 nM (36) have been reported using conventional receptor-binding assays. Lollo et al. showed that the affinity of LFA-1 for ICAM-1 on T cells activated by phorbol esters was approximately 400 nM (42). These values are of the same order of magnitude as the 500 nM measured in this study. Surface plasmon resonance technology has a great advantage in that we can analyze both the association phase and dissociation phase of the interaction; thus, this is the first kinetic analysis of the interaction of ICAM-1 with LFA-1 using a BIAcore biosensor.

Recent studies have provided affinity and kinetic data on the interactions of CD2 with its ligands CD48 (43) and CD58 (44, 45). These studies have concluded that monomeric CD2 binds 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 ($k_{diss} \geq 1.6$ 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 s^{-1}). The extremely fast k_{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.

Table I. *Comparison of published affinity and kinetic data*

Interaction	K_d (μM)	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	Ref.
CD2–CD58	9–22	≥400,000	≥4	44
CD28–CD80	4	≥660,000	≥1.6	46
CD62L–GlyCAM-1	108	≥100,000	≥10	47
ICAM-1–LFA-1	0.5	200,000	0.1	This study

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_{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.

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