Structure and Regulation of the Leukocyte Adhesion Receptor LFA-1 and Its Counterreceptors, ICAM-1 and ICAM-2

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Immune responses hinge on appropriate interactions between T lymphocytes and antigen-presenting cells (APC) or target cells. Although immunological specificity is mediated by antigen receptors (Bjorkman et al. 1987), antigen-specific cytotoxicity and helper-T-lymphocyte responses require lymphocyte function associated-1 (LFA-1) and other "accessory molecules" (Springer et al. 1987). LFA-1 mediates adhesion between lymphocytes and other cells and, in the process, may inform the cell of its environment via signal transduction mechanisms (Springer et al. 1987; van Noesel et al. 1988), as do other integrins (Menko and Boettinger 1987). Therefore, we will refer to LFA-1 as an adhesion receptor. LFA-1 mediates adhesion by binding to cell-surface molecules on other cells that include ICAM-1 and ICAM-2 (Marlin and Springer 1987; Makgoba et al. 1988b; Staunton et al. 1989a). We will refer to these ligand molecules as counterreceptors, based on the idea that they may also be capable of transducing signals.

In the first part of this paper, we present an overview of recent work on LFA-1 and ICAM structure. In the second part, we present data that address another key problem: How is the LFA-1/ICAM mechanism regulated in interactions of T lymphocytes with other cells? The interaction of lymphocyte adhesion receptors and their counterreceptors has no intrinsic immunological specificity. This raises the question of how adhesion mechanisms function within the context of the immune response; that is, how is T-lymphocyte adhesion regulated? ICAM-1 expression is regulated by cytokines and differentiation with changes occurring over a period of several hours to days (Clark et al. 1986; Dustin et al. 1986). Increasing ICAM-1 expression at sites of inflammation is a powerful mechanism for increasing the potential for interaction of local cells with T lymphocytes. However, these changes are too slow to account for the kinetics of antigen-specific cytolytic T-lymphocyte interactions with target cells in which contact is followed by a brief period of strong adhesion and then detachment from the target cell (Martz 1977; Poenie et al. 1987); we will refer to the latter process as de-adhesion. A clue to a more dynamic mode of regulation was provided by the observation of phorbol-ester-stimulated aggregation of lymphocytes. Phorbol-ester-stimulated aggregation occurs within 1 hour, is blocked by LFA-1 monoclonal antibody (MAb) and ICAM-1 MAb, but is not accompanied by a change in LFA-1 or ICAM-1 expression (Patarroyo et al. 1985; Rothlein and Springer 1986b; Rothlein et al. 1986). Here, we have further examined phorbol ester stimulation of adhesion in such a way that it was possible to determine whether LFA-1 or ICAM activity is regulated. These observations were extended to examine the effects of stimulation through the T-cell antigen receptor (TCR). Our results show that LFA-1 avidity for ICAM-1 is profoundly increased by phorbol-ester- or TCR-mediated stimulation. The TCR-stimulated increase in avidity is transient, suggesting a mechanism for de-adhesion after execution of TCR-triggered regulatory or effector functions. On the basis of these observations and previous studies that have shown that LFA-1 requires energy for function (Marlin and Springer 1987), we propose that the TCR acts as a switch controlling use of cellular energy to convert LFA-1 from a low-avidity to a high-avidity state.

METHODS

Monoclonal antibodies. The following monoclonal antibodies were used as ascites or purified IgG: TS2/4 (native LFA-1α, IgG1), TS1/18 (native LFA-1β, IgG1), TS1/22 (native or denatured LFA-1α, IgG1) (Sanchez-Madrid et al. 1983), RR1/1 (ICAM-1, IgG1) (Rothlein et al. 1986), OKT3 (CD3, IgG2a) (Kung et al. 1979), and Leu4 (CD3, IgG1, a generous gift from R. Evans) (Evans et al. 1981).

LFA-1α and β cDNAs. cDNAs have been described previously (Kishimoto et al. 1987; Larson et al. 1989). Amino acid residue numbering is for the mature protein.

RNase mapping and identification of leukocyte adhesion deficiency (LAD) mutations. The location of mutations in β subunits of LAD patients 2 and 14 were determined by forming hybrids of patient RNA with a radiolabeled antisense cDNA probe and digesting the hybrids with RNase A and RNase T1 (A.J. Wardlaw et al., in prep.). This method is sensitive to single mismatches. cDNA libraries prepared with antisense β

oligonucleotides as primers for reverse transcriptase were generated from patient RNA, and clones were isolated using the normal β subunit cDNA as a probe. Dideoxy sequencing of patient cDNAs was used to identify mutations, the locations of which were revealed by RNase mapping (A.J. Wardlaw et al., in prep.). The mutations were introduced into the normal β cDNA in pCDM8 by subcloning of fragments from the mutant β subunit or by site-directed mutagenesis. Sequencing was used to confirm that these were the only mutations.

Oligonucleotide-directed mutations. Point mutations of cDNA inserts were generated in the CDM8 vector according to the method of Kunkel (1985).

Genomic cloning of ICAM-2. A human genomic library (5×10^5 colonies) in the cosmid vector pWE15 (kindly donated by G.A. Evans, Salk Institute, La Jolla, California) on nitrocellulose filters was probed with the human ICAM-2 cDNA by standard methods (J. Garcia-Aguilar and T.A. Springer, in prep.). Three identical 40-kb cosmid clones were obtained. The ICAM-2 gene was in a 9-kb *Sma* fragment that was subcloned into pGEM-7 and sequenced using oligonucleotide-primed dideoxy sequencing.

Transfections. COS cells were transfected with cDNA clones in the CDM8 vector using a standard DEAE-dextran transfection method (Aruffo and Seed 1987). Transient expression was examined by indirect immunofluorescence flow cytometry on day 3 or 4. Patient and control B-lymphoblastoid cell lines (B-LCL) were transfected by electroporation with the β-subunit cDNA in an episomally replicating vector containing an Epstein-Barr virus (EBV) origin of replication and a hygromycin-resistance marker constructed by fusing parts of CDM8 and p205118a (kindly provided by B. Seed, Massachusetts General Hospital, Boston, Massachusetts) (M.L. Hibbs et al., in prep.). Cells were grown in 200 μg/ml hygromycin for 6 weeks to obtain stable lines expressing transfected β subunit.

Adhesion receptor purification. ICAM-1 was immunoaffinity purified from spleens of patients with hairy cell leukemia or from JY B-LCL (Marlin and Springer 1987). LFA-1 was immunoaffinity purified from SKW3 T-lymphoma lysates using the TS2/4 LFA-1 MAb and elution at pH 11.5 in the presence of 2 mM $MgCl_2$ (Dustin and Springer 1989). Monoclonal antibodies were coupled to Sepharose as described previously (March et al. 1974). SKW3 lysates, immunoaffinity isolates, and eluted material were prepared as described previously (Kürzinger and Springer 1982). In batch experiments (described in Fig. 2, lanes 1–10), LFA-1 bound to TS2/4-Sepharose was treated for 30 minutes at 4°C with elution buffer (50 mM triethylamine [pH 11.5], 0.15 M NaCl, 1% octylglucoside [OG] detergent with or without divalent cations). Material remaining bound to the beads was eluted with SDS and subjected to SDS-PAGE. The neutralized pH 11.5 eluates were subjected to a second round of immunoaffinity isolation with anti-β-Sepharose, and the bound material was also eluted with SDS and subjected to SDS-PAGE (Laemmli 1970).

Adhesion receptor reconstitution and binding assays. Purified proteins in 1% OG detergent were combined with phospholipids and liposomes formed by dialysis as described previously (Brian and McConnell 1984). Glass-supported planar membranes were formed on 5-mm coverslips glued to the bottom of 96-well microplates. Alternatively, purified LFA-1 and ICAM-1 in 1% OG were adsorbed to polystyrene microtiter plate wells by addition of 5 μl of the detergent-solubilized protein to 45 μl of 25 mM Tris (pH 8.0), 0.15 M NaCl, 2 mM $MgCl_2$ (TSM). After a 16-hour incubation at 4°C, the plates were incubated for 1 hour at room temperature in 1% BSA/TSM and then washed with assay media. The amount of protein bound was quantitated by radiometric assay with directly iodinated monoclonal antibody at 10 μCi/μg. Binding assays were performed in RPMI 1640, 10% fetal bovine serum (FBS), 25 mM HEPES (pH 7.4) (assay media). Cells labeled with $Na_2{}^{51}CrO_4$ ($\sim 5 \times 10^4$) were pretreated with blocking monoclonal antibody and then centrifuged onto adhesion-molecule-coated surfaces at 10*g* for 5 minutes and then washed after 5–15 minutes as specified. Alternatively, cells were allowed to settle at 1*g* for 1 hour before washing. Washing consisted of aspirating media to 50 μl (glass-supported planar membranes) or completely (plastic-adsorbed protein) and adding 200 μl of fresh media eight (planar membranes) or three (plastic-adsorbed protein) times. Resting T cells were isolated from whole blood by plastic adherence and nylon wool filtration and were used within 24 hours of drawing blood. In binding assays, resting T lymphocytes were washed by flicking media from the plates eight times with 100 μl added between each wash. Flicking was more effective for thoroughly removing unbound peripheral blood lymphocytes (PBL-T), which were more difficult to remove by aspiration due to their small size. It was also quicker, allowing more careful kinetic analysis (see below). Since COS cells bind to plastic under standard conditions, different conditions were used to measure binding of transfected COS cells. Binding assays were performed using phosphate-buffered saline (PBS) buffer with 5% FBS, 2 mM $MgCl_2$, and 0.025% NaN_3 for 60 minutes at 24°C, followed by four washes using a 26 ga. needle (ICAM-1 transfectants) or an 18 ga. needle (LFA-1 transfectants) for aspiration. The smaller needle used with ICAM-1 transfectants results in slower aspiration and lower shear force.

Conjugate formation assays. Resting T lymphocytes purified as above were labeled with sulfofluorescein diacetate, and LFA-1$^-$ B-LCL were labeled with hydroethidine as described previously (Luce et al. 1985). Resting T lymphocytes were pretreated with CD3 MAb at 4°C and washed. Resting T lymphocytes and B-LCL were mixed at a 1:2 ratio with the appropriate mono-

clonal antibody or anti-Ig added and allowed to settle for 1 hour at 4°C or centrifuged for 5 minutes at 10*g* at 24°C before they were incubated 5 minutes at 37°C. The pellets were resuspended by vortexing and analyzed on a Coulter Epics V using filters and data collection modes described previously (Luce et al. 1985).

RESULTS

Leukocyte Adhesion Receptors

Elucidation of the primary structure of LFA-1 and ICAMs has linked these molecules to two adhesion receptor families and a large body of existing knowledge about the function of other members of these families. LFA-1 is a noncovalent heterodimer with an α subunit (CD11a) of 180 kD and a β subunit (CD18) of 95 kD. LFA-1 shares its β subunit polypeptide with two other cell-surface heterodimers, Mac-1 (CD11b) and p150,95 (CD11c). All of these molecules are implicated in adhesion. Elucidation of the primary structure of the β subunit led to the definition of a new adhesion receptor family based on sequence comparison to a previously published chicken fibronectin receptor subunit (Tamkun et al. 1986; Kishimoto et al. 1987). This group is now referred to as the integrin family, after the important concept that these molecules integrate the outside (extracellular matrix and other cells) and inside (cytoskeleton) of cells (Hynes 1987). Currently, three subfamilies are characterized by their distinctive β subunits: β_1, the VLA or fibronectin receptor subfamily; β_2, the leukocyte integrin subfamily that includes LFA-1; and β_3, the IIbIIIa/vitronectin receptor subfamily (Hynes 1987). This organization may require revision on the basis of the recent identification of additional β subunits that associate with previously known α subunits (Cheresh et al. 1989; Kajiji et al. 1989). There is indirect evidence that the concept of cytoskeletal integration may apply to LFA-1 (Kupfer and Singer 1989). Lymphocyte utilization of adhesion receptors that are shared with virtually all other cells in the body suggests that generation of form in the immune system has much in common with morphogenesis in other multicellular systems.

LFA-1 Structure: β Subunit

The primary structure of the β_2 subunit was of particular interest for two reasons: (1) It is shared between all three leukocyte adhesion receptors and (2) it is defective in LAD, a disease characterized by absence of all three leukocyte integrins and by recurrent life-threatening bacterial infections (Anderson and Springer 1987). It was subsequently found that the β subunits are more highly conserved than the α subunits both between species and between subfamilies. The most striking aspect of this conservation is the perfect alignment of the 56 cysteine residues between β subunits (Kishimoto et al. 1987). The concentration of cysteine residues in 4 repeats between residues 449 and 628 (Fig. 1) suggests a relatively rigid structure in terms of mechanical flexibility.

The most highly conserved region among β_1, β_2, and β_3 (53–63% identity) is between residues 100 and 341. Three LAD mutations, in which the β_2 subunit is synthesized but fails to associate with α subunits, fall into this region, suggesting that it is important for $\alpha\beta$ subunit association. The first LAD mutation characterized was caused by a point mutation in a splice-acceptor site resulting in aberrant splicing out of an exon and deletion of residues 310–339 (Kishimoto et al. 1989). We have characterized mutations in two other LAD patients that result in single amino acid substitutions (A.J. Wardlaw, in prep.). Mutations detected by RNase mapping and sequencing of indicated regions in patient cDNA clones cause substitution of glycine 147 to arginine (G147R) in patient 2 and leucine 127 to proline (L127P) in one allele from patient 14 (Fig. 1). Studies on other integrins implicate this highly conserved region of the β subunit in ligand binding. Peptides containing the sequence RGD act as ligand analogs for several integrins (although not for LFA-1). Cross-link-

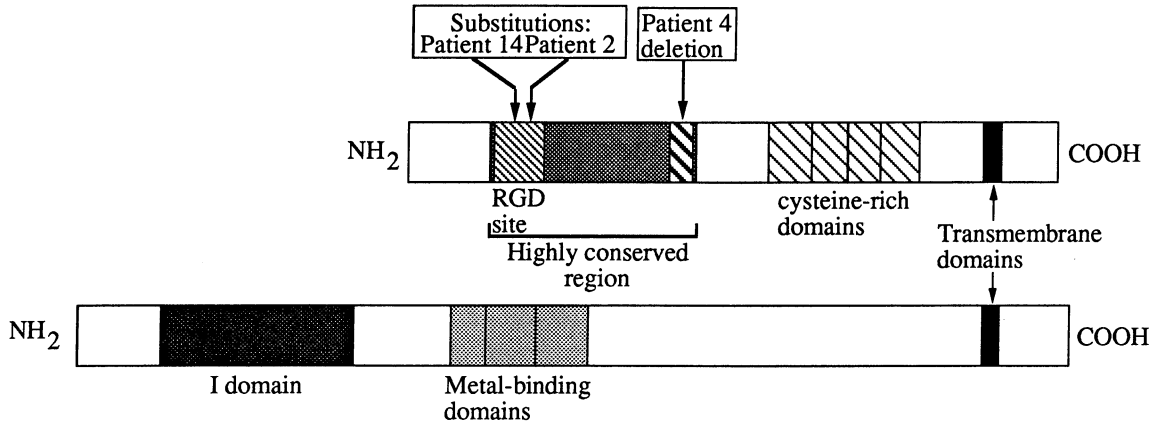

Figure 1. Schematic of LFA-1 α and β subunits. Regions are drawn to scale using boundaries defined previously (Kishimoto et al. 1987, 1989; Larson et al. 1989). The "RGD site" is the smallest gp IIbIIIa peptide to which an RGD peptide was cross-linked (D'Souza et al. 1988) and is highly homologous to the indicated region of the LFA-1 β subunit.

ing of photoaffinity analogs of RGD peptides to a platelet fibrinogen receptor (gpIIbIIIa), followed by isolation and sequencing of labeled fragments (D'Souza et al. 1988), suggests that the highly conserved region (residues 109–171) has a role in ligand binding (Fig. 1). Combination of data about different integrins thus suggests that the ligand-binding region may be one of close association between α and β.

LFA-1 Structure: α Subunit

Although the β subunit is implicated in binding to ligand by the RGD peptide cross-linking studies just mentioned and antibody-blocking experiments, it is clear that different α subunits impart different ligand-binding specificities when associated with the same β subunit. cDNA cloning of LFA-1α (α_L) (Larson et al. 1989) and a number of other α subunits shows that these proteins are structurally related transmembrane proteins (Fig. 1). A striking feature of integrin α subunits including LFA-1 is the presence of either three or four putative divalent cation binding sites that are similar to Ca^{++}/Mg^{++} binding sequences in calmodulin, troponin C, and parvalbumin. Notably, LFA-1 function is dependent on divalent cations, primarily Mg^{++} (Rothlein and Springer 1986). LFA-1, the other leukocyte integrins, and the collagen receptor VLA-2 all have a 200-amino-acid inserted domain (I domain) toward the amino-terminal end of the α subunit (Fig. 1) that is strikingly homologous to the three A domains of von Willebrand factor, two domains of cartilage matrix protein, and a single domain in the complement components C2 and factor B (Corbi et al. 1988; Pytela 1988; Larson et al. 1989; Takada and Hemler 1989). The A1 and A2 domains of von Willebrand factor have been shown to be functional domains in binding of heparin, collagen, and platelet glycoprotein Ib (Girma et al. 1987). The presence of I domains in the leukocyte integrins and VLA-2, but not in four other integrins, suggests that integrins containing the I domain may have more complex ligand recognition, incorporating recognition elements of the I domains as well as recognition elements common to all integrins.

Purification of LFA-1 in Functional Form

We made use of putative divalent cation-binding sites in the α subunit of LFA-1 to purify it in a functionally active form. LFA-1 α and β subunits dissociate under previously described elution conditions (Kürzinger and Springer 1982; Larson et al. 1989) and, as such, are inactive in adhesion assays (M.L. Dustin and T.A. Springer, unpubl.). To test the ability of divalent cations to stabilize LFA-1 subunit interactions, Mg^{++} or Ca^{++} were included in the pH 11.5 buffer used to elute LFA-1 from MAb-Sepharose. This yielded intact $\alpha\beta$ complexes as shown by coprecipitation of the α and β subunits using either β-chain-specific monoclonal antibody (Fig. 2, lanes 6 and 7) or α-chain-specific monoclonal antibody (Fig. 2, lanes 9 and 10). The subunits

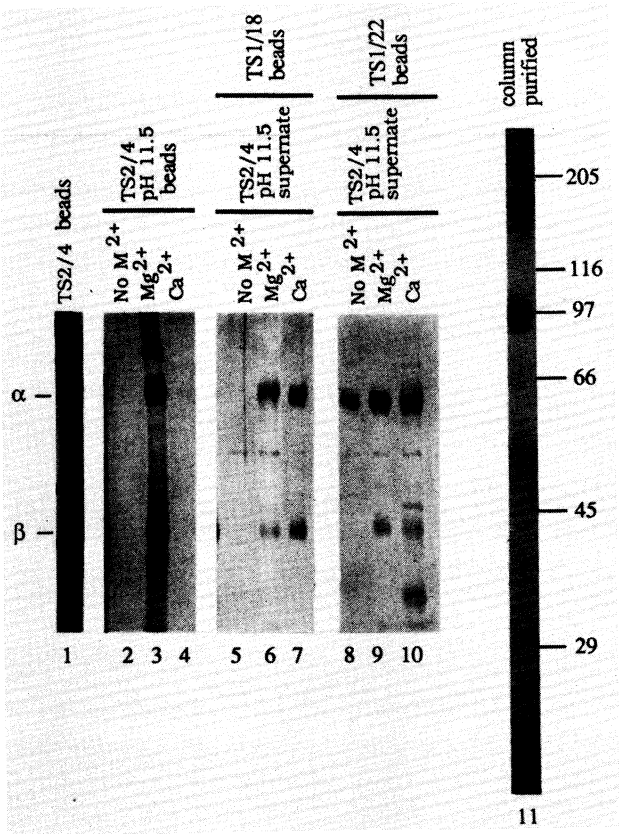

Figure 2. Divalent cation requirements for elution of intact LFA-1 from TS2/4 MAb-Sepharose and SDS-PAGE of purified LFA-1. TS2/4 beads with bound LFA-1 (lane *1*) or material remaining bound to beads after treatment at pH 11.5 in the presence of indicated divalent cation (lanes *2–4*); material re-isolated from neutralized pH 11.5 eluates with TS1/18 MAb (lanes *5–7*) or TS1/22 MAb (lanes *8–10*). Purified LFA-1 (1 μg) (lane *11*). Proteins were heated to 100°C with reducing sample buffer and run on SDS-7%-PAGE. Lanes *1–10* and lane *11* were from different gels.

were dissociated in the absence of divalent cations, resulting in precipitation of only the α subunit alone with α-chain-specific monoclonal antibody and no precipitation with β-chain-specific monoclonal antibody (Fig. 2, lanes 5 and 8). We should point out that removal of divalent cations from LFA-1 solubilized in Triton X-100 at pH 7–8 does not result in dissociation, since intact LFA-1 is readily obtained from cell detergent lysates prepared with EDTA (Sanchez-Madrid et al. 1983). Apart from providing a method for purifying intact LFA-1, these experiments provide the first evidence that divalent cations bind to LFA-1.

Intact LFA-1 purified as described above was tested for cell binding. LFA-1 was reconstituted into unilaminar liposomes at different lipid:protein ratios, and glass-supported planar membranes were formed that had a range of LFA-1 densities. L428 Reed-Sternberg cells and SKW3 T-lymphoma cells bound to these membranes in an LFA-1 density-dependent manner (Fig. 3). This binding was blocked efficiently by LFA-1 monoclonal antibody. Prior cell–cell adhesion studies

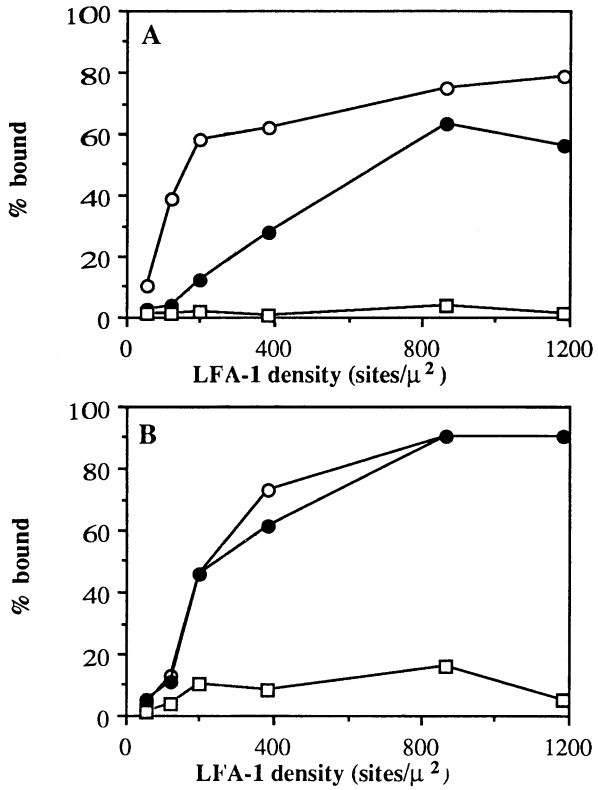

Figure 3. Binding of L428 (*A*) or SKW3 (*B*) cells to LFA-1 in planar membranes. Binding of ^{51}Cr-labeled SKW3 or L428 cells was measured by incubating cells on LFA-1 planar membranes with the indicated density of LFA-1 for 60 min at 37°C and then washing eight times by aspiration. Cells were incubated in the presence of 50 μg/ml control IgG (○), ICAM-1 MAb (●), or LFA-1 MAb (□).

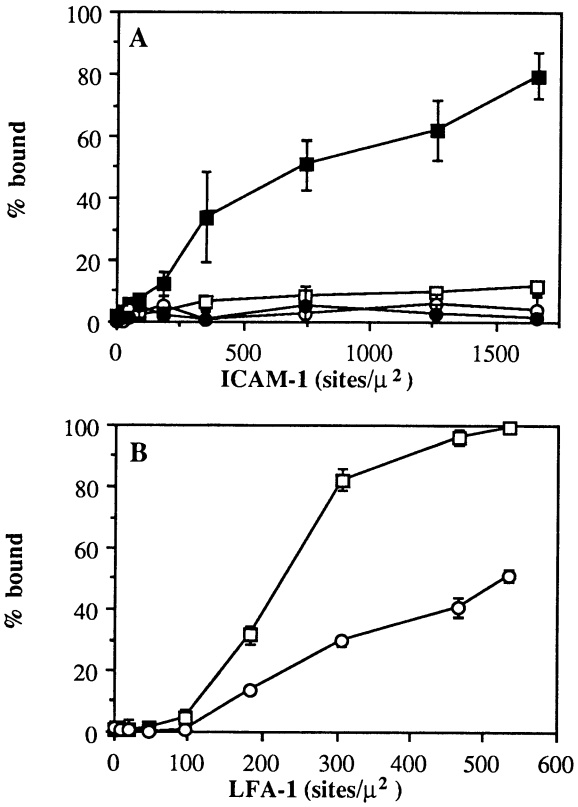

Figure 4. Binding of JY cells to LFA-1 or ICAM-1 adsorbed to plastic at 37°C or 4°C. Binding of ^{51}Cr-labeled JY cells was measured by centrifuging (10*g* for 5 min) cells onto the bottom of microtiter wells coated with the indicated density of purified LFA-1 or ICAM-1 at 4°C and incubating for 10 min at 37°C (squares) or 4°C (circles). Similar results were obtained if cells were allowed to settle for 1 hr at 4°C. Binding to LFA-1 was performed in the presence (filled symbols) and absence (open symbols) of 50 μg/ml ICAM-1 MAb. Binding to ICAM-1 was performed in the presence (filled symbols) or absence (open symbols) of 50 ng/ml PMA. Cells were pretreated with PMA for 30 minutes at 37°C prior to cooling to 4°C for centrifugation. This was required to obtain efficient binding of cells to ICAM-1.

indicated the presence of LFA-1 counterreceptors besides ICAM-1 on several hematopoietic cell lines and endothelial cells. These studies showed that L428 cells have both ICAM-1-dependent and -independent components, whereas SKW3 has only the ICAM-1-independent component (Makgoba et al. 1988a; Rothlein et al. 1986). The ICAM-1 on L428 is only required for binding when LFA-1 is at a relatively low density as indicated by blocking with ICAM-1 monoclonal antibody; at higher LFA-1 density, the other LFA-1 counterreceptor(s) is sufficient for efficient binding. In contrast, binding of SKW3 to LFA-1 is not blocked by ICAM-1 monoclonal antibody at any LFA-1 density, suggesting that ICAM-1 is not involved in this binding. These experiments confirmed the presence of an LFA-1 counterreceptor distinct from ICAM-1 and provided a powerful approach to its functional cloning (see below).

Adhesion mechanisms are susceptible to inhibition at low temperatures; this can indicate a requirement for membrane fluidity (lateral mobility) or metabolic energy. As described previously (Marlin and Springer 1987), binding of JY cells to ICAM-1 substrates is temperature-sensitive with no adhesion observed at 4°C, even when cells are pretreated with phorbol esters at 37°C (Fig. 4A). However, there is significant binding of JY cells to LFA-1 substrates at 4°C (50% reduced compared to 37°C) (Fig. 4B). The relative contributions of ICAM-1 and other ICAMs to low-temperature binding could not be determined without antibodies to other ICAMs. These reciprocal experiments show that the temperature sensitivity of the LFA-1/ICAM mechanism is due to cell-surface LFA-1. The loss of function of cell-surface LFA-1 at low temperature must be related to a cellular property, since purified LFA-1 can bind cell-surface ICAM-1 at 4°C. Furthermore, purified LFA-1 binds to purified ICAM-1 equally well at 4°C and 37°C (Dustin and Springer 1989).

Expression of Recombinant LFA-1

An important approach to understanding LFA-1 structure/function relationships is to express recombinant LFA-1 molecules in animal cells. When LFA-1 α

and β subunits were transfected into COS cells, the chains were assembled properly, and LFA-1 was expressed on the cell surface (not shown) (R.S. Larson et al., in prep.). LFA-1 expressed in COS cells was functional, based on binding of transfected COS cells to ICAM-1 substrates (R.S. Larson et al., in prep.). Transfection of the α subunit alone did not lead to expression of α chain, as would be expected from the LAD patients who lack β_2 and fail to express α. However, transfection of β_2 alone did result in expression of small amounts of human β_2 epitopes. Whether α subunits normally associated with β_1 can complement expression or whether β_2 can be expressed on its own is unknown. The COS cell system was used to express β_2 cDNAs containing the mutations found in LAD patients 2 and 14 (A.J. Wardlaw et al., in prep.). Coexpression in COS cells of the α subunit and β_2 subunit with the L149P mutation led to weak expression of α chain and two β_2 chain epitopes but no expression of the TS1/18 β_2 chain epitope, which requires subunit association, and no detectable ICAM-1-binding activity (not shown). The expression results are consistent with the phenotype of patient 14, who had a moderate deficiency that resulted from two mutant alleles, only one of which has been sequenced. There was no expression in COS cells of α or β epitopes when β subunit with the G169R mutation was cotransfected with the α subunit (not shown). Reversion of these mutant cDNAs to wild type by site-directed mutagenesis restored expression in COS cells. These results confirmed that the changes found in LAD cDNAs can account for the LAD phenotype.

Wild-type β subunit cDNA expressed in EBV-transformed B-LCL derived from LAD patients using an episomally replicating EBV-based vector rescued expression of the LFA-1 complex (Table 1) (M.L. Hibbs et al., in prep.). The LFA-1 was functional, since these cells showed strong phorbol-ester-stimulated adhesion to ICAM-1-coated plates (Table 1 and see below). The ability to rescue LFA-1 expression in LAD patient-derived cell lines provides conclusive evidence that the defect is in the β_2 subunit, a model for future gene therapy, and a powerful system in which to analyze the function of in vitro mutagenized β subunits.

ICAM-1

ICAM-1 is a single-chain glycoprotein of about 90 kD with a deglycosylated size of 55 kD. The identity of ICAM-1 as an LFA-1 counterreceptor was proven by binding of LFA-1$^+$ cells to purified ICAM-1 with

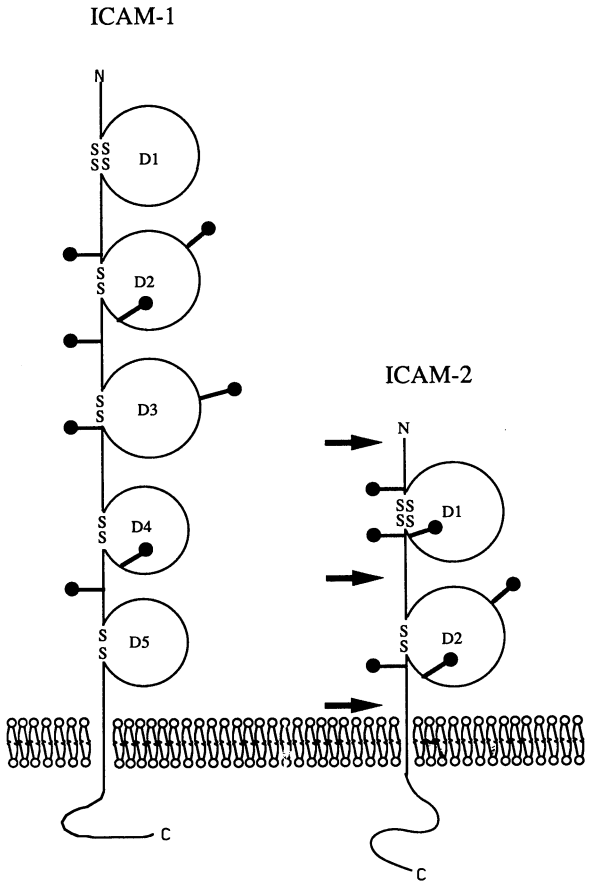

Figure 5. Schematic representation of ICAM-1 and ICAM-2. Loop motifs represent Ig-like domains. The number of SS pairs in each loop represents the number of predicted disulfide bridges between cysteine residues/domain. Domains 4 and 5 of ICAM-1 are missing a β strand present in the other domains. The arrows indicate the positions of exon boundaries determined by genomic cloning of ICAM-2. Each Ig domain is in a single exon.

Table 1. Transfection of LAD Patient B-lymphoblastoid Cells with β Subunit cDNA Restores LFA-1 Surface Expression and Function

LFA-1 Expression (mean fluorescence)		Binding to ICAM-1 substrates (%) Expt. 1	Expt. 2
Control	82	48 ± 2	78 ± 2
Patient 2	1	1.3 ± 0.1	
Patient 2 + β	53	30 ± 2	
Patient 12	0		2.8 ± 0.5
Patient 12 + β	44		31 ± 3

LFA-1 expression was determined by indirect staining and immunofluorescence flow cytometry. The mean fluorescence is given in linear units. Binding of chromium-labeled cells to LFA-1 adsorbed to plastic was performed as in Methods.

specific inhibition by LFA-1 monoclonal antibody (Marlin and Springer 1987). ICAM-1 is a member of the immunoglobulin superfamily and was the first member of this family to be shown to interact with an integrin (Simmons et al. 1988; Staunton et al. 1988). As shown by Greve et al. (1989) and confirmed by Staunton et al. (1989b), ICAM-1 also is the receptor for the major group of rhinoviruses, which are responsible for about 50% of common colds. Coordinate effects of ICAM-1 monoclonal antibody on LFA-1 binding and rhinovirus binding suggest similar interaction sites (Staunton et al. 1989b). Deletion studies were undertaken to localize the LFA-1 and rhinovirus-14 binding sites of ICAM-1 to the smallest region this approach would allow. Sequence homologies predict that ICAM-1 is folded into 5 Ig-like domains (Fig. 5). ICAM-1 with 1 or 2 domains deleted by oligonucleotide-directed mutagenesis was expressed in COS cells (D.E. Staunton et al., in prep.). ICAM-1 constructs with domain 3 or domains 4 + 5 deleted are well expressed, based on immunofluorescence, and are functional in binding to LFA-1 and rhinovirus 14 (Fig. 6). The low rhinovirus-14 binding to the ICAM-1 domain 4 + 5 deletion may be due to decreased distance of the binding domains from the cell membrane, leading to greater steric hindrance from the glycocalyx. However, this binding is significant, suggesting that this construct does retain the binding site for the rhinovirus 14. On the basis of these data, the LFA-1 and rhinovirus-14 binding site on ICAM-1 binding site can be localized to domain 1 and/or 2.

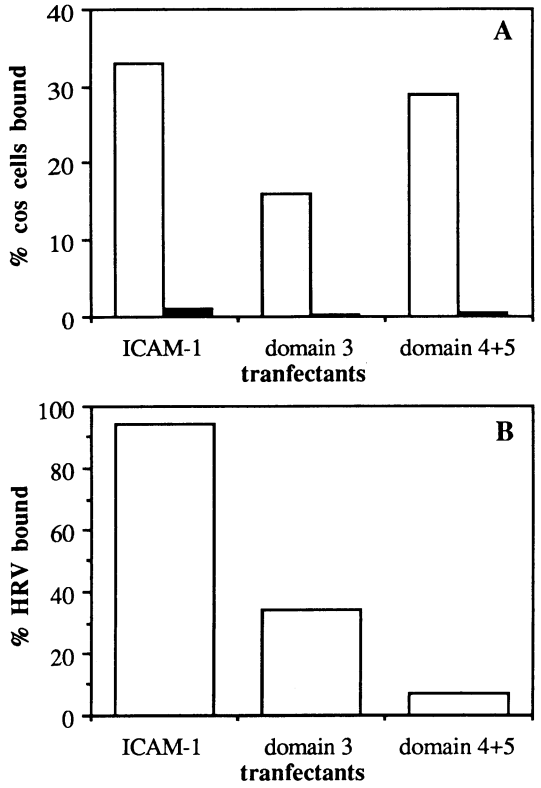

Figure 6. Binding of COS cell transfectants expressing ICAM-1 and ICAM-1 deletion mutants to LFA-1 on plastic. Forms of ICAM-1 with deletions between residues 185 and 284 (domain 3^-) and residues 284 and 489 (domain $4 + 5^-$) were generated by oligonucleotide-directed mutagenesis. (*A*) Binding of ^{51}Cr-labeled cells was measured in the presence (filled bars) and absence (open bars) of 10 μg/ml ICAM-1 MAb. (*B*) Binding of ^{35}S-labeled rhinovirus 14 (HRV) was performed as described previously (Staunton et al. 1989b). COS cell and rhinovirus binding is normalized for variations in COS cell expression of ICAM-1 forms using mean linear fluorescence intensity determined by indirect immunofluorescence flow cytometry with the RR1/1 MAb.

Identification of ICAM-2

Recently, a second LFA-1 counterreceptor, ICAM-2, has been cloned using functional screening in COS cells (Staunton et al. 1989a). A cDNA was isolated that conferred on COS cells the ability to bind to purified LFA-1 coated on petri dishes in the presence of ICAM-1 monoclonal antibody. ICAM-2 is smaller than ICAM-1 with two predicted Ig-domains that are 34% homologous to the two most amino-terminal domains of ICAM-1 (Fig. 5). Although ICAM-1 and ICAM-2 might appear to account for all of the functional data on LFA-1 counterreceptors, the existence of other counterreceptors cannot be ruled out. The ability of ICAM-2 to bind LFA-1 while having only two Ig-like domains is consistent with mapping of the LFA-1-binding site of ICAM-1 to domains 1 and 2. Genomic cloning of ICAM-2 (J. Garcia-Aguilar and T.A. Springer, in prep.) shows that each of the predicted Ig-like domains of ICAM-2 is encoded in a separate exon (Fig. 5), as has been found for most other members of the Ig superfamily. Other members, such as NCAM, have two exons per domain.

Regulation of the LFA-1/ICAM-1 Adhesion Pathway

Understanding regulation of the LFA-1/ICAM adhesion mechanism is crucial to understanding how adhesion receptors can participate in antigen-specific interactions in the immune system. Early experiments with peritoneal exudate cytotoxic T lymphocytes (CTL) demonstrated a fivefold preference of CTL to bind to specific antigen-expressing targets rather than to an identical cell type not expressing the appropriate antigen (Berke and Levey 1972). Prior to knowledge of adhesion receptors, it was suggested that this adhesion was mediated by the TCR binding to specific antigen on target cells. Generation of function-blocking monoclonal antibody to CTL led to discovery of the "accessory molecules" LFA-1 and Lyt-2, which were proposed to act with antigen receptors to mediate adhesion (Springer et al. 1982). It was subsequently speculated that LFA-1 might be involved in a TCR-regulated "adhesion strengthening" step (Martz et al. 1983). However, this speculation received little acceptance because no experimental evidence in support of it was obtained. Quite the opposite idea became accepted—that interaction mediated by LFA-1 preceded antigen

recognition based on studies using cloned human CTL, which showed that these cells adhere strongly to a wide range of target cell types regardless of antigen recognition (Shaw et al. 1986; Spits et al. 1987). These results are at odds with the numerous results obtained with murine CTL (Martz 1987). We believe that this discrepancy may be related to the manner in which the human CTL were maintained in vitro or the time period after restimulation at which they were used. Both murine and human CTL are able to recycle (kill multiple targets); they must be able to "de-adhere" from targets. Therefore, we examined the possibility that the LFA-1/ICAM adhesion mechanism could be involved in antigen-specific adhesion and de-adhesion.

We became interested in the question of LFA-1 regulation when it was observed by us and other investigators that phorbol-ester-stimulated leukocyte homotypic adhesion was dependent on LFA-1 (Patarroyo et al. 1985; Rothlein and Springer 1986). The first issue we addressed was whether LFA-1 avidity, ICAM avidity, or some general cellular property, such as ability to spread on substrates, was modulated by phorbol ester activation. An ideal system in which to study this question is binding of cells that express both LFA-1 and ICAMs to purified LFA-1 or purified ICAM-1 on inert surfaces. These reciprocal studies allowed the effects of phorbol esters or other stimuli on single cell-surface adhesion receptors to be determined. The SKW3 T lymphoma, the JY B-LCL, and resting T cells adhere to ICAM-1-coated substrates very poorly, if at all (Fig. 7A,C). In contrast, binding of the same cells to LFA-1 is highly efficient (Fig. 7B,D), as was observed in the planar membrane system. This result alone suggests a difference in the nature of cell-surface ICAM-1 and LFA-1: The former is constitutively active on most cells, whereas the latter is not. Addition of phorbol ester resulted in a dramatic increase in adhesion of all three cell types to ICAM-1, but no change or a twofold shift in the concentration dependence of binding to LFA-1. This result clearly demonstrates that the activity of cell-surface LFA-1 is profoundly regulated by phorbol esters, but the activity of cell-surface ICAMs is not. The shift in the LFA-1 density-dependence of binding of JY or SKW3 cells in the presence of phorbol myristic acetate (PMA) was correlated with increased spreading, which may be related to a nonspecific effect of PMA on cell deformability. It is also clear that ICAMs expressed on SKW3 or resting T cells (ICAM-2?) as well as ICAM-1 expressed on JY cells do not show avidity regulation, but it is not known at this point whether cell-surface LFA-1 avidity for ICAM-2 is regulated as it is for ICAM-1. Purified ICAM-2 will be required for these studies.

TCR Regulation of LFA-1 Avidity

Is regulation of cell-surface LFA-1 avidity physiologically relevant to antigen-specific T lymphocyte cell–cell interactions? The ability of TCR ligation to trigger increased LFA-1 avidity was tested. TCR cross-linking on resting T cells with mouse CD3 monoclonal antibody and anti-mouse Ig is required to obtain a rapid increase in phosphatidylinositol (PtdIns) turnover and intracellular Ca^{++} (Lerner et al. 1988). Resting T cells were used, since they had low basal adhesion to ICAM-1 and are clearly a relevant model for cells taking part in the initiation of immune responses. CD3 monoclonal antibody of the IgG subclass alone had no effect on adhesion to ICAM-1, but addition of anti-Ig caused a dramatic increase in T-cell adhesion to ICAM-1 (Fig. 7C). In contrast, there was no effect of TCR cross-linking on binding to LFA-1 (Fig. 7D). This process had specificity for CD3, since cross-linking a CD5 monoclonal antibody, which bound to the same number of sites per cell, induced only a twofold increase in binding (not shown). The TCR-triggered adhesion to ICAM-1 was completely blocked by LFA-1 monoclonal antibody or ICAM-1 monoclonal antibody (not shown).

Does the effect of TCR cross-linking on LFA-1 avidity occur through direct communication or signaling pathways? TCR-mediated signaling, including PtdIns-turnover and tyrosine phosphorylation, are blocked by elevation of cytoplasmic cAMP prior to receptor cross-linking (Kaibuchi et al. 1982; Klausner et al. 1987; Lerner et al. 1988). Treatment of resting T cells with the cell-permeable cAMP analog, dibutyryl cAMP (dbcAMP), prior to TCR cross-linking strongly inhibited stimulation of binding to ICAM-1 substrates. A similar effect was seen with the adenylate cyclase activator forskolin (25 μM) in combination with the phosphodiesterase inhibitor IBMX (0.5 mM) (not shown). However, dbcAMP or forskolin/IBMX had no effect on PMA-stimulated adhesion (Fig. 7C), indicating that inhibition of the TCR-stimulated adhesion was not due to toxicity. This suggests that the TCR effect on LFA-1 occurs via cytosolic second messengers and that LFA-1 is a transducer of signals from the cytosol to the extracellular space; i.e., inside-out signaling.

The kinetics of the TCR-stimulated increase in LFA-1 avidity was examined with the hope that its reversal could provide a mechanism for the de-adhesion of antigen-specific T lymphocytes. Strikingly, when anti-Ig was added to CD3 monoclonal-antibody-treated resting T cells in suspension and variable amounts of time were allowed to elapse before centrifuging cells onto ICAM-1 substrates, adhesion to ICAM-1 peaked at 10 minutes and decreased to basal levels by 30 minutes (Fig. 7E). In contrast, cells treated with PMA in suspension remained in the high LFA-1 avidity state for at least 40 minutes. There was no change in LFA-1 density at 0, 5, or 30 minutes after cross-linking as determined with fluorescein isothiocyanate (FITC)-LFA-1 monoclonal antibody and immunofluorescence flow cytometry (not shown). Furthermore, cells treated with CD3 monoclonal antibody and anti-Ig for 30 minutes and then treated with phorbol ester again adhered efficiently to ICAM-1, suggesting that 30 minutes after TCR cross-linking, the adhesion machinery was still intact but was no longer activated (not shown).

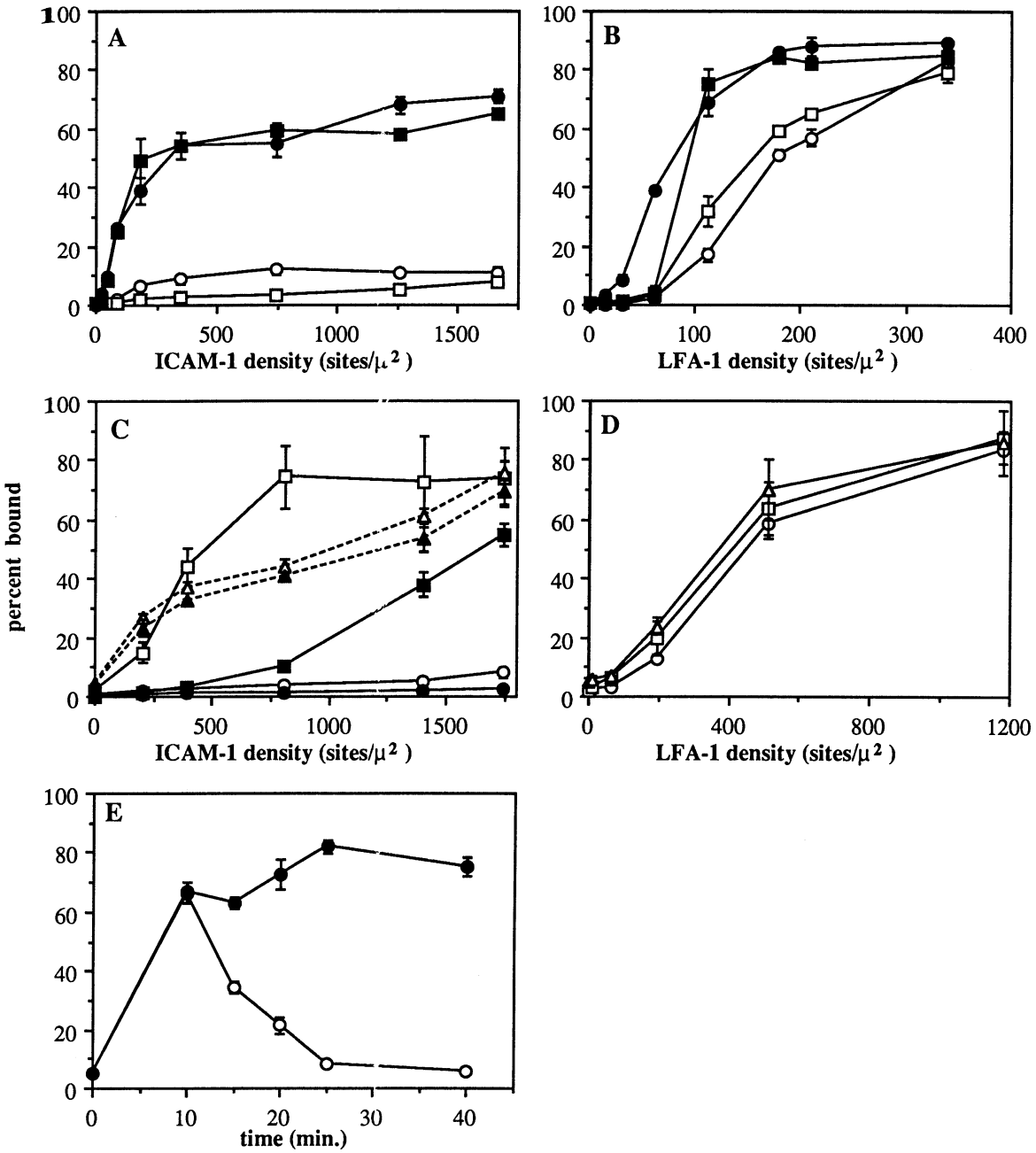

Figure 7. Effect of activation on binding of lymphocytes to ICAM-1 or LFA-1. Binding of JY cells (circles) or SKW3 cells (squares) to ICAM-1 (*A*) or LFA-1 (*B*) on plastic at indicated density in the absence (open symbol) or presence (filled symbol) of 50 ng/ml PMA. Resting T-lymphocyte binding to ICAM-1 (*C*) or LFA-1 (*D*) with no addition (circles), 50 ng/ml PMA (triangles), or mouse CD3 MAb and anti-mouse IgG (squares) all in the absence (open symbols) or presence (filled symbols) of 1 mM dbcAMP. (*E*) Kinetics of PMA or CD3 MAb-stimulated binding to ICAM-1 (1000 sites/μ^2). PMA or anti-mouse IgG was added to untreated or CD3 MAb-pretreated cells in suspension, respectively, and the cells were centrifuged onto the plate at the indicated period of time. Included in the time shown is the 5-min centrifugation and 6-min incubation on the ICAM-1 substrate.

Does TCR cross-linking have a similar effect on cell–cell adhesion in the presence of other adhesion pathways? Furthermore, are other adhesion mechanisms regulated by the TCR? These are important questions, since the interaction of cloned CTL with target cells is not only dependent on the LFA-1/ICAM mechanism, but also on the CD2/LFA-3 adhesion mechanism. Conjugate formation between resting T lymphocytes and LFA-1$^-$ B-LCL was determined by two-color fluorescence flow cytometry (Luce et al. 1985). In this system, a low level of spontaneous conjugate formation was observed (Fig. 8) in contrast to the high level (40–80%) observed with cloned CTL adhering to B-LCL targets. The efficiency of conjugate formation was

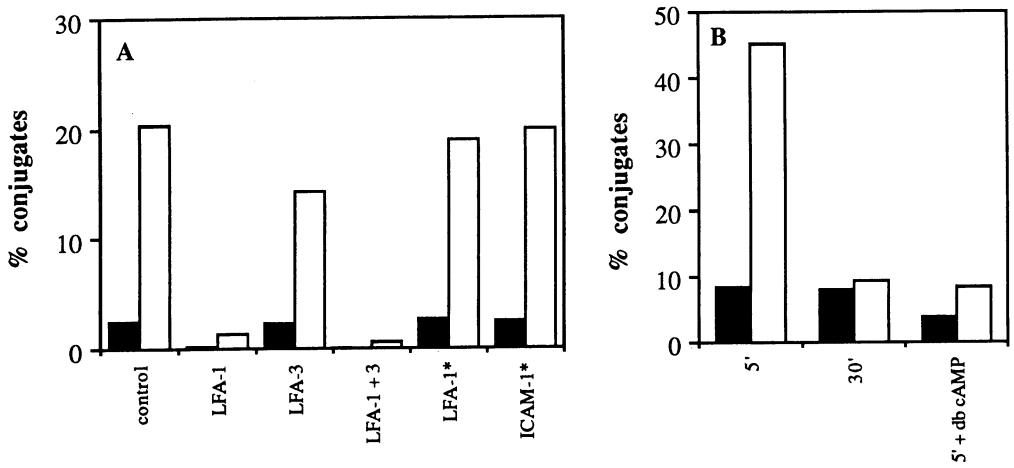

Figure 8. Conjugate formation between resting T lymphocytes and B-LCL. (*A*) Resting T lymphocytes labeled with sulfofluorescein diacetate were preincubated without (filled bars) or with (open bars) OKT3 CD3 MAb at 4°C and washed three times. Pretreated T lymphocytes (1.7×10^6) were allowed to co-sediment with hydroethidine-labeled B-LCL (3.3×10^6) in 50 μl for 1 hr at 4°C in the presence of 5 μg/ml anti-mouse IgG2a and the indicated IgG1 MAb. The pellet was incubated at 37°C for 6 min, resuspended by vortexing, and analyzed by fluorescence flow cytometry. (*B*) Similar to above, except T and B cells were co-centrifuged 20*g* for 5 min at 24°C. Resting T cells were pretreated 30 min at 37°C with anti-mouse IgG2a or 15 min at 24°C with 1 mM dbcAMP where indicated. The centrifugation was needed to allow kinetic study and study of dbcAMP effect under conditions used for binding to purified adhesion receptors.

dramatically increased by cross-linking the TCR, and the stimulated adhesion was completely inhibited by LFA-1 monoclonal antibody but only partially inhibited by CD2 or LFA-3 monoclonal antibody (Fig. 8A), and showed the same transience as adhesion to substrate with basal adhesion 30 minutes after cross-linking (Fig. 8B). TCR-stimulated cell–cell adhesion was also inhibited by dbcAMP. Thus, regulation of LFA-1 avidity is distinct from regulation of CD2 (Selvaraj et al. 1987). Furthermore, these results show that the LFA-1 adhesion mechanism has a prominent role in regulating interactions with targets expressing both ICAM-1 and LFA-3.

DISCUSSION

The current model for structure of LFA-1 and other integrins is not highly refined, but homologies, analysis of mutations affecting subunit association, and chemical cross-linking studies with RGD peptides point to potentially important regions of α and β subunits. The most striking features of the α subunit are the I domain and the putative divalent cation repeats. The I domain may be a relatively independent folding unit, based on its appearance in many different contexts. It is attractive to speculate that the I domain may have a critical role in binding to counterreceptors; this is consistent with the ligand-binding role of the homologous "A" domains in vWF. However, the general requirement for divalent cations in all integrin-mediated interaction suggests that the putative metal-binding repeats may have an important role in binding or in maintaining an active conformation of other sites (Hynes 1987). Direct binding of divalent cations to two other integrins has been demonstrated previously (Gailit and Ruoslahti 1988). The most direct evidence for the function of putative divalent cation-binding sites in LFA-1 is the ability of Mg^{++} and Ca^{++} to stabilize LFA-1 subunit interactions at high pH (this paper and M.L. Dustin and T.A. Springer, in prep.). This explains the Mg^{++} requirement of LFA-1/ICAM interaction. The identification of two point mutations that can cause the LAD phenotype in a highly conserved region of the β subunit suggests that the region of β implicated in RGD binding by photoaffinity labeling (D'Souza et al. 1988) may also be a critical region of the β subunit for contact with α. This is consistent with α and β coming together to form a binding pocket, the conformation of which may be profoundly affected by divalent cations bound to the α subunit. The conformation of this hypothetical pocket could also be a target for regulation of LFA-1 avidity by phorbol esters or TCR signaling. Systems to express in vitro mutated α and β subunits are available to test these hypotheses.

ICAM-1 structure is more easily modeled, since ICAM-1 is homologous to immunoglobulins, for which high-resolution crystal structures are available. The deletion studies suggest that the minimal unit for expression and function comprises the two amino-terminal Ig-like domains. This is supported by the two-domain structure of ICAM-2.

We have succeeded in resolving LFA-1- and ICAM-mediated adhesion and have strong evidence that LFA-1 avidity for ICAM-1 is regulated. Adhesion of lymphocytes to purified ICAM-1 adsorbed to plastic was dramatically increased by phorbol ester treatment, whereas adhesion of the same cell types to purified LFA-1 on plastic was slightly affected by phorbol ester treatment, the most dramatic effect being an increase in cell spreading. A role of LFA-1 avidity regulation in

antigen-specific interactions was revealed by TCR cross-linking, which increases LFA-1 avidity without affecting cell-surface ICAMs or CD2, the latter in a cell–cell adhesion system. The TCR stimulation of LFA-1 avidity appeared to be mediated by intracellular signals, since it was blocked by dbcAMP or agents that increase cytoplasmic cAMP by endogenous mechanisms. A striking characteristic of the TCR-stimulated increase in LFA-1 avidity is that it peaks at 5–10 minutes after cross-linking and returns to basal levels by 30 minutes. This transience is consistent with the ability of T lymphocytes to de-adhere from antigen-presenting cells or target cells expressing specific antigens. The kinetics of the increase and subsequent decrease in LFA-1 avidity is very similar to the kinetics of CTL/target interactions (Martz 1977; Poenie et al. 1987). The kinetics of LFA-1 avidity changes and adhesion may be influenced by the extent of TCR cross-linking and the density of ICAMs. In this regard, lymphokines generated by T cells are potent up-regulators of ICAM-1 on several cell types (Dustin et al. 1988) and such that products of T-cell activation could influence adhesion kinetics over the course of an immune response.

Regulation of LFA-1 avidity by the TCR provides a mechanism for adhesion molecule function within the context of specific immune recognition. We would propose that LFA-1 is in a low-avidity state or inactive on resting T lymphocytes. When T lymphocytes encounter antigen-expressing target cells, ligation of the TCR results in conversion of LFA-1 to a high-avidity state leading to strong adhesion. A subsequent change in a second-messenger profile may cause the return of LFA-1 to the low-avidity state allowing de-adhesion.

The mechanism by which LFA-1 avidity is regulated by PMA or the TCR is not known but should be attacked from many angles in the next few years. A change in LFA-1 conformation or redistribution in the membrane seems most likely. A striking property of cell-surface LFA-1-dependent adhesion is the requirement for elevated temperature and energy, which is not seen for cell-surface ICAMs (above and not shown). We propose that the conversion of LFA-1 from the low- to high-avidity states triggered by the TCR requires input of metabolic energy. Phosphorylation of the cytoplasmic domain of LFA-1, or of another protein with which it interacts, may be the switch regulated by the TCR. The energy input, utilized to drive the equilibrium between adherence and nonadherence toward adherence, may be analogous to the energy used to drive an otherwise unfavorable biochemical reaction. Avidity regulation is not unique to LFA-1. Another well-characterized example is the integrin gpIIbIIIa on platelets, which does not bind fibrinogen on inactivated platelets but shows high-affinity binding (29–45 mM K_d) after treatment of platelets with ADP (Di Minno et al. 1983). Another potential example of transient avidity regulation is stimulation of Mac-1-dependent binding of neutrophils to bind iC3b opsonized erythrocytes, endothelial cells, or each other by PMA or chemotactic peptides, which has similar kinetics to the increase in LFA-1 avidity reported here (Wright and Meyer 1986; Buyon et al. 1988; Lo et al. 1989). However, these experiments are less well controlled than those described here, since stimulation of neutrophil aggregation may also involve up-regulation of the counter-receptor, which is not defined. Mac-1 is present in an intracellular pool in monocytes and granulocytes, which may be up-regulated or exchanged with cell-surface Mac-1 (there is no intracellular pool of LFA-1 in lymphocytes), and PMA has drastic effects on monocytes and granulocytes, resulting in strong activation of the respiratory burst, degranulation, vacuolation, and proteolytic removal of surface proteins (Wright and Meyer 1986; Huizinga et al. 1988). Phorbol esters induce transient effects in neutrophils while producing sustained effects in lymphocytes; it is possible that transience in neutrophils reflects mechanisms different from those defined here. Mac-1 has been shown to cluster in the plane of the membrane upon stimulation with kinetics similar to those of iC3b binding (Detmers et al. 1987). One possible mechanism for avidity increase and clusterings for integrins is interactions with cytoskeletal proteins such as talin (Burridge and Connell 1983). In fact, talin has been shown to colocalize with LFA-1 to sites of adhesion in antigen-specific helper T-lymphocyte interactions with B cells, suggesting that this putative interaction may be regulated by the TCR (Kupfer and Singer 1989). However, this redistribution has not been correlated with a change in LFA-1 avidity or the efficiency of adhesion. It is intriguing that talin/LFA-1 co-redistribution was more sensitive to antigen dose than the reorientation of the Golgi apparatus and microtubule organizing center, which are associated with directed lymphokine secretion by helper T cells and lethal hit delivery by CTL.

We have defined the primary structure of the adhesion receptor LFA-1 and its counterreceptors ICAM-1 and ICAM-2. We also present a mechanism by which these molecules can be used to dynamically regulate lymphocyte adhesion. The inside-out signaling through LFA-1 may be a general capability of integrins and could be of general importance in regulating cell–cell and cell–matrix interactions during development or tissue remodeling. TCR stimulation of T lymphocytes led to a temporal gradient of LFA-1 activity. The same mechanisms acting at different parts of a cell could generate spatial gradients of adhesion receptor avidity. In the model studied here, the TCR was ligated all over the cell with a soluble monoclonal antibody, whereas in the physiological interactions, TCR would be engaged only at the site of cell contact, and avidity enhancement might only apply to LFA-1 molecules in or recruited to that area, generating a spatial gradient of LFA-1 avidity. Intrinsic or chemotactic factor-driven spatial gradients of integrin avidity from high at the leading edge of a cell to low at the trailing edge could provide a means for de-adhesion at the trailing edge (Bretscher 1988) and could drive cell migration.

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