Involvement of the "I" Domain of LFA-1 in Selective Binding to Ligands ICAM-1 and ICAM-3

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Abstract. To analyze the binding requirements of LFA-1 for its two most homologous ligands, ICAM-1 and ICAM-3, we compared the effects of various LFA-1 activation regimes and a panel of anti-LFA-1 mAbs in T cell binding assays to ICAM-1 or ICAM-3 coated on plastic. These studies demonstrated that T cell binding to ICAM-3 was inducible both from the exterior of the cell by Mn^{2+} and from the interior by an agonist of the "inside-out" signaling pathway. T cells bound both ICAM ligands with comparable avidity. A screen of 29 anti-LFA-1 mAbs led to the identification of two mAbs specific for the α subunit of LFA-1 which selectively blocked adhesion of T cells to ICAM-3 but not ICAM-1. These two mAbs, YTH81.5 and 122.2A5, exhibited identical blocking properties in a more defined adhesion assay using LFA-1 transfected COS cells binding to immobilized ligand. Blocking was not due to a steric interference between anti-LFA-1 mAbs and N-linked carbohydrate residues present on ICAM-3 but not ICAM-1. The epitopes of mAbs YTH81.5 and 122.2A5 were shown to map to the I domain of the LFA-1 α subunit. A third I domain mAb, MEM-83, has been previously reported to uniquely activate LFA-1 to bind ICAM-1 (Landis, R. C., R. I. Bennett, and N. Hogg. 1993. *J. Cell Biol.* 120:1519–1527). We now show that mAb MEM-83 is not able to stimulate binding of T cells to ICAM-3 over a wide concentration range. Failure to induce ICAM-3 binding by mAb MEM-83 was not due to a blockade of the ICAM-3 binding site on LFA-1. This study has demonstrated that two sets of functionally distinct mAbs recognizing epitopes in the I domain of LFA-1 are able to exert differential effects on the binding of LFA-1 to its ligands ICAM-1, and ICAM-3. These results suggest for the first time that LFA-1 is capable of binding these two highly homologous ligands in a selective manner and that the I domain plays a role in this process.

THE integrin lymphocyte function-associated antigen (LFA)[1]-1 mediates adhesion events between leukocytes and a variety of other cell types which express one or more of its ligands, namely intercellular adhesion molecule (ICAM)-1 (Simmons et al., 1988; Staunton et al., 1988), ICAM-2 (Staunton et al., 1989), and ICAM-3 (Fawcett et al., 1992; Vazeux et al., 1992; de Fougerolles et al., 1993). The ICAM ligands are members of the immunoglobulin superfamily (IgSF), with ICAM-1 and ICAM-3 displaying particular similarity. Both are composed of five extracellular C2-like Ig domains and exhibit ~50% amino acid identity rising to 77% in the second domain. LFA-1 is a typical integrin composed of a unique α subunit (CD11a) (Larson et al., 1989) and a common β_2 subunit (CD18) (Kishimoto et al., 1987; Law et al., 1987). The α subunit contains a 197-amino acid "inserted" (or "I") domain located between repeated domains 2 and 3. A closely related I domain is present in the other two β_2-type integrins, Mac-1 and p150,95, and also two members of the β_1 integrins, very late antigen (VLA)-1 and VLA-2 (Corbi et al., 1987, 1988; Ignatius et al., 1990; Takada and Hemler, 1989). Comparisons of the intron/exon boundaries of p150,95 with the platelet integrin GPIIbIIIa suggest that the I domain has been inserted as an evolutionarily conserved unit into the basic integrin structure (Corbi et al., 1990; Heidenreich et al., 1990). Similar domains are present in a number of unrelated proteins and the integrin I domains are now recognized as belonging to a larger family of so-called type "A" domain proteins (for review see Colombatti and Bonaldo, 1991).

LFA-1 mediates leukocyte adhesion events at diverse stages of the immune response, including adhesion to endothelium and extravasation into inflamed tissues, priming

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1. *Abbreviations used in this paper*: APC, antigen-presenting cells; ICAM, intercellular adhesion molecule; IgSF, immunoglobulin superfamily; LFA, lymphocyte function-associated antigen; LIB, ligand-induced binding; PdBu, phorbol dibutyrate; VLA, very late antigen.

of T cells by antigen presenting cells (APCs) and killing of target cells by effector T cells (for review see Harvey et al., 1993). The details of which ICAM ligand is used at each of these steps is still unclear, but it is currently thought that ICAMs-1 and -2 are principally required for leukocyte adhesion to endothelium, whereas ICAMs-1 and -3 are involved in formation of APC:T cell couples. During the APC:T cell interaction ICAM-1 appears to be the favored ligand on memory T cells whereas ICAM-3, which is expressed at higher levels on resting T cells, may be initially favored during early stages of primary antigenic responses (de Fougerolles and Springer, 1992).

LFA-1 is not constitutively avid for ICAM-1 (Dustin and Springer, 1989; van Kooyk et al., 1989) but rapidly acquires high avidity binding in response to one of three basic types of signals: (1) agonists that trigger a so-called "inside-out" signal in leukocytes, which include phorbol esters or mAbs against other surface receptors, such as CD3, CD2, and CD31 (for review see Pardi et al., 1992); (2) changes in the extracellular concentrations of the divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} (Dransfield et al., 1992; Dransfield and Hogg, 1989); and (3) special "activating" anti-LFA-1 mAbs which directly induce the ligand-binding conformation on the receptor (Andrew et al., 1993; Landis et al., 1993; Robinson et al., 1992; Keizer et al., 1988). The activation epitope for one of these mAbs, MEM-83, has been mapped and shown to localize to the I domain, thereby suggesting a regulatory role for this domain in LFA-1 activation. Support for such a role has been provided by the finding that a novel Mac-1 "activation-reporter" epitope is also located in the I domain (Diamond and Springer, 1993). No information is available on the potential role of the I domain in the binding of ICAM-2 or ICAM-3 by LFA-1.

Stable interaction of LFA-1 with ICAM-1 has been shown to be dependent not only upon stimulation of LFA-1 by agonist but also by prior ligation with ICAM-1, referred to as the "ligand induced binding" (LIB) interaction, which represents a necessary step leading to the full activation of LFA-1 (Cabañas and Hogg, 1993). A similar event has been identified in the integrin GPIIbIIIa, in which case stable binding of ligand fibrinogen was shown to be dependent upon a prior interaction with the RGD motif contained within fibrinogen (Du et al., 1991). These findings suggest that a common feature of integrin activation may be participation by ligand in acquisition of the high avidity–binding conformation.

In the present study we have examined whether LFA-1 is able to bind selectively to its most homologous ligands, ICAM-1 and ICAM-3. We demonstrate that two functionally distinct sets of anti-LFA-1 mAbs recognizing epitopes in the I domain are able to exert differential effects on the binding of T cells or LFA-1-transfected COS cells to immobilized ICAM-1 and ICAM-3. These results therefore suggest for the first time that LFA-1 is capable of binding selectively to ICAMs-1 and -3 and that the I domain has a role in this process.

Materials and Methods

Preparation of ICAM-1 and ICAM-3–coated Plates

ICAM-1 and ICAM-3 fusion proteins, consisting of the first two extracellular domains fused to a human IgG1 Fc fragment (ICAM-Fcs) have been previously described (Fawcett et al., 1992). Each preparation of protein A–Sepharose purified ICAM-Fc was checked for purity by SDS-PAGE analysis and found to migrate as a single band under reducing conditions of 70 kD in the case of ICAM-1 and a diffuse band of between 75–80 kD in the case of ICAM-3. ICAM-3-Fc was deglycosylated at 100 μg/ml in buffer containing 20 mM phosphate, pH 8.0 plus 25 mM EDTA by sequential incubations for 20 and 16 h at 37°C in the presence of 4 and 2 U/ml N-Glycosidase F (Boehringer-Mannheim, UK). For T cell adhesion assays, ICAM-Fcs were added at 500 ng/well in PBS to Immulon 1 96-well plates (Dynatech Laboratories Inc., Chantilly, VA). For LFA-1/COS cell assays, ICAM-Fcs were added at 500 ng/well to Immulon 3 plates precoated with 1 μg/well goat-anti-human-Fc Ig (Sigma Chemical Company Ltd., Dorset, U.K.). Plates were coated overnight at 4°C and non-specific sites were blocked before the adhesion assay with 2.5% BSA (Fraction V, Sigma) in PBS for 2 h at room temperature. All ICAM-Fc constructs were coated to plates at saturating levels, as determined by ELISA and T cell binding assays.

Monoclonal Antibodies

Anti-LFA-1 α subunit mAbs MEM-83 and MEM-94 were generously provided by Dr. V. Horejsi (Prague, Czech Republic), mAb 122.2A5 by Dr. R. Vilella (Barcelona, Spain) and mAb YTH81.5 by Serotec (Oxford, U.K.) (Bazil and Horejsi, 1990; Schmidt, 1989). Anti-ICAM-1 mAb RR1/1 was a gift from Dr. R. Rothlein (Ridgefield, CT) (Rothlein et al., 1986). The remaining mAbs, obtained from Dr. Reinhold Schmidt, were from the Leukocyte Typing Workshop IV (Schmidt, 1989) and included the anti-CD11a mAbs MHM24, 122.2A5, MEM-95, MEM-30, MEM-25, 2F12, F110.22, GRS3, HI111, M10, 0501, 25.3.1, 459, YTH 81.5, 1524, TMD3-1, CC5 1D7, and the anti-CD18 mAbs H-52, MHM23, TS1/18, M232, IC11, CLB-54, IB-4, YFC 51.1, YFC 118.3, and GRF1. Purified anti-CD11a mAbs MEM-83, 38 and YTH81.5 were used in T cell adhesion assays at final concentrations of 2–5 μg/ml and in immuno-precipitation assays at 30 μg/ml. mAb 122.2A5 was used as ascitic fluid diluted 1:300 in T cell adhesion assays and at 1:50 in immunoprecipitation assays. Purified anti-ICAM-1 mAb RR1/1 was used in T cell binding assays at 10 μg/ml and the immunoprecipitation negative control CD8 mAb 14 was used at 30 μg/ml. All other CD11a and CD18 mAbs were used as ascitic fluids in T cell adhesion assays at saturating concentrations ranging between 1:100 and 1:1,000 dilutions depending on FACS titre.

T Cell Adhesion Assay

Human T cells were expanded in culture from unstimulated peripheral blood mononuclear cells and served as a model for inducible adhesion to immobilized ICAM-1 as previously described (Dransfield et al., 1992). Briefly 5×10^7 cultured T cells were labeled with 25 μCi [^{3}H]methyl thymidine and washed three times in the appropriate assay medium supplemented with 0.2% BSA (Fraction V, Sigma). The medium employed in the binding assay was either RPMI 1640 or a defined Mn^{2+}-containing medium, consisting of 20 mM Hepes, 140 mM NaCl, 2 mg/ml D-glucose, and 400 μM Mn^{2+}. ^{3}H-labeled T cells were added to ICAM-Fc-coated plates in the presence or absence of phorbol dibutyrate (PdBu; 200 nM) at 2.5×10^5 cells/well in a 100-μl vol. Cells were preincubated on ice for 20 min in the presence of saturating titres of anti-LFA-1 mAbs in a 50-μl vol, centrifuged at 30 *g* for 1 min and incubated an additional 30 min at 37°C. ICAM-3 binding was carried out in the presence of mAb RR1/1 (5 μg/ml) to eliminate LFA-1/ICAM-1 dependent clustering of T cells. Cells that remained bound to ICAM-Fc plates after 5 washes with prewarmed RPMI were lysed and the incorporated radioactivity quantitated using a Betaplate counter (LKB Instruments Inc., Bromma, Sweden). Stimulation index is defined as: Cell binding in the presence of agonist (PdBu or Mn^{2+})/binding in the absence of agonist.

LFA-1 Transfection of COS Cells and Adhesion Assay

COS cells were maintained in D-MEM medium supplemented with 10% FCS and passaged at confluence by trypsinization. COS cells were transiently transfected by DEAE Dextran using CD11a and CD18 cDNAs cloned into the expression vector pcDNA/AMP (In Vitrogen, U.K.). Briefly, COS cells were grown to confluence in 15-cm petri dishes and transfected with 50 μg of each cDNA for 2–4 h at 37°C in the presence of 1 mg/ml DEAE dextran, followed by a 2-min shock in 10% DMSO. LFA-1 transfected COS cells were used in adhesion assays 48 h after transfection and were routinely 20% LFA-1 positive by FACScan analysis (Becton Dickinson Immuno-

cytometry Systems, Mountain View, CA). The adhesion assay was performed similarly as described for T cells. Briefly, LFA-1/COS cells were labeled overnight with [^{3}H]methyl thymidine at 25 μCi per 2 $\times$ 10^7 cells and harvested before the adhesion assay by ice-cold PBS supplemented with 2 mM EDTA. Cells were washed three times in assay medium, consisting of RPMI supplemented with 2.5% FCS, and added to ICAM-Fc plates in a 30-μl vol at 3 $\times$ 10^4 cells/well. Cells were preincubated on ice for 20 min in the presence of 50 ng/ml PMA and saturating titres of anti-LFA-1 mAbs in a 25-μl vol, followed by an additional 30-min incubation at 37°C. Cells that remained bound after five washes with prewarmed RPMI were lysed and counted for incorporation of radioactivity as described above.

cDNA Constructs of the LFA-1 α Subunit

The construction of the CD11a deletion series used for epitope mapping has been previously described (Landis et al., 1993). The "nested" series of constructs contained successive domain deletions starting from the NH_2 terminus of the LFA-1 α subunit, with each fragment terminating in domain 7 (designated as fragments N, 2, I, 3 and 4). Thus, the construct spanning from the NH_2 terminus to domain 7 was labeled N, the construct spanning from domain 2 to 7 was labeled 2, and so on. A control fragment, which included the region between the end of domain 7 and the transmembrane domain, was designated C. A previously undescribed fragment, which consisted only of the I domain plus domain 2 (amino acids 71-323), was designated 2-I. PCR amplifications were carried out using a GeneAmp™ DNA amplification kit with Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions.

In Vitro-Translation and Immunoprecipitation

Transcription and translation of the nested CD11a cDNA deletion series was carried out using the one-step TNT™ T7 coupled reticulocyte lysate system (Promega Corp., Madison, WI) according to manufacturer's instructions. 1 μg of cloned DNA in Bluescript™ KS$^+$ phagemid served as the template for the reaction, which was carried out for 2 h at 30°C, in a 50-μl vol in the presence of [^{35}S]methionine (Amersham) and rabbit reticulocyte lysate (50% by volume). After translation, 25 μl of 2× EIA buffer (500 mM NaCl, 100 mM Hepes pH 7.0, 0.2% NP-40) was added to an equal volume of translation product and the total volume of the precipitation made up to 200 μl with 1× EIA buffer. The primary incubation was carried out in the presence of 30 μg purified mAbs at 4°C for 3–5 h, before the addition of 20 μl (packed volume) Sepharose Protein G beads. Immunoprecipitations were incubated an additional 3–5 h at 4°C before harvesting the Sepharose beads by a 30-s microcentrifugation. 10 μl of the supernate was removed for monitoring of the translation efficiency of each fragment by SDS-PAGE. The remaining lysate was discarded and the beads washed three times with 1× EIA buffer plus 0.1% NP-40, and twice with 1× EIA buffer alone. Approximately 25 μl of electrophoresis reducing buffer was added to the beads before SDS-PAGE analysis on a 9% acrylamide separating gel. Gels were treated with ENHANCE™ (New England Nuclear Research Products, Boston, MA) before autoradiography.

Results

Comparative Binding of T Cells to ICAM-1 and ICAM-3

To determine whether ICAM-3 was recognized by LFA-1 in the same regulated manner as ICAM-1, we examined the effect of two LFA-1-activation regimes on T cell binding to immobilized ICAM-1 and ICAM-3. In these experiments, the high affinity conformation of LFA-1 was triggered either from the exterior of the cell by the addition of Mn^{2+} (Dransfield et al., 1992) or from the interior of the cell by the addition of PdBu, an agonist of the inside-out pathway of LFA-1 activation (Rothlein et al., 1986). Fig. 1 shows that ICAM-1 and ICAM-3 supported comparable levels of T cell adhesion stimulated through PdBu or Mn^{2+}. The index of stimulation was also equivalent between the two ligands, with an average stimulation index of 2.9 for ICAM-1 and 3.1 for ICAM-3. These results demonstrate that LFA-1 exhibited

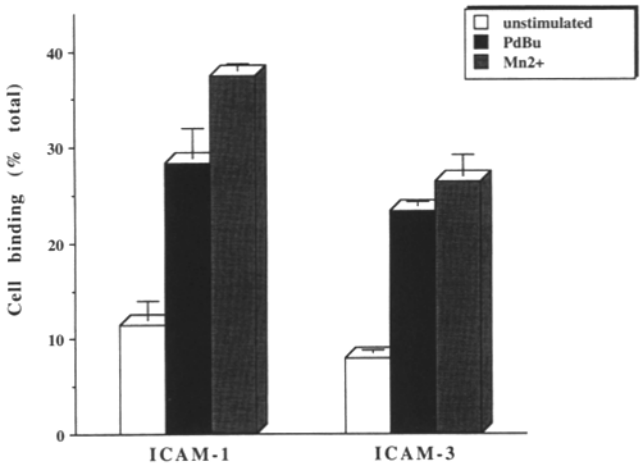

Figure 1. T cell binding to ICAM-3 is induced by phorbol ester or Mn^{2+}. ^{3}H-labeled T cells (2.5 $\times$ 10^5) were added to ICAM-1 or ICAM-3 coated 96-well plates (Immulon 1, Dynatech) and stimulated either by the addition of PdBu in RPMI-1640 medium or by washing cells into Hepes buffered medium containing Mn^{2+}. ICAM-3 binding was carried out in the presence of anti-ICAM-1 mAb RR1/1 in order to eliminate LFA-1/ICAM-1 dependent clustering of T cells. Cell binding was quantitated after a 30-min incubation at 37°C by measuring the radioactivity of the bound fraction of cells. Results are expressed as the average ± SD of triplicate incubations from a single experiment, representative of four similar experiments.

the same basic features of avidity-regulation towards its two most homologous ligands, ICAM-1 and ICAM-3.

Effect of anti-LFA-1 mAbs on Adhesion to ICAM-1 and ICAM-3

To determine whether LFA-1 could recognize ICAM-1 and ICAM-3 differentially, we screened a panel of anti-LFA-1 mAbs (consisting of 19 mAbs against the α subunit and 10 against the β_2 subunit) for blocking effects on T cell binding to either ligand. In this assay, T cells were preincubated with the anti-LFA-1 mAbs indicated in Fig. 2, followed by a 30-min binding step in the presence of PdBu to immobilized ICAM-1 or ICAM-3. Although the blocking footprint of the mAb panel was broadly similar for each of the ligands, the screen identified two mAbs specific for the α subunit of LFA-1 which exhibited differential blocking between the two ligands. These two mAbs, YTH 81.5 and 122.2A5, specifically inhibited T cell binding to ICAM-3 but not ICAM-1 (Fig. 2).

Having screened the panel of mAbs for selective effects on T cell adhesion to ICAMs-1 and -3, we subsequently examined the blocking properties of mAbs YTH81.5 and 122.2A5 in greater detail. To exclude donor variability, Fig. 3 *a* was compiled from eight different donors and confirmed that these mAbs exhibited good blocking of T cells to ICAM-3 compared to a control blocking mAb 38, but no blocking to ICAM-1. Selective blocking was maintained under conditions which raised the affinity of LFA-1, using 3 mM Mg^{2+} and 1 mM EGTA (to chelate Ca^{2+}), alone or in combination with phorbol ester (data not shown). These T cell assays, however, suffered from a potential drawback related to the high levels of expression of both ICAM-1 and ICAM-3

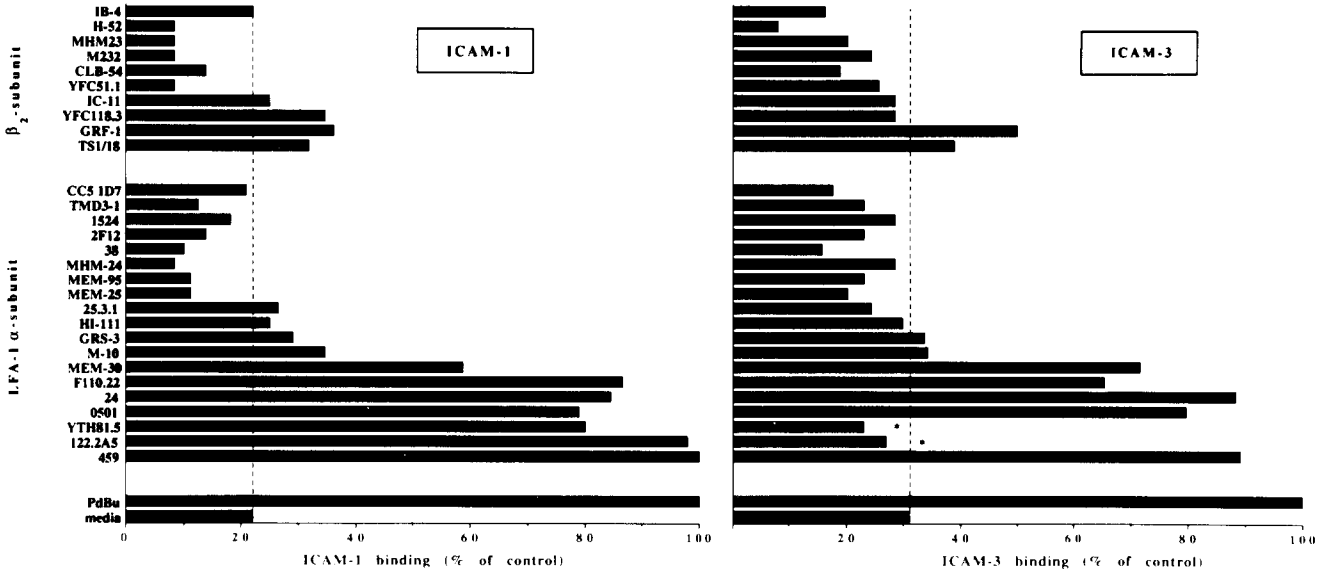

Figure 2. Effect of anti-LFA-1 panel of mAbs on T cell adhesion to ICAM-1 and ICAM-3. T cells were preincubated on ice for 20 min in the presence of PdBu, and saturating doses of the anti-LFA-1 mAbs, specific for the α and β_2 subunits of LFA-1, as indicated. ICAM-1 and ICAM-3 binding assays were carried out as described in the legend to Fig. 1 and results expressed as% adherence relative to control binding in the presence of PdBu only.

on the T cell surface (data not shown). Since PdBu activated T cells have been shown to aggregate via an LFA-1/ICAM-1 dependent pathway (Rothlein et al., 1986), ICAM-3 binding assays were routinely carried out in the presence of a blocking anti-ICAM-1 mAb RR-1/1 to prevent ICAM-1 dependent clustering between cells. This introduced the possibility that the behavior of T cell LFA-1 was being affected by anti-ICAM-1 mAb. Therefore, to completely rule out any involvement of cell surface ICAMs from the assay, we repeated the experiment using COS cells transfected with human CD11a and CD18 cDNAs (LFA-1/COS). Fig. 3 *b* shows that mAbs YTH81.5 and 122.2A5 blocked binding of LFA-1/COS cells to ICAM-3 but not ICAM-1, in a manner analogous to that previously observed with T cells, and that the control mAb 38 inhibited binding to both ICAMs. These experiments therefore demonstrated unequivocally that mAbs YTH81.5 and 122.2A5 selectively blocked adhesion of LFA-1 to ICAM-3.

Effect of Deglycosylation on ICAM-3 Ligand Function

ICAM-3 is an extensively glycosylated molecule which expresses five potential N-linked glycosylation sites on domain 1 which are absent from the corresponding region of ICAM-1 (Fawcett et al., 1992; Vazeux et al., 1992; de Fougerolles et al., 1993). It is therefore possible that anti-LFA-1 mAbs YTH81.5 and 122.2A5 could be mediating their selective blocking effects indirectly, via a steric interaction with bulky carbohydrate residues present on ICAM-3 but not ICAM-1. To address this issue directly and to gain further insight into the role of glycosylation on ligand function, ICAM-3 was deglycosylated at the asparagine glycosylamine linkage by treatment with N-Glycosidase F. Fig. 4 *a* shows that treatment with N-Glycosidase F under non-denaturing conditions fully converted ICAM-3Fc from a diffuse band migrating at 75–80 kD to a sharper band at 55 kD. No further removal of carbohydrate could be achieved by SDS-denaturing before N-Glycosidase F digestion, suggesting that ICAM-3Fc was fully deglycosylated under non-denaturing conditions (data not shown). The structural integrity of deglycosylated ICAM-3 was verified by a panel of 14 anti-ICAM-3 mAbs, all of which demonstrated reactivity by ELISA assay with deglycosylated antigen coated onto plates (Littler, A., unpublished observations). Fig. 4 *b* demonstrates that deglycosylated ICAM-3 served as a ligand for LFA-1 on T cells and that blocking by anti-LFA-1 mAbs YTH81.5 and 122.2A5 remained unaffected. This experiment therefore excluded a possible role for N-linked carbohydrates on ICAM-3 in mediating the selective blocking effects by the anti-LFA-1 mAbs. These data also demonstrated that glycosylation was not an essential prerequisite for ligand function by ICAM-3, although a drop in ligand function between 35–50% was consistently observed. Deglycosylated ICAM-1 supported LFA-1 binding equally well as native ICAM-1 and exhibited unchanged blocking profiles with anti-LFA-1 mAbs YTH81.5 and 122.2A5 (data not shown).

Mapping of the YTH81.5 and 122.2A5 Epitopes

To identify regions within the extracellular domain of the LFA-1 α subunit that might be involved in the blocking of ICAM-3 binding, we mapped the epitopes of YTH81.5 and 122.2A5. This was accomplished by immunoprecipitation of in vitro-translated protein fragments of the LFA-1 α subunit. A cDNA deletion series of the LFA-1 α subunit was constructed, which consisted of five successive single domain deletions beginning from the NH_2-end and terminating in domain 7 (each fragment labeled according to its NH_2 terminus as N, 2, I, 3, 4), plus a control fragment (C) which included the rest of the α subunit terminating at the transmembrane domain (see Materials and Methods). Radiolabeled proteins corresponding to these constructs were immunoprecipitated by the anti-LFA-1 mAbs of interest or an irrelevant anti-CD8 mAb (mAb 14) and resolved by SDS-

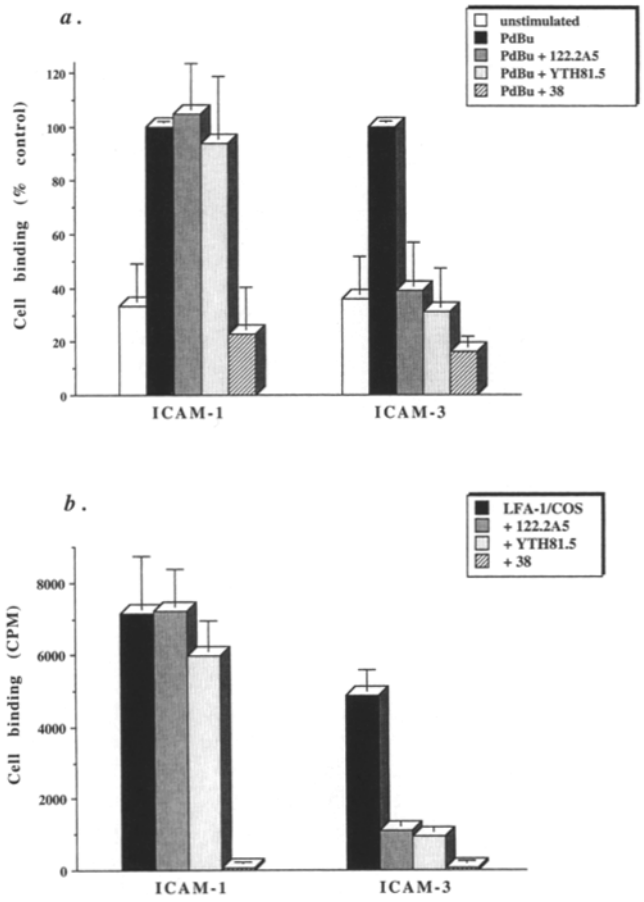

Figure 3. mAbs YTH81.5 and 122.2A5 selectively block adherence of T cells and LFA-1/COS cells to ICAM-3. (*a*) ^{3}H-labeled T cells were adhered to ICAM-1 or ICAM-3 coated 96-well plates in the presence or absence of PdBu and anti-LFA-1 α subunit mAbs indicated. Results represent the average ± SD % of input T cells adhering to plates from eight independent experiments. (*b*) LFA-1 transfected COS cells were assessed as in *a* for binding to ICAM-1 and ICAM-3 plates. The histogram depicts average ± SD adherence of LFA-1/COS cells from a single experiment, each condition performed in septuplicate, representative of four similar experiments.

PAGE. The upper panel of Fig. 5 *a* demonstrates that mAbs YTH81.5 and 122.2A5 immunoprecipitated protein fragments which contained the I domain (fragments N, 2 and I), but not fragments from which the I domain had been deleted (3, 4, and C). Nonspecific binding by the control mAb 14 was minimal. The lower panel of Fig. 5 *a* represents the nonprecipitated fraction and shows that efficiency of translation and incorporation of radiolabel within each protein fragment was comparable across the entire deletion series. Although in vitro translation generated truncated protein fragments, only full length translation products (marked by an *arrow*) were immunoprecipitated. To provide further evidence that the I domain was sufficient for recognition by these mAbs, the LFA-1 α subunit was deleted from the COOH-terminal end of the I domain, creating a fragment consisting only of the I domain plus domain 2 (2-I). Fig. 4 *b* demonstrates that mAb YTH81.5 could recognize this construct, thereby confirming that it mapped to the I domain of LFA-1.

Effect of mAb MEM-83 on ICAM-1 vs ICAM-3 Binding

We have previously identified a unique LFA-1 activation epitope, recognized by mAb MEM-83, which is also located in the I domain of the LFA-1 α subunit (Landis et al., 1993). It was therefore of interest to determine whether this mAb could induce binding of LFA-1 to ICAM-3 in a manner similar to that previously reported for ICAM-1. Fig. 6 demonstrates that mAb MEM-83 was not able to stimulate binding of T cells to ICAM-3 over a wide concentration range, in contrast to the induced binding to ICAM-1.

Although differential binding to ICAM-1 in the presence of mAb MEM-83 might be due to selective effects on the LFA-1 activation process, it is equally possible that it is simply due to blocking by mAb of the ICAM-3 binding site on LFA-1. To test this latter possibility, mAb MEM-83 was assessed for its ability to inhibit binding of LFA-1 to ICAM-3 when LFA-1 was activated via another stimulus. Fig. 7 shows that mAb MEM-83 does not block Mn^{2+}-induced binding to either ICAM-1 or ICAM-3, while reiterating the fact that it could selectively induce de novo binding to ICAM-1. These results demonstrate that mAb MEM-83 can influence LFA-1 activation in favor of ICAM-1-binding in the absence of any intrinsic ICAM-3 blocking properties. Taken in conjunction with the selective blocking properties of mAbs YTH81.5 and 122.2A5, these results with mAb MEM-83 demonstrate that epitopes exist within the I domain of LFA-1 which can regulate the binding to different ICAM ligands.

Discussion

We have demonstrated that the integrin LFA-1 can bind selectively to its two most homologous ligands, ICAM-1 and ICAM-3, and that the I domain has a critical role in this process. The experimental approach was to screen a panel of anti-LFA-1 mAbs for differential effects on T cell binding to ICAM-1 vs ICAM-3. By this means, we identified three mAbs specific for the α subunit of LFA-1 which allow discrimination of LFA-1 binding to ICAM-1 and ICAM-3. mAb MEM-83 induced LFA-1 to bind ICAM-1 but not ICAM-3, whereas mAbs YTH81.5 and 122.2A5 exhibited selective blocking of ICAM-3. Differential blocking was confirmed in an independent adhesion assay which employed LFA-1-transfected COS cells binding to immobilized ligand. Importantly, the COS cell assay was free from any possible interference by cell surface ICAMs. This was relevant because the T cells used in our studies expressed cell-surface ICAM-1 and ICAM-3 at equivalent levels (data not shown).

A trivial explanation for the selective blocking effects by mAbs YTH81.5 and 122.2A5 might be invoked if the affinity of LFA-1 is greater for ICAM-1 than for ICAM-3, in which case binding to the latter ligand might be more easily blocked. Several facts argue against this possibility. Firstly, such differential inhibition was not observed with any other mAbs studied in the panel. Secondly, avidity of binding to ICAM-1 and ICAM-3 was comparable using either activated T cells or LFA-1-transfected COS cells. These results confirm a previous observation that T cells are able to bind ICAM-1 and ICAM-3 with comparable avidity (Campanero et al., 1993) but are not in accordance with an earlier report that ICAM-1 is the favored ligand for LFA-1 (de Fougerolles and Springer, 1992). Apparent differences could depend on

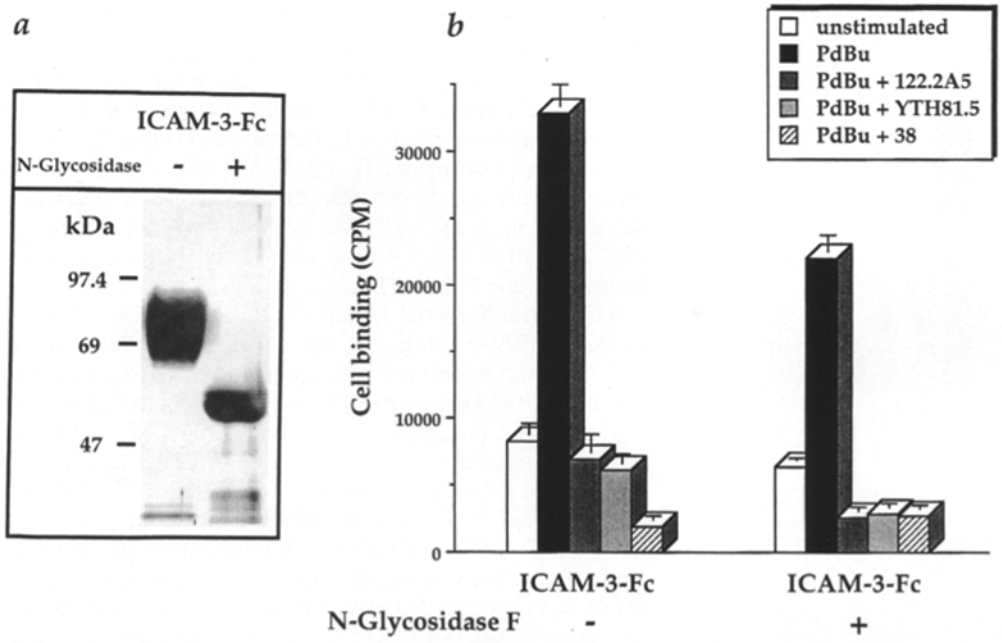

Figure 4. Selective blocking by mAbs YTH81.5 and 122.2A5 is not dependent upon N-linked carbohydrates on ICAM-3. (*a*) ICAM-3 Fc was digested under non-denaturing conditions with N-Glycosidase F and compared with a mock-treated control for completeness of deglycosylation on a 7.5% SDS-PAGE gel (4 μg/lane) visualized by silver-stain. (*b*) Plates were coated with mock-treated or deglycosylated ICAM-3 Fc and T cell binding was assessed as described in the legend to Fig. 3. Results represent mean adhesion ± SD of triplicate incubations from a single experiment, representative of three similar experiments.

a number of distinctive factors between the adhesion assays employed in these studies, such as cell type, source of ICAM-3, activation regimes and inversion of the binding assay (i.e., binding of ICAM-expressing cells to immobilized LFA-1). It should be noted that none of these adhesion assays are suitable for obtaining monomolecular affinities between receptor/ligand pairs and that such a determination for ICAM-1 and ICAM-3 will require competition and equilibrium binding experiments of the type used to measure the affinity constant for mouse LFA-1 to ICAM-1 (Lollo et al., 1993).

ICAM-3 contains five potential asparagine-linked glycosylation sites in domain 1 which are absent from the corresponding domain of ICAM-1. As the LFA-1 binding site of ICAM-3 has been localized to domain 1 and part of domain 2 (Holness, C., manuscript submitted for publication), it could be argued that mAbs YTH81.5 and 122.2A5 might be artefactually preventing access to the binding site via a steric interaction with bulky carbohydrate residues selectively expressed on domain 1 of ICAM-3. To address this possibility, ICAM-3 Fc was deglycosylated by treatment with N-Glycosidase F, which has previously been shown to remove asparagine-linked oligosaccharides under non-denaturing conditions from a variety of proteins (Tarentino et al., 1985). The resultant deglycosylated ICAM-3Fc retained reactivity with a panel of 14 mAbs specific for the first two domains of ICAM-3 and still supported binding of LFA-1 expressed on T cells. These results suggest that the extensive glycosylation on domain 1 is not essential for maintaining conformation or ligand function of ICAM-3. The ability of deglycosylated ICAM-3 to support binding of LFA-1 was similar to results obtained using ICAM-1 (data not shown) and a previous report on ICAM-1 ligand function (Diamond et al., 1991). Furthermore, experiments using deglycosylated ICAM-3 ligand ruled out carbohydrates as possible mediators of the selective blocking effects by anti-LFA-1 mAbs YTH81.5 and 122.2A5.

The third anti-LFA-1 mAb MEM-83 selectively induced binding of T cells to ICAM-1 but not ICAM-3, which provided a second line of evidence that binding of LFA-1 to these two ligands was distinct. Again there are several possible explanations as to how this mAb might be working. One possibility is that mAb MEM-83-activated cells failed to bind ICAM-3 simply because the mAb blocked the high affinity ICAM-3 binding site. This was not the case, however, as MEM-83 did not block the LFA-1/ICAM-3 interaction when the high affinity state of LFA-1 was induced by Mn^{2+}. An alternative possibility is that mAb MEM-83 could be exerting its effects at an earlier phase of the LFA-1/ICAM interaction. The high affinity phase of ICAM-1 binding is dependent not only upon agonist stimulation of LFA-1 but also a preceding ligation with ICAM-1 via the so-called "LIB" interaction, which represents a necessary step leading to the full activation of LFA-1 (Cabañas and Hogg, 1993). It is not yet certain whether the LIB interaction with ICAM-1 is required to stabilize a transiently activated LFA-1 conformer or whether ICAM-1 directly induces a higher affinity state of LFA-1. Thus, mAb MEM-83 could be directly inducing/stabilizing an active ICAM-1 binding conformer of LFA-1 or alternatively preventing the alteration necessary for ICAM-3 binding or both. A similar argument may also be applied to explain the selective blocking effects of mAbs YTH81.5 and 122.2A5. The role of LIB interactions with ICAM-3 in the LFA-1 activation process is currently under investigation.

There are examples in other systems of differential binding by one integrin of multiple ligands. For example, VLA-2 exhibits cell-type specific adhesion towards ligands collagen and laminin (Elices and Hemler, 1989; Kirchhofer et al., 1990) and VLA-4 can bind the IgSF ligand VCAM-1 and the CS1 region of fibronectin depending on the cellular context in which they are expressed (Elices et al., 1990). These binding patterns can be influenced not only by the local cell environment but also by treatment with divalent cations and activating mAbs (Chan and Hemler, 1993; Matsumoto and Hemler, 1993). In contrast, the present results suggest that recognition of the two IgSF ligands ICAM-1 and ICAM-3 by

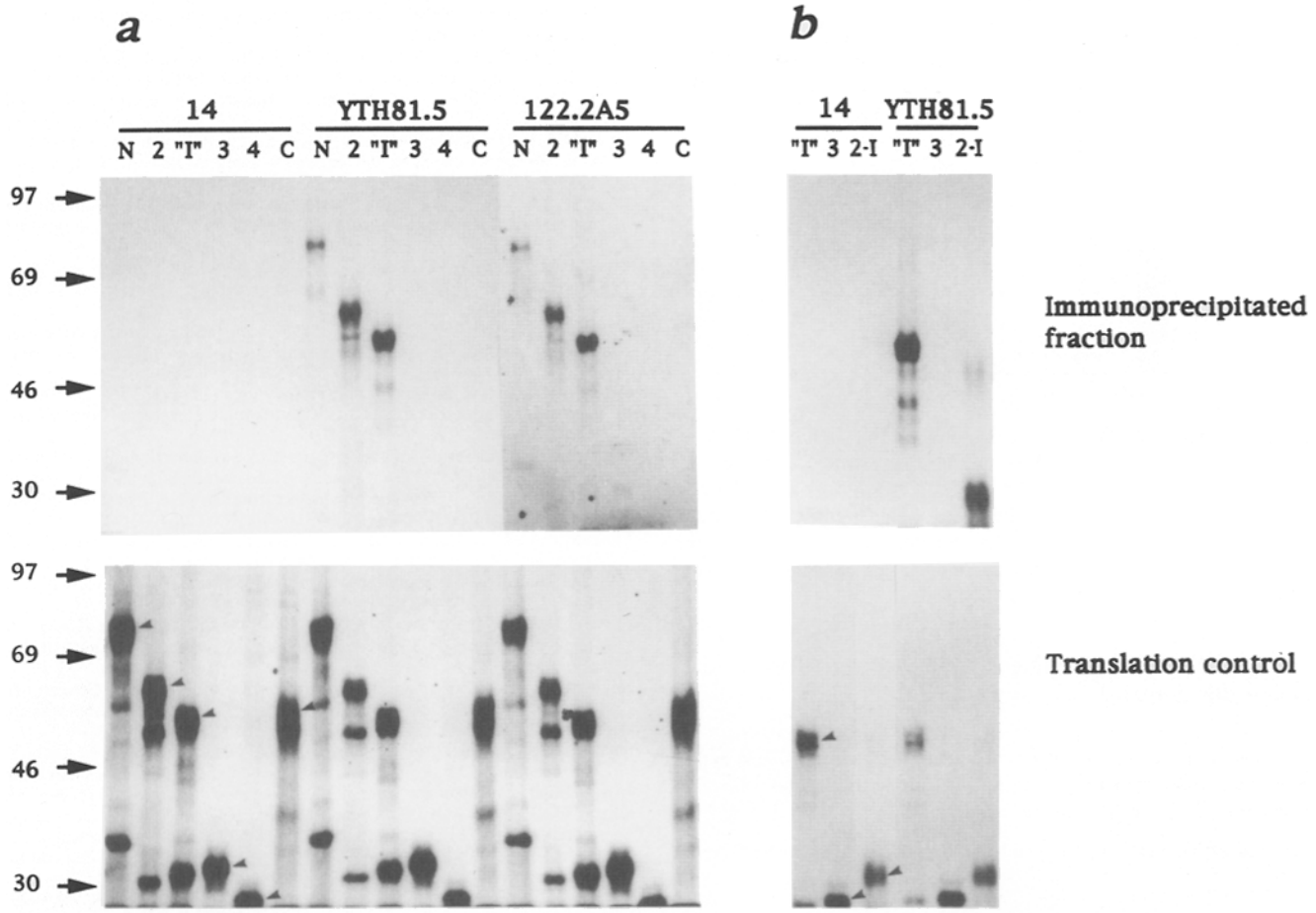

Figure 5. mAbs YTH81.5 and 122.2A5 map to the I domain of LFA-1. (*a*) The lower panel depicts the migration on an SDS-PAGE gel of six [^{35}S]methionine-labeled protein fragments of the LFA-1 α subunit, created by transcription/translation of a cDNA deletion series. The cDNA series consisted of five consecutive single-domain deletion fragments, beginning from the NH_2 terminus and ending in domain 7 of the LFA-1 α subunit (labeled according to the NH_2 terminus as *N*, *2*, *I*, *3*, *4*), plus a control fragment (*C*) spanning from the end of domain 7 to the transmembrane region. Arrows indicate the expected molecular size for full length protein translation products. The upper panel depicts immunoprecipitation of translation products by anti-LFA-1 mAbs YTH81.5 and 122.2A5 or a control anti-CD8 mAb 14. Only full length translation products (*arrows*) containing the I domain were immunoprecipitated by mAbs YTH81.5 and 122.2A5. (*b*) Depicts immunoprecipitation by mAb YTH81.5 of the NH_2-terminal deletion fragments N and 3, plus a COOH-terminal deletion fragment (labeled *2-I*), consisting only of the I domain plus domain 2. Autoradiograms from a single experiment are shown, representative of four similar experiments.

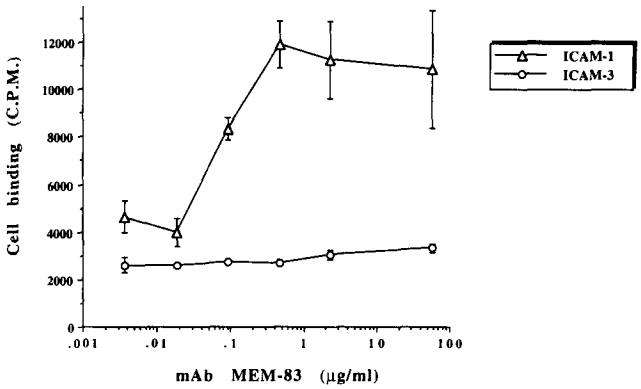

Figure 6. mAb MEM-83 induces binding of T cells to ICAM-1 but not ICAM-3. T cells were preincubated on ice for 20 min with the designated doses of mAb MEM-83, before binding to ICAM-1- and ICAM-3-coated plates. The results show adherence ± SE of quadruplicate incubations from a single experiment, representative of three similar experiments.

LFA-1 is not discriminated by different cell environments (comparing T cells and COS cells) or regimes of LFA-1-activation (comparing PdBu, Mn^{2+}, or Mg^{2+}-data not shown), but may be distinguished by activating antibodies directed against the LFA-1 α subunit.

Finally, epitope mapping studies revealed that the two ICAM-3-inhibiting mAbs YTH81.5 and 122.2A5 and the LFA-1 activating mAb MEM-83 (Landis et al., 1993) all map to the I domain of the LFA-1 α subunit. Based on competition binding experiments, the three mAbs appeared to recognize a cluster of closely related epitopes (data not shown). All three mAbs can recognize a recombinant I domain fusion protein, which was also shown to contain a ligand binding site for ICAM-1 (Randi and Hogg, 1994). An attractive speculation would be that alternative forms of the LFA-1-binding site exist normally on the cell surface after activation and that mAbs used in this study are able to induce/stabilize (MEM-83) or interfere with (YTH81.5 and 122.2A5) one of these forms. Function-affecting mAbs to the I domain may

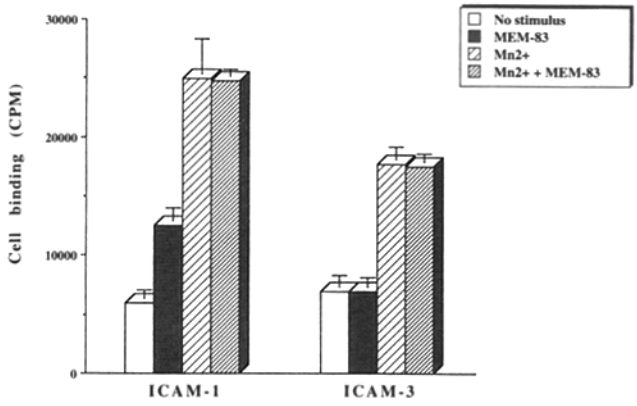

Figure 7. mAb MEM-83 does not block Mn^{2+}-induced ICAM-3 binding. ICAM-1 and ICAM-3 binding-assays were carried out in the presence or absence of mAb MEM-83 (2 μg/ml) using T cells in RPMI-1640 medium or washed into a Hepes buffered medium containing 400 μM Mn^{2+}. Results represent average ± SD adhesion of quadruplicate incubations from a single experiment, representative of three similar experiments.

therefore be directly influencing the ligand binding site within this domain.

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References

Andrew, D., A. Shock, E. Ball, S. Ortlepp, J. Bell, and M. Robinson. 1993. KIM185, a monoclonal antibody to CD18 which induces a change in the conformation of CD18 and promotes both LFA-1 and CR3-dependent adhesion. *Eur. J. Immunol.* 23:2217–2222.

Bazil, V., I. Stefanova, I. Hilgert, H. Kristofova, S. Vanek, and V. Horejsi. 1990. Monoclonal antibodies against human leukocyte antigens IV. Antibodies against the LFA-1 (CD11a/CD18) leukocyte-adhesion glycoprotein. *Folio Biologica (Praha).* 36:41–50.

Cabañas, C., and N. Hogg. 1993. Ligand ICAM-1 has a necessary role in the activation of integrin LFA-1. *Proc. Natl. Acad. Sci. USA.* 90:5838–5842.

Campanero, M. R., M. A. del Pozo, A. G. Arroyo, P. Sanchez-Mateos, T. Hernandez-Caselles, A. Craig, R. Pulido, and F. Sanchez-Madrid. 1993. ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway. *J. Cell Biol.* 123:1007–1016.

Chan, B. M. C., and M. E. Hemler. 1993. Multiple functional forms of the integrin VLA-2 can be derived from a single $\alpha 2$ cDNA clone: interconversion of forms induced by an anti-$\beta 1$ antibody. *J. Cell Biol.* 120:537–543.

Colombatti, A., and P. Bonaldo. 1991. The superfamily of proteins with von Willebrand factor type A-like domains: one theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defense mechanisms. *Blood.* 77:2305–2325.

Corbi, A. L., J. Garcia-Aguilar, and T. A. Springer. 1990. Genomic structure of an integrin α subunit, the leukocyte p150,95 molecule. *J. Biol. Chem.* 265:2782–2788.

Corbi, A. L., T. K. Kishimoto, L. J. Miller, and T. A. Springer. 1988. The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) α subunit. *J. Biol. Chem.* 263:12403–12411.

Corbi, A. L., L. J. Miller, K. O'Connor, R. S. Larson, and T. A. Springer. 1987. cDNA cloning and complete primary structure of the α subunit of a leukocyte adhesion glycoprotein, p150,95. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4023–4028.

de Fougerolles, A. R., and T. A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med.* 175:185–190.

de Fougerolles, A. R., L. B. Klickstein, and T. A. Springer. 1993. Cloning and expression of intercellular adhesion molecule 3 reveals strong homology to other immunoglobulin family counter-receptors for lymphocyte function associated antigen 1. *J. Exp. Med.* 177:1187–1192.

Diamond, M. S., and T. A. Springer. 1993. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J. Cell Biol.* 120:545–556.

Diamond, M. S., D. E. Staunton, S. D. Marlin, and T. A. Springer. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell.* 65:961–971.

Dransfield, I., C. Cabañas, A. Craig, and N. Hogg. 1992. Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J. Cell Biol.* 116:219–226.

Dransfield, I., and N. Hogg. 1989. Regulated expression of Mg^{2+} binding epitope on leukocyte integrin α subunits. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3759–3765.

Du, X., E. F. Plow, A. L. Frelinger, T. E. O'Toole, J. C. Loftus, and M. H. Ginsberg. 1991. Ligands "activate" integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Cell.* 65:409–416.

Dustin, M. L., and T. A. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature (Lond.).* 341:619–624.

Elices, M. J., and M. E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906–9910.

Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-1/fibronectin binding site. *Cell.* 60:577–584.

Fawcett, J., C. L. L. Holness, L. A. Needham, H. Turley, K. C. Gatter, D. Y. Mason, and D. L. Simmons. 1992. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. *Nature (Lond.).* 360:481–484.

Harvey, J. E., N. Hogg, and R. C. Landis. 1993. LFA-1 and the ICAMs. *In* Lymphocyte Adhesion Molecules. Y. Shimizu, editor. R.G. Landes Company, Austin. 26–54.

Heidenreich, R., R. Eisman, S. Surrey, K. Delgrosso, J. S. Bennett, E. Schwartz, and M. Poncz. 1990. Organisation of the gene for platelet glycoprotein IIb. *Biochemistry.* 29:1232–1244.

Ignatius, M. J., T. H. Large, M. Houde, J. W. Tawil, A. Barton, F. Esch, S. Carbonetto, and L. F. Reichardt. 1990. Molecular cloning of the rat integrin α_1-subunit: a receptor for laminin and collagen. *J. Cell Biol.* 111:709–720.

Keizer, G. D., W. Visser, W. Vliem, and C. G. Figdor. 1988. A monoclonal antibody (NKI-L16) directed against a unique epitope on the α-chain of human leukocyte function-associated antigen-1 induces homotypic cell-cell interactions. *J. Immunol.* 140:1393–1400.

Kirchhofer, D., L. R. Languino, E. Ruoslahti, and M. D. Pierschbacher. 1990. $\alpha_2\beta_1$ integrins from different cell types show different binding specificities. *J. Biol. Chem.* 265:615–618.

Kishimoto, T. K., N. Hollander, T. M. Roberts, D. C. Anderson, and T. A. Springer. 1987. Heterogeneous mutations in the β subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. *Cell.* 50:193–202.

Landis, R. C., R. I. Bennett, and N. Hogg. 1993. A novel LFA-1 activation epitope maps to the I domain. *J. Cell Biol.* 120:1519–1527.

Larson, R. S., A. L. Corbi, L. Berman, and T. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1 α subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* 108:703–712.

Law, S. K. A., J. Gagnon, J. E. K. Hildreth, C. E. Wells, A. C. Willis, and A. J. Wong. 1987. The primary structure of the β-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:915–919.

Lollo, B. A., K. W. H. Chan, E. M. Hanson, V. T. Moy, and A. A. Brian. 1993. Direct evidence for two affinity states for lymphocyte function-associated antigen 1 on activated T cells. *J. Biol. Chem.* 268:21693–21700.

Matsumoto, A., and M. E. Hemler. 1993. Multiple activation states of VLA-4. Mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule. *J. Biol. Chem.* 266:228–234.

Michishita, M., V. Videm, and M. A. Arnaout. 1993. A novel divalent cation-binding site in the A domain of the $\beta 2$ integrin CR3 (CD11b/CD18) is essential for ligand binding. *Cell.* 72:857–867.

Pardi, R., L. Inverardi, and J. R. Bender. 1992. Regulatory mechanisms in leukocyte adhesion: flexible receptors for sophisticated travelers. *Immunol. Today.* 13:224–230.

Randi, A. M., and N. Hogg. 1994. The I domain of the $\beta 2$ integrin LFA-1 contains a binding site for ligand ICAM-1. *J. Biol. Chem.* 269:12395–12398.

Robinson, M. K., D. Andrew, H. Rosen, D. Brown, S. Ortlepp, P. Stephens, and E. C. Butcher. 1992. Antibody against the Leu-CAM β-chain (CD18) promotes both LFA-1- and CR3-dependent adhesion events. *J. Immunol.* 148:1080–1085.

Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer. 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* 137:1270–1274.

Schmidt, R. E. 1989. Non-lineage/natural killer section report: new and previously defined clusters. *In* Leukocyte Typing IV. White cell differentiation antigens. W. Knapp, B. Dorken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. Kr. von dem Borne, editors. Oxford University Press, Oxford. 517–574.

Simmons, D., M. W. Makgoba, and B. Seed. 1988. ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature (Lond.).* 331:624–627.

Staunton, D. E., M. L. Dustin, and T. A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature (Lond.).* 339:61–64.

Staunton, D. E., S. D. Marlin, C. Stratowa, M. L. Dustin, and T. A. Springer. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell.* 52: 925–933.

Takada, Y., and M. E. Hemler. 1989. The primary structure of the VLA-1/collagen receptor α^2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109: 397–407.

Tarentino, A., C. Gomez, and T. Plummer. 1985. Deglycosylation of asparagine linked glycans by peptide:N-glycosidase F. *Biochemistry.* 24:4665–4668.

van Kooyk, Y., P. van de Wiel-van Kemenade, P. Weder, T. W. Kuijpers, and C. G. Figdor. 1989. Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature (Lond.).* 342:811–813.

Vazeux, R., P. A. Hoffmann, J. K. Tomita, E. S. Dickinson, R. L. Jasman, T. S. John, and W. M. Gallatin. 1992. Cloning and characterization of a new intercellular adhesion molecule ICAM-R. *Nature (Lond.).* 360:485–488.