Regulation of Adhesion to ICAM-1 by the Cytoplasmic Domain of LFA-1 Integrin β Subunit

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Interactions between cytotoxic lymphocytes and their targets require the T cell antigen receptor (TCR) and the integrin lymphocyte function-associated molecule–1 (LFA-1, CD11a/CD18). LFA-1 is not constitutively avid for its counterreceptors, intercellular adhesion molecules (ICAMs)–1 and –2. Cross-linking of the TCR transiently converts LFA-1 to a high avidity state and thus provides a mechanism for regulating cellular adhesion and de-adhesion in an antigen-specific manner. Truncation of the cytoplasmic domain of the β, but not the α, subunit of LFA-1 eliminated binding to ICAM-1 and sensitivity to phorbol esters. Thus, LFA-1 binding to ICAM-1 was found to be regulated by the cytoplasmic domain of the β subunit of LFA-1.

THE ABILITY OF TCR CROSS-LINKing to promote adhesion of LFA-1–bearing cells to ICAM-1 substrates, the inhibition of this process with agents that increase the intracellular concentration of adenosine 3′,5′-monophosphate (cAMP), and the stimulation of this process with agents that activate intracellular protein kinase C, all support the hypothesis that signals from the cytosol are transduced across the cell membrane to generate changes in the extracellular domain of LFA-1 (*1, 2*). We have tested the hypothesis that the cytoplasmic domain of LFA-1 is important in regulating adhesion to ICAM-1. Mutants of the α or β subunit of LFA-1 with serially truncated cytoplasmic domains were constructed by site-directed mutagenesis (Fig. 1). The mutant α and β subunits were expressed in COS cells by cotransfection with a wild-type β or α subunit construct, respectively. All β subunit mutants and wild-type β subunits were expressed equally well in LFA-1 αβ complexes, as shown by labeling with a monoclonal antibody (MAb) (TS1/22) to the LFA-1 α subunit, a MAb (NKI-L16) to the α subunit activation epitope (Fig. 2), and a MAb to the β subunit (*3*). The β subunit is absolutely required for expression of the LFA-1 α subunit in COS cells (*4*). Most of the mutant LFA-1 α subunits were expressed on the surface of COS cells equivalently to the wild-type α subunit (Fig. 2B). The exceptions were α1096*, which contains only three cytoplasmic amino acids and was not expressed, and α1129*, which reproducibly showed lower expression than the other mutants.

COS cells that transiently expressed LFA-1 with truncated α or β subunits were examined for their ability to bind purified ICAM-1 (Fig. 3). Wild-type LFA-1 expressed in COS cells is functionally active in this system (*4*). Whereas COS cells that expressed wild-type LFA-1 could bind to ICAM-1 substrates, COS cells that expressed the LFA-1 α subunit in association with either truncated β subunits or a β subunit with an altered COOH-terminal sequence (Fig. 1A) were either unable to bind ICAM-1 or showed impaired binding (Fig. 3A). Specificity was demonstrated by inhibition with a MAb to ICAM-1. Thus, the cytoplasmic domain of the β subunit (even the five most COOH-terminal residues) is essential for the function of LFA-1. Surprisingly, the function in COS cells of the longest truncation mutant was less diminished than the function of shorter truncation mutants (but see results with lymphoid cells described below). The binding to ICAM-1 of all β subunit cytoplasmic domain mutants and wild-type LFA-1 could be stimulated with NKI-L16, a MAb to LFA-1 that stimulates LFA-1–dependent adhesion (*5*). This stimulated adhesion was specific and was inhibited with a blocking MAb to the LFA-1 α subunit (Fig. 3A). NKI-L16 did not fully overcome the effect of cytoplasmic domain deletion, suggesting that this MAb may not induce signals that are equivalent to those transduced by the LFA-1 β subunit cytoplasmic domain.

In contrast to the results with truncated β subunits, all mutants with truncated LFA-1 α subunits—with the exception of mutant α1096*, which was not expressed on the surface of COS cells—could bind to ICAM-1 (Fig. 3B). Mutant α1129* showed lower binding to ICAM-1 than the other α subunit mutants, and this lower binding

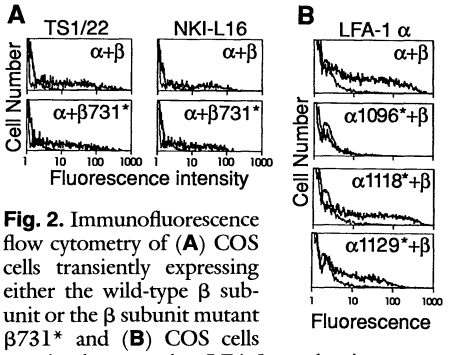

Fig. 2. Immunofluorescence flow cytometry of (**A**) COS cells transiently expressing either the wild-type β subunit or the β subunit mutant β731* and (**B**) COS cells transiently expressing LFA-1 α subunit mutants. In (A) the indicated transfectants were labeled with either TS1/22 (MAb to LFA-1 α) or NKI-L16 (MAb to LFA-1 α activation epitope) (thick lines), or the nonbinding control antibody X63 (thin line). In (B) the indicated transfectants were labeled with either TS1/22 (thick line) or X63 (thin line). All β subunit mutants were expressed to the same degree; mutant β731* is a representative example. The level of expression of the α subunit mutants α1107* and α1139* was identical to that represented by mutant α1118*. The cDNAs encoding the wild-type (*4*) or mutant LFA-1 α subunits and the wild-type (*6*) or mutant β subunits were transfected into COS cells with the use of DEAE-dextran (*24, 25*). Cell surface expression was quantitated 3 to 4 days after transfection (*6*).

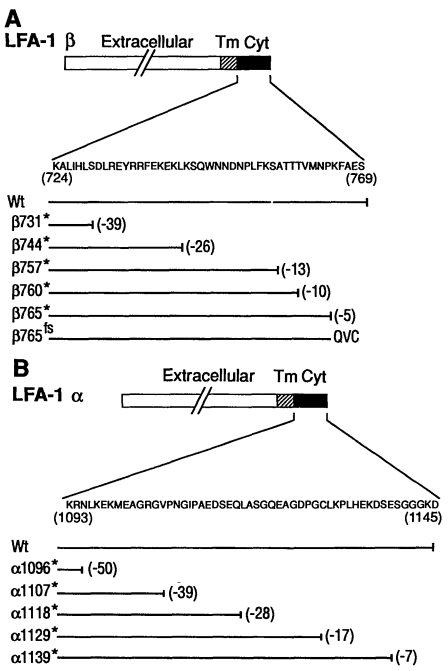

Fig. 1. Schematic representation of the wild-type (Wt) LFA-1 β (**A**) and α (**B**) subunits, depicting extracellular, transmembrane (Tm), and cytoplasmic (Cyt) domains and their mutants. Nomenclature for the mutants is as follows: β731* indicates that the nucleotide sequence encoding the amino acid at position 731 in the unprocessed β subunit has been mutated to a stop codon. Mutants of the α and β subunits in CDM8 (*4, 6*) were prepared by oligonucleotide-directed mutagenesis (*23*). All mutations were verified by nucleotide sequencing of the region encoding the cytoplasmic domain; the entire coding region of β731* was sequenced. An additional mutant was generated ($\beta765^{fs}$) in which an extra cytosine had been inserted at nucleotide 2365 that resulted in a change in the reading frame beginning at Lys^{765}. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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correlated with lower surface expression (Fig. 2B).

Stable transfectants that expressed mutant β subunits were generated with an episomal Epstein-Barr virus (EBV)–based vector in an EBV-transformed B lymphoblastoid cell line that was derived from an individual with leukocyte adhesion deficiency (LAD). Functional LFA-1 αβ heterodimers can be reconstituted in cell lines derived from individuals with LAD by transfection with a wild-type β subunit construct, and binding of these lymphoblastoid cell lines to ICAM-1 is up-regulated by phorbol esters (*6*). Phorbol ester stimulation of the binding of lymphoid cell lines to ICAM-1 is not accompanied by any increase in surface expression of LFA-1 (*1, 7*). LAD lymphoid cell lines are thus physiologically relevant recipients for study of the effect of mutation of the β subunit on LFA-1 avidity regulation; in contrast, binding of COS cells transfected with LFA-1 is not enhanced by phorbol esters (*4*). LFA-1 αβ heterodimers were reconstituted in equivalent amounts on the surface of LAD B lymphoblastoid cells after transfection with either wild-type or mutant β subunit constructs (Fig. 4A).

The stably transfected LAD lymphoid cell lines were tested for their ability to bind to purified ICAM-1 that was immobilized on plastic (Fig. 4B). Control EBV-transformed B lymphoblastoid cells from a healthy individual (CO3) and transfectants expressing wild-type LFA-1, but not untransfected LAD cells, were able to bind to ICAM-1; this adhesion could be increased more than twofold in the presence of phorbol 12-myristate 13-acetate (PMA). MAbs to ICAM-1 inhibited this interaction. In contrast, lymphoblastoid cells that expressed either truncated β subunits or a β subunit with an altered COOH-terminal sequence showed little or no binding to ICAM-1, either in the presence or absence of PMA. In addition, in homotypic adhesion assays, the lymphoblastoid cells that expressed mutant β subunits did not aggregate in response to PMA, whereas PMA induced formation of large aggregates of lymphoblastoid cells that expressed the wild-type β subunit (*3*). These results demonstrate that cell binding to ICAM-1–coated plastic is representative of cell-cell adhesion.

Our results demonstrate the importance of the cytoplasmic domain of the β subunit in regulating adhesion of LFA-1 to ICAM-1, and they therefore support the hypothesis that signals transduced from inside the cell regulate the activity of the extracellular domain of LFA-1 in binding ICAM-1 (*1*). Truncation of the cytoplasmic domain of the β subunit of LFA-1 both diminished binding to ICAM-1 and inhibited stimulation of binding by PMA. Our data indicate that the β subunit is a final target for the cellular machinery that regulates the avidity of LFA-1. The way in which the cytoplasmic domain of the β subunit regulates LFA-1 avidity is unclear at present, but phosphorylation of the cytoplasmic domain or its interaction with a cytoskeletal protein may bring about a conformational change in the ICAM binding site—such mechanisms may possibly be linked to association of LFA-1 with itself or with other membrane proteins.

The nature of the cellular proteins that interact with the cytoplasmic domain of the β subunit and of intermediates in the signaling pathway remains unclear, although pharmacological studies suggest that protein kinase C and cAMP-dependent protein kinase have agonistic and antagonistic effects, respectively (*1*). An interaction between the cytoplasmic domain of the β subunit and the cytoskeletal component talin (*8*) or some other protein may contribute to the LFA-1 avidity change. The cytoplasmic domain of the β1 integrin subunit is necessary for the localization of β1 integrins to focal contacts in fibroblasts (*9–11*). We have reproducibly and unexpectedly observed in COS fibroblastoid cells that the mutant β731* binds appreciably to ICAM-1, whereas other mutant β subunits that have shorter truncations bind less well (Fig. 3A). In lymphoblastoid cells, neither β731* nor the other truncation

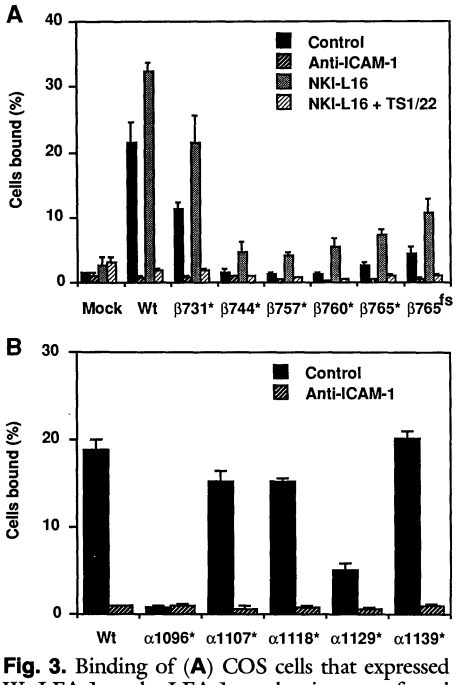
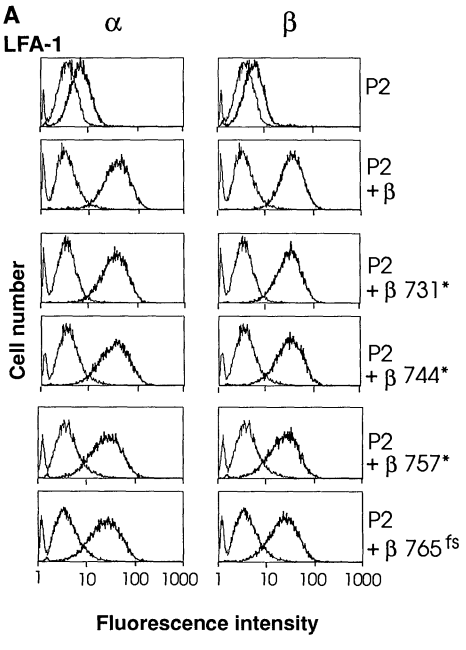


Fig. 3. Binding of (**A**) COS cells that expressed Wt LFA-1 or the LFA-1 α subunit cotransfected with the indicated β subunit mutants or vector alone (mock), and (**B**) COS cells expressing Wt LFA-1 or the LFA-1 β subunit cotransfected with the indicated α subunit mutants to ICAM-1–coated plastic. Binding was performed in the presence of a MAb to histocompatibility class I antigens A and B (control; 1/4 dilution of supernatant), a MAb to ICAM-1 (anti–ICAM-1; 1/4 dilution of supernatant), NKI-L16 (1/8 dilution of supernatant), or NKI-L16 plus TS1/22 (1/8 dilution of NKI-L16 supernatant and 1/400 dilution of TS1/22 ascites). Results are expressed as mean ± SD and are representative of three experiments. ICAM-1 was purified from lysates of splenocytes from individuals with hairy cell leukemia and absorbed to plastic (*6*). The binding of cells to ICAM-1 substrates was measured as described (*6*), with the exception that 2×10^4 COS cells were added to each well. COS cell transfectants were subjected to flow cytometry before each binding assay to ensure that the levels of LFA-1 expressed by the different transfectants were comparable.



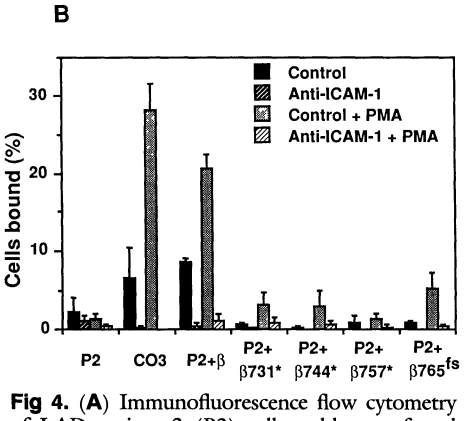


Fig 4. (**A**) Immunofluorescence flow cytometry of LAD patient 2 (P2) cells stably transfected with β subunit mutants. The indicated cell lines were stained with either TS1/22 or a MAb to LFA-1 β subunit (TS1/18) (thick lines), or the X63 MAb (thin line). The cDNAs encoding the Wt and mutant β subunits were subcloned into the episomal EBV-based vector p205118a, and the EBV-transformed B lymphoblastoid cell line derived from LAD patient 2 was transfected by electroporation (*6*). (**B**) Binding of B lymphoblastoid cells from a healthy individual (CO3) or of cells from patient 2 that had been stably transfected with the indicated β subunits, in the presence or absence of PMA, to ICAM-1–coated plastic. Binding was performed as described in Fig. 3.

mutants bind ICAM-1 (Fig. 4B). Our findings in COS cells with the LFA-1 β subunit, integrin β2, are paralleled by the effect of cytoplasmic domain truncation on integrin β1 localization to focal contacts in fibroblasts (*10*) and suggest the presence of both positive and negative regulatory regions in β subunit cytoplasmic domains that can be recognized in fibroblastic cells. Cytoplasmic domain truncation of several immunoglobulin superfamily adhesion molecules has no effect on their adhesiveness (*12*), whereas such truncation ablates adhesiveness of a member of the cadherin family (*13, 14*). Cadherins participate in Ca^{2+}-dependent cell-cell adhesion, and there is no evidence for cellular regulation of their avidity, in contrast to integrins such as LFA-1.

Our results show that the cytoplasmic domain of the β subunit, but not the α subunit, of LFA-1 is important for the regulation of adhesion. This finding correlates with the strong conservation during evolution of the cytoplasmic domain of the LFA-1 β subunit compared to that of the α subunit. The cytoplasmic domains of the human and mouse leukocyte integrin β (CD18) subunits are 96% conserved (*15–17*), whereas the human and mouse LFA-1 α (CD11a) subunit cytoplasmic domains show only 52% amino acid identity (*18, 19*). Similarly, the mouse and human Mac-1 α (CD11b) subunit cytoplasmic domains are only 68% identical (*20–22*). It therefore seems likely that the β subunit would also function in adhesion regulation of not only LFA-1 but also of the other leukocyte integrins that share it, Mac-1 and p150,95.

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Permeation of Calcium Ions Through Non-NMDA Glutamate Channels in Retinal Bipolar Cells

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The conduction of calcium ions through glutamate-gated channels is important in the induction of long-term potentiation and may trigger other cellular changes. In retinal bipolar cells, which lack the *N*-methyl-D-aspartate (NMDA) type of glutamate-gated channel, calcium permeability through non-NMDA channels was examined. Changes in extracellular calcium concentration unexpectedly affected the reversal potential for glutamate-induced currents in a manner consistent with these channels being highly permeable to calcium. External magnesium ions promote desensitization of these non-NMDA channels in a voltage-independent way. Thus, in addition to non-NMDA channels that conduct only sodium and potassium, there is a class that is also permeable to calcium.

RECEPTORS FOR GLUTAMATE IN THE vertebrate central nervous system (CNS) are classified according to whether NMDA, kainate, or quisqualate acts as an agonist. Channels gated by NMDA differ from non-NMDA channels in several physiologically important ways. NMDA channels are permeable to Ca^{2+} ions (*1–4*) but are blocked by Mg^{2+} ions (*5–7*), whereas non-NMDA channels are thought to have neither property (*2–4, 8*) but have faster kinetics (*9*).

Glutamate is the neurotransmitter at the synapse between the photoreceptors and bipolar cells of the vertebrate retina (*10*). Using the patch-clamp technique (*11*) in the whole-cell mode, we have examined the permeability of bipolar cell channels opened by glutamate.

L-Glutamate and the agonists kainate and quisqualate, when rapidly applied (*12*) to bipolar cells isolated from the salamander retina, elicited a conductance increase in 66% of cells examined (n = 642). These cells are thought to be the hyperpolarizing or off-center bipolar cells (*13*). As in other neurons (*9, 14, 15*), quisqualate-induced currents decayed over a time course of tens of milliseconds, whereas kainate-induced currents showed no desensitization (Fig. 1, A and B). In cells that responded to glutamate, kainate, or quisqualate, NMDA (100 to 200 μM) evoked no responses (Fig. 1C) (n = 28 cells) in the presence of glycine (1 to 5 μM), a potentiator of the effect of NMDA (*16*), and in the absence of external Mg^{2+}, which exerts a voltage-dependent block of NMDA-induced currents (*6, 7*). As a further check that NMDA receptors were absent from our cells, we compared the magnitude of glutamate responses in the presence and absence of 1 or 5 μM glycine (*17*). Of 24 cells examined, 21 showed less than 5% change in glutamate-evoked current in the presence of glycine, 2 showed small (<15%) increases, and 1 showed a small (<10%) decrease. The non-NMDA receptor blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (*18*), at 10 μM blocked a majority of the glutamate current. Since the residual current in CNQX could be seen at −60 mV in the presence of 0.5 mM external Mg^{2+}, it is not an NMDA channel current. On the basis of these results, we concluded that NMDA channels are absent from salamander bipolar cells.

Responses to L-glutamate itself (200 μM) resembled those to quisqualate in that a large transient current rapidly decayed to a sustained current. Time constants (mean ± SD) (*19*) for desensitization between 5 and 13 ms (10.1 ± 1.8; n = 24) were recorded, but are likely to be overestimates since drug application was not instantaneous (*12*). Responses to glutamate, quisqualate, and kai-

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