The α Subunit Cytoplasmic Domain Regulates the Assembly and Adhesiveness of Integrin Lymphocyte Function-Associated Antigen-1[1]

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The integrin LFA-1 mediates activation-dependent leukocyte adhesion. The β subunit cytoplasmic domain has been demonstrated previously to modulate the adhesiveness of LFA-1. To investigate whether the α subunit cytoplasmic domain is also involved in the regulation of LFA-1-adhesive function, we stably expressed cytoplasmic domain truncated forms of the α subunit in a Jurkat mutant (Jurkat-$\beta_{2.7}$) deficient in the endogenous LFA-1 α subunit and in K562 cells. Clones expressing similar levels of cell surface LFA-1 were tested for their ability to bind to immobilized ICAM-1. Truncation of the α subunit cytoplasmic domain before, but not after, the conserved GFFKR sequence motif resulted in constitutive ICAM-1 binding of both Jurkat-$\beta_{2.7}$ and K562 transfectants. However, truncation after the GFFKR motif reduced sensitivity to stimulation by PMA or stimulatory Abs. Internal deletion of the GFFKR motif, or point mutations of the Gly (G), the two Phe (F), or the Arg (R) in the GFFKR motif to Ala (A) rendered LFA-1 constitutively active. Mutation of the Lys (K) did not affect LFA-1 adhesion to ICAM-1. These findings indicate that the GFFKR motif maintains the low adhesive state of LFA-1, possibly by restraining the receptor conformation. We further demonstrate that the α subunit cytoplasmic domain and the conserved GFFKR motif are also required for efficient formation of LFA-1 $\alpha\beta$ heterodimers. *The Journal of Immunology,* **1997, 159: 268–278.**

Lymphocyte function-associated antigen-1 is a member of the leukocyte integrin subfamily. Members of this subfamily share a common β subunit (the β_2 integrin or CD18 subunit) and have distinct α subunits, α_L (CD11a), α_M (CD11b), and α_X (CD11c) for LFA-1, Mac-1, and p150,95, respectively (1, 2). Recently, a new member, $\alpha_D\beta_2$, has been identified (3). The biologic importance of the leukocyte integrins has been illustrated in leukocyte adhesion deficiency. Leukocyte adhesion deficiency patients have defective expression of leukocyte integrins on the cell surface due to mutations in the common β subunit (4). The disease causes an inability of phagocytic cells to bind to and migrate across the endothelium into sites of inflammation, resulting in severe bacterial and fungal infections (5).

LFA-1 is expressed on all leukocytes and is the receptor for three Ig superfamily members, ICAM-1, -2, and -3 (6–8). Interaction of LFA-1 with ICAMs requires LFA-1 activation. On resting T lymphocytes, LFA-1 binds only weakly to cell surface expressed or purified ICAM-1. TCR cross-linking rapidly (within minutes) converts LFA-1 to a high affinity state (9, 10). The enhanced adhesion is transient, and by 30 min after TCR stimulation, cells lose their ability to bind to ICAM-1. This may provide a mechanism for regulating T cell adhesion and de-adhesion with APC and target cells. Activation of LFA-1 can also be triggered by phorbol esters that activate protein kinase C (11). Nevertheless, the level of surface LFA-1 does not change with the increase in adhesion (9–11). It has been, therefore, hypothesized that signals from the cytosol are transduced across the plasma membrane to generate conformational changes in the extracellular domain of LFA-1 (the "inside-out" signaling) (9, 10). This hypothesis is supported by the existence of Abs that specifically recognize activated integrins (12–17). However, how cytoplasmic signals result in conformational changes and ligand binding in the extracellular domains of integrins still remains unknown.

In addition to the activation by intracellular signals, divalent cations can directly modulate ligand-binding function of integrins (13, 14, 18–20). Interaction of LFA-1 with ICAM-1 is Mg^{2+} dependent (6, 14). Mn^{2+} can directly convert LFA-1 to an active form (13, 14, 18). Ca^{2+} affects LFA-1-dependent lymphocyte adhesion by modulating LFA-1 cell surface distribution (21). Binding of Mn^{2+} or Mg^{2+} has been documented to directly induce an active conformation in integrins (14, 19, 22, 23). Activation of integrins can also be mimicked by certain Abs that bind to the α or β subunit (24–28). α subunit-specific mAb, NKI-L16, and β_2-specific mAbs, KIM127, KIM185, and CBR LFA-1/2, have been described to activate LFA-1-dependent cell aggregation and adhesion to ICAM-1 (12, 18, 29, 30).

The integrin cytoplasmic domains, especially the highly conserved membrane-proximal GFFKR sequence in the α subunit cytoplasmic domains, are potential targets for mediating integrin affinity modulation. Results from mutagenesis studies have demonstrated the importance of the cytoplasmic domains for integrin function. Whereas partial deletion of LFA-1 α cytoplasmic domain does not affect the ability of transfected COS cells to bind to ICAM-1, truncation of the LFA-1 β subunit cytoplasmic domain, or mutations of the three contiguous threonines at amino acid residues 758–760 or the phenylalanine at residue 766, eliminates ICAM-1 binding and prevents stimulation of LFA-1 by PMA, implicating a crucial role of the β cytoplasmic domain in regulating LFA-1 adhesiveness (31, 32). Deletion of the α_4 and α_6 cytoplasmic domains results in a lack of ligand binding of $\alpha_4\beta_1$ and $\alpha_6\beta_1$ integrins, respectively (33, 34). Truncation of the α_{IIb} cytoplasmic domain before, but not after, the conserved GFFKR sequence, or deletion of this sequence from the α_L cytoplasmic

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Received for publication October 29, 1996. Accepted for publication April 1, 1997.

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0022-1767/97/$02.00

domain connected to the extracellular and transmembrane domains of α_{IIb} renders the $\alpha_{II}\beta_3$ integrin constitutively active, implicating an important role of this conserved sequence in integrin affinity regulation (35, 36). Expression of the cytoplasmic domain of the β_1 subunit joined to the extracellular and transmembrane domains of an unrelated membrane protein shows that the autonomously expressed β_1 cytoplasmic domain can act as a *trans*-dominant inhibitor of $\alpha_5\beta_1$ function (37, 38); and this presumably occurs through competitive interactions with cytoplasmic factors that are required for $\alpha_5\beta_1$ adhesive function. These studies suggest that physiologic activation signals may be transmitted through integrin cytoplasmic domains.

Although partial truncation of the LFA-1 α subunit cytoplasmic domain has no effect on the constitutive activity of LFA-1 in COS cells (31), it is not known whether the α cytoplasmic domain or the conserved GFFKR sequence regulates LFA-1 adhesiveness in cells that normally express LFA-1. Recently, a Jurkat mutant, Jurkat-$\beta_{2.7}$, that lacks the endogenous LFA-1 α subunit protein, but expresses an intracellular β_2 subunit precursor, has been generated. Transfection of this mutant with LFA-1 α cDNA reconstitutes functional LFA-1 $\alpha\beta$ heterodimer on the surface (39). In this study, we have examined the role of the α subunit cytoplasmic domain and the conserved GFFKR sequence in the regulation of LFA-1-adhesive activity in a physiologic cellular environment by expressing α subunit mutants in Jurkat-$\beta_{2.7}$ cells. In parallel, we have also expressed these α subunit mutants with the wild-type β subunit in K562 cells. We demonstrate that truncation of the α cytoplasmic domain before, but not after, the conserved GFFKR sequence, or internal deletion of the GFFKR motif results in constitutively active forms of LFA-1 in a cell-type-independent manner, and that the glycine (G), the two phenylalanines (FF), and the arginine (R) in the GFFKR motif are important for LFA-1 function. We also demonstrate that the α cytoplasmic domain and the conserved GFFKR motif are required for efficient dimerization of LFA-1 α and β subunits.

Materials and Methods

Cell lines and Abs

Jurkat (human T lymphoma cell line), K562 (human erythroleukemia cell line), and COS-7 cells (SV40-transformed monkey kidney fibroblasts) were maintained in RPMI 1640 supplemented with 10% FBS and 50 μg/ml of gentamicin (complete medium).

mAbs TS1/22, TS1/18, CBR LFA-1/2, NKI-L16, and CBRLFA-1/7, and mAb 24 have previously been described (12, 13, 18, 23, 40).

Construction of LFA-1 mutant α subunits

Mutant αGPI[3] was generated by replacing the α subunit cDNA (41) encoding the transmembrane and cytoplasmic domain with a signal sequence for GPI anchor addition. A 1.2-kb *Afl*II-*Kpn*I fragment that contains the GPI anchor addition signal sequence from LFA-3 was excised from IC1/GPI1.4 (42). A unique *Afl*II restriction site was introduced into the α subunit cDNA at the junction of the extracellular and transmembrane domain by PCR. The PCR product was then digested with *Xba*I and *Afl*II, and the resultant *Xba*I-*Afl*II fragment containing the α subunit cDNA from bases 2561 to 3352 was gel purified. The wild-type α subunit cDNA in Ap^{r}M8 (Ap^{r}M8-LFA-1 α) (43) was digested completely with *Kpn*I and partially digested with *Xba*I, and the 6.8-kb *Kpn*I-*Xba*I fragment that contains α cDNA bases 1 to 2562 was isolated. A triple ligation of the above three isolated fragments was performed. The amino acid sequence of the resultant construct at the point of fusion is:

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LFA-1:LFA-3
YEKN:LSPS
YEKG:CIPS
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[3] Abbreviation used in this paper: GPI, glycosylphosphatidylinositol.

where amino acids in the construct and in wild-type LFA-1 and LFA-3 are shown above and below, respectively. The residue to which the GPI anchor is predicted to be attached is the serine (S).

Mutants α1090* and α1095* were constructed by truncating the α subunit cytoplasmic domain after amino acid position 1090, just before the conserved GFFKR sequence, and after position 1095, just after the GFFKR sequence. PCR primers were designed to include a stop codon at the desired position, followed by a *Sph*I recognition sequence to facilitate subcloning. The upstream primer for these PCR reactions corresponds to α subunit cDNA bases 2522 to 2542. The PCR-amplified fragments were digested with *Xba*I corresponding to base 2561 and *Sph*I, and ligated with the 7.8-kb *Sph*I-*Xba*I fragment from Ap^{r}M8-LFA-1 α that contains α subunit cDNA bases 1 to 2560.

Mutant $\alpha\Delta$GFFKR that contains an internal deletion of the highly conserved GFFKR sequence was generated by overlap extension PCR (44, 45). The inner complementary PCR primers (5′-CTTCTCCTTCAGGTTA ACTTTGTACAGCACT-3′ and 5′-AACCTGAAGGAGAAGATGGAGG CT-3′) were designed to omit the GFFKR sequence. The outer left and right primers were 5′-GCACGCCAATGTGACCTGTAA-3′ and 5′-ACATGCATGCTAGCGAATTTGGTCTTTGGC-3′, corresponding to α subunit cDNA bases 2770 to 2791 and 4085 to 4114, respectively. The outer right primer contains a *Sph*I sequence at the 5′ end and a *Nhe*I site immediately upstream to the *Sph*I sequence. The *Nhe*I site was introduced to facilitate identification of mutant clones. The product of the second round PCR reaction was digested with *Bst*XI and *Sph*I, and the *Bst*XI-*Sph*I fragment corresponding to α cDNA bases 3291 to 4109 was inserted into Ap^{r}M8-LFA-1 α after the corresponding wild-type fragment was removed. The point mutants αG1090A, αFF1091-2AA, αK1093A, and αR1094A, in which individual amino acid residues in the conserved GFFKR sequence were replaced by alanine residues, were constructed with the same method as for $\alpha\Delta$GFFKR. Mutations at the desired positions were introduced into the inner complementary PCR primer pair.

The wild-type α subunit cDNA in Ap^{r}M8 was used as the template for all PCR reactions. All mutations and the surrounding regions that were amplified by PCR and introduced into the wild-type α subunit cDNA were verified by DNA sequencing.

cDNA transfections

For transient expression in COS cells, cDNA encoding LFA-1 wild-type or mutant α subunits were subcloned from Ap^{r}M8 into the *Xba*I site of the transient expression vector pEF-BOS, which carries the promoter of the human EF-1α gene (46), and cotransfected with the β subunit cDNA contained in plasmid CDM8 (47), as previously described (43). Transfected COS cells were detached with PBS/5 mM EDTA on day 3 after transfection, and analyzed for LFA-1 surface expression and binding to immobilized ICAM-1 (see below).

For generating stable cell lines that express LFA-1, the wild-type or mutant α subunit cDNA was subcloned into the *Xba*I site of pEFpuro (kindly provided by Dr. S. Orkin, Harvard Medical School, Boston, MA), which is a modification of the pEF-BOS expression vector that contains the puromycin-resistance gene (48). Twenty micrograms of pEFpuro containing α subunit cDNA was linearized with *Pvu*I and introduced into 1×10^7 Jurkat-$\beta_{2.7}$ cells in 800 μl of PBS by electroporation at 180 V and 960 μF using 0.4-cm path-length cuvettes (Bio-Rad Laboratories, Hercules, CA). For stable expression in K562 cells, 2 μg of linearized pEFpuro containing the α cDNA was cotransfected with 40 μg of *Nhe*I-linearized CDM8 containing the β subunit cDNA by electroporation at 250 V and 960 μF. After culture for 48 h in complete medium, cells were selected for resistance to 3 μg/ml of puromycin (Sigma Chemical Co., St. Louis, MO). Cells that expressed surface LFA-1 were selected first by panning on TS1/22 mAb-coated plates (49) and further subcloned by limiting dilution. All stable cell lines were maintained in complete medium supplemented with 3 μg/ml of puromycin.

Flow cytometry

Cells were washed twice with L15 medium plus 2.5% FBS (L15/FBS), and 10^6 cells in 50 μl were incubated with an equal volume of primary Ab (20 μg/ml of purified mAb, 1/100 dilution of mAb ascites or 1/2 dilution of hybridoma supernatant in PBS) on ice for 30 min. mAb 24 was diluted in L15/FBS and incubated with cells for 25 min at 37°C. Cells were then washed three times with L15/FBS and incubated with FITC-conjugated goat anti-mouse IgG (heavy and light chain; Zymed Laboratories, San Francisco, CA) for 30 min on ice. After washing, cells were resuspended in cold PBS and analyzed on a FACScan (Becton Dickinson, San Jose, CA). Surface LFA-1 levels were expressed as mean fluorescence intensity of cells.

Binding of transfectants to immobilized ICAM-1

ICAM-1 was purified from tonsil by immunoaffinity chromatography, as described previously (50). Purified ICAM-1 (50 μl of 5 μg/ml in 20 mM Tris-HCl, pH 9, 150 mM NaCl, and 2 mM $MgCl_2$) was adsorbed to each well of flat-bottom 96-well polystyrene plates (Flow Laboratories, McLean, VA) by incubation overnight at 4°C for 2 h at room temperature. Nonspecific binding sites were blocked with 1% heat-treated BSA for 1 h at room temperature. Cells were labeled with 2′,7′-bis-(carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), as previously described (18), and resuspended to 1×10^6/ml in L15/FBS. For cytochalasin D treatment, cells were preincubated with 10 μM of cytochalasin D for 30 min at 37°C with gentle agitation. Fifty microliters of cell suspension was mixed in ICAM-1-coated wells with an equal volume of L15/FBS containing PMA (100 ng/ml) and mAb (20 μg/ml of purified mAb, 1/100 dilution of ascites, or 1/2 dilution of hybridoma supernatant). For testing the effect of Mn^{2+} on cell binding to ICAM-1, BCECF-labeled cells were washed three times with 20 mM HEPES, pH 7.5, 140 mM NaCl, 2 mg/ml glucose, and 1 mM $MnCl_2$ (HEPES/NaCl/Mn), and resuspended in the same buffer to 1×10^6 cells/ml, and 100-μl cell suspension was added to ICAM-1-coated wells. The cells in the 96-well plates were spun down at 200 × *g* for 2 min at 4°C and incubated at 37°C for 20 min for Jurkat-$\beta_{2.7}$ transfectants and COS transfectants, and 30 min for K562 transfectants. At the end of the incubation, unbound cells were removed on a Microplate Autowasher (Bio-Tek Instruments, Winooski, VT), with a wash program setup of 250 μl dispense volume and 100 μl volume remaining after aspiration. The pressure applied to the autowasher was 2 psi, and the wash buffer was HBSS. Three wash cycles were performed. The fluorescence content of total input cells (before washing) and the bound cells (after washing) in each well was quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, ME). The bound cells were expressed as a percentage of total input cells per sample well. The washing procedure was programmed such that binding of mock-transfected cells or binding in the presence of LFA-1-blocking mAb was below 5% of input.

Aggregation assay

Jurkat-$\beta_{2.7}$ transfectants were washed twice with complete medium and resuspended to 3×10^6/ml in the same medium. One hundred microliters of the cell suspension was mixed with an equal volume of complete medium containing 100 ng/ml PMA, 20 μM cytochalasin D, or mAb (1/2 dilution of hybridoma supernatant or 20 μg/ml purified mAb), and added to each well of flat-bottom, tissue culture-treated 96-well plates. The plate was incubated at 37°C for 4 h, and the degree of aggregation was scored as previously described (11).

Radiolabeling and immunoprecipitation

For pulse-chase labeling with [^{35}S]cysteine, 2×10^7 cells were washed once with cysteine-free RPMI containing 15% dialyzed FBS, and resuspended to 5×10^6/ml in the same medium. After incubation for 45 min at 37°C, 500 μCi of [^{35}S]cysteine (ICN Biochemicals, Irvine, CA) was added to the cells and incubation was continued for 1 h. One-half of the labeled cells were harvested, and the remaining cells were chased for 16 h by addition of unlabeled L-cysteine to a final concentration of 250 μg/ml and an equal volume of fresh complete medium. For metabolic labeling with [^{3}H]palmitic acid, 2×10^7 cells were resuspended in 2 ml of RPMI 1640, 15% dialyzed FBS, and 5 mM sodium pyruvate, and labeled with 1 mCi [^{3}H]palmitic acid (Amersham, Arlington Heights, IL) for 16 h at 37°C. Labeled cells were washed twice with PBS, and 10^7 cells were lysed in 500 μl lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM iodoacetamide, 1 mM PMSF, 0.24 trypsin inhibitor unit (TIU)/ml aprotinin, and 10 μg/ml each of pepstatin A, antipain, and leupepstin) by incubation on ice for 30 min with occasional vortexing. Nuclei were removed by spinning at 12,000 × *g* for 10 min at 4°C, and cell lysates were precleared by incubation with 60 μl mouse IgG-coupled Sepharose beads (50% suspension, 3 mg Ab/ml beads) for 2 h at 4°C, with agitation. One-third of the precleared lysate was diluted to 500 μl with lysis buffer and incubated with TS1/22 or TS1/18-coupled Sepharose beads (20 μl of 50% suspension, 3 mg mAb/ml beads) for 1 h at 4°C, with agitation. Beads were washed three times with lysis buffer and one time with lysis buffer without detergent. The bound proteins were eluted in 50 μl of Laemmli sample buffer by heating for 5 min at 100°C, and the immunoprecipitates were analyzed by 7.5% SDS-PAGE (51). The gel was fixed, soaked in EN^{3}HANCE (DuPont, Wilmington, DE), dried, and exposed to Kodak X-OMAT XAR-5 film at −70°C for fluorography, or exposed to phosphor screen for quantitation of radioactivity of protein bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

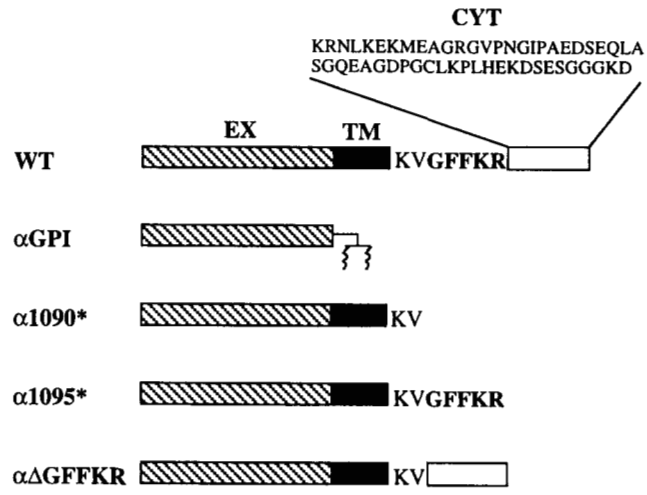

FIGURE 1. Schematic diagram of LFA-1 α subunit constructs. The wild-type α subunit is composed of the extracellular (EX), transmembrane (TM), and cytoplasmic (CYT) domains. The conserved cytoplasmic GFFKR sequence corresponding to amino acids 1090–1094 is adjacent to the transmembrane domain. Mutant αGPI was created by replacing the transmembrane and cytoplasmic domains with the LFA-3 sequence that signals reanchoring with GPI. Mutants α1090* and α1095* were generated by truncating the cytoplasmic domain before and after the conserved GFFKR sequence, respectively. Mutant αΔGFFKR contained an internal deletion of the GFFKR sequence.

Results

Expression of LFA-1 α subunit mutants in Jurkat-$\beta_{2.7}$ and in K562 cells

To examine whether the cytoplasmic domain of the α subunit is involved in the assembly of the LFA-1 αβ heterodimer and in the regulation of LFA-1-adhesive activity, we generated several α subunit mutants (Fig. 1). Mutant αGPI was constructed by replacing the α subunit transmembrane and cytoplasmic domains with the GPI-anchoring signal derived from LFA-3 (47). Mutant α1090* and α1095* were truncated before and after the highly conserved GFFKR sequence, respectively. In addition, an internal deletion of the GFFKR sequence (mutant αΔGFFKR) was generated. The wild-type α and mutant α subunit cDNAs were stably transfected into Jurkat-$\beta_{2.7}$ cells, which are deficient in the endogenous LFA-1 α subunit (39). To examine whether the α subunit specifies cell-type-specific affinity modulation of LFA-1, we also expressed the wild-type α and mutant α subunits in association with the wild-type β subunit in K562 erythroleukemia cells. LFA-1 surface expression in the transfected cells was evaluated by flow cytometry.

Despite several transfection attempts, no surface LFA-1 expression was observed in mutant αGPI-transfected cells, although intracellular GPI-anchored α subunit protein was detected (see below). Mutant α1090*-, α1095*-, and αΔGFFKR-transfected Jurkat-$\beta_{2.7}$ and K562 cells, after selection for surface expression of LFA-1 by panning on LFA-1 mAb-coated dishes, exhibited lower surface LFA-1 expression than the wild-type α-transfected cells, as shown by staining with mAbs specific for both the LFA-1 α (TS1/22) and β subunit (TS1/18) (Table I). Mutant α1090*-transfected cells expressed only 30% of the wild-type levels. The wild-type α and mutant α transfectants were therefore cloned, and clones that expressed comparable levels of surface LFA-1 were selected for additional experiments (Fig. 2). Clones of mutant αGPI transfectants were selected by their expression of intracellular α subunit protein.

Table I. *Flow cytometric analysis of LFA-1 surface expression on transfected cells*[a]

	Mean Fluorescence Intensity			
	Jurkat-β2.7 transfectants		K562 transfectants	
α Constructs	TS1/22	TS1/18	TS1/22	TS1/18
Mock	4	3	5	4
WT	117	105	107	96
α1090*	37	28	34	31
α1095*	73	61	79	66
$\alpha\Delta$GFFKR	50	45	63	59

[a] The indicated α subunit-transfected cells, after selection for LFA-1 surface expression by panning on LFA-1 mAb-coated dishes, and mock-transfected cells were stained with mAbs specific for LFA-1 α (TS1/22) and β (TS1/18), followed by incubation with FITC-conjugated goat anti-mouse IgG. Surface LFA-1 levels are expressed as mean fluorescence intensity.

Cytoplasmic domain and the conserved GFFKR sequence of the α subunit are important for efficient assembly of LFA-1 heterodimers

Although mutant αGPI-transfected cells did not express surface LFA-1, an αGPI protein with a molecular mass 6 to 7 kDa smaller than the wild-type α subunit precursor was immunoprecipitated from pulse-labeled Jurkat-$\beta_{2.7}$ transfectants with anti-α mAb TS1/22 (Fig. 3*A*, *lane 1* vs *lane 4*). The αGPI protein could be labeled metabolically with [^{3}H]palmitic acid (3*A*, *lane 8*), showing that a GPI anchor was added. By contrast, the wild-type subunit was not labeled with palmitate (Fig. 3*A*, *lane 7*). The mature form of the wild-type α subunit with a m.w. slightly higher than the precursor was precipitated after a 16-h chase (Fig. 3*A*, *lane 2*), and dimerized with the endogenous β subunit, as shown by coimmunoprecipitation with the TS1/18 mAb (Fig. 3*A*, *lane 3*) that recognizes the mature β subunit in $\alpha\beta$ heterodimers (40). In contrast, the αGPI protein did not dimerize with the endogenous β subunit, as no detectable $\alpha\beta$ complex was immunoprecipitated from chased cells with either anti-α (TS1/22) or anti-β (TS1/18) mAb (Fig. 3*A*, *lanes 5* and *6*). In the absence of dimerization, the α subunit is not matured and transported to the surface (52, 53). Thus, exchange of the transmembrane and cytoplasmic domains of the α subunit with a GPI anchor results in a lack of LFA-1 α and β subunit association.

The observation that mutant α1090*-, α1095*-, or $\alpha\Delta$GFFKR-transfected cells expressed reduced cell surface levels of LFA-1 (Table I) suggests that these mutations might affect the stability of the mutant proteins or the efficiency of α and β subunit dimerization. To address this, clones of transfected Jurkat-$\beta_{2.7}$ cells selected for comparable surface expression were pulse chase labeled with [^{35}S]cysteine, and LFA-1 α and β subunits were immunoprecipitated and quantitated on a PhosphorImager. Consistent with the data from flow cytometry (Fig. 2*A*), the levels of the mature α and β subunits immunoprecipitated by TS1/18 mAb from pulse-chased cells expressing mutant α1090*, α1095*, or $\alpha\Delta$GFFKR were similar and comparable with that of the mature wild-type α and β subunits (Fig. 3*B*). However, the levels of the α1090*, α1095*, and $\alpha\Delta$GFFKR precursors precipitated by TS1/22 mAb from pulse-labeled cells were 7.3-, 4.1-, and 5.5-fold, respectively, higher than the wild-type α precursor (Fig. 3*B* and data not shown). These results indicate that to express similar levels of LFA-1 heterodimer on the cell surface, cells need to synthesize more mutant α precursor than the wild-type α precursor. After 16-h chase, about 40% of the labeled wild-type α precursor was processed to the mature higher m.w. form. By contrast, only a small percentage (<10%) of the labeled mutant α precursors was processed to the mature forms (Fig. 3*B*, chase-TS1/22). Most of the labeled mutant α subunit proteins remained as unassociated precursor forms even after a longer chase period (38 h) that chased all of the wild-type α precursors to the mature form (data not shown). The mutations did not appear to affect the stability of the mutant α subunit protein, as no significant degradation of the mutant α precursors was observed after chase (Fig. 3*B*). In a further experiment, wild-type α subunit-transfected Jurkat-$\beta_{2.7}$ cells (clone 26), and mutant α1090* transfectants (clone 29) that expressed 4.9-fold less cell surface LFA-1, as determined by flow cytometry (data not shown), were pulse chase labeled. Although the levels of the mature α and β subunits precipitated from pulse-chased cells expressing mutant α1090* were 4.3-fold lower than cells expressing wild-type LFA-1 (Fig. 3*C*, chase-TS1/18), the level of the mutant α1090* precursor precipitated from pulse-labeled cells was 1.9-fold higher than that of the wild-type α precursor (Fig. 3*C*, pulse-TS1/22). The concentration of the endogenous β subunit was not limiting, because free β precursor was precipitated with mAb CBRLFA-1/2, which recognizes both free and complexed β, from pulse-chased cells expressing mutant α1090* (Fig. 3*C*, chase-CBRLFA-1/2). Furthermore, the ratio of precursor β to mature β was higher for α1090* than for wild type. Thus, truncation of the α subunit cytoplasmic domain or deletion of the conserved GFFKR sequence directly affects α and β subunit dimerization, resulting in reduced surface expression of the LFA-1 heterodimer.

Truncation of the α subunit cytoplasmic domain before the GFFKR sequence or deletion of this sequence results in constitutively active LFA-1

The adhesive properties of Jurkat-$\beta_{2.7}$ and K562 cells expressing the wild-type α or mutant α subunits were assessed by their ability to bind to purified ICAM-1 that was immobilized on plastic. In the absence of stimulation, Jurkat-$\beta_{2.7}$ cells transfected with the wild-type α subunit showed low basal binding to ICAM-1 (Fig. 4*A*). Truncation of the α cytoplasmic domain after the GFFKR sequence (α1095*) did not alter ICAM-1 binding of the transfectants (Fig. 4*A*). In contrast, Jurkat-$\beta_{2.7}$ cells that expressed mutant α1090* that was truncated before the GFFKR sequence exhibited more than fourfold higher basal control binding than the wild-type transfectants. This result suggests that the GFFKR sequence is an important regulator of LFA-1 adhesiveness. An internal deletion of this sequence ($\alpha\Delta$GFFKR) also resulted in constitutively high binding of transfectants to ICAM-1, further demonstrating the importance of this highly conserved sequence motif. ICAM-1 binding of Jurkat-$\beta_{2.7}$ cells expressing the wild-type α or mutant α1095* subunits could be increased by PMA or Mn^{2+}. However, the degree of stimulation by PMA was significantly lower for mutant α1095* transfectants than for the wild-type α transfectants ($p < 0.001$), suggesting that maximal stimulation of ICAM-1 binding by PMA requires the intact α cytoplasmic domain. By comparison, PMA and Mn^{2+} did not stimulate additional ICAM-1 binding of mutant α1090* and $\alpha\Delta$GFFKR transfectants. Treatment with cytochalasin D, which inhibits actin polymerization (54), did not significantly affect basal ICAM-1 binding of the wild-type α- or mutant α-transfected Jurkat-$\beta_{2.7}$ cells. However, PMA-stimulated binding of the wild-type α or mutant α 1095* transfectants was inhibited partially by cytochalasin D (Fig. 4*A*).

The effect of α subunit cytoplasmic domain truncation and deletion of the GFFKR sequence was also examined in K562 erythroleukemia transfectants. The results on binding to ICAM-1 were similar to those with Jurkat-$\beta_{2.7}$ transfectants, except that there was little to no basal control binding of unstimulated wild-type α- and mutant α1095*-transfected cells (Fig. 4*B*). In other words,

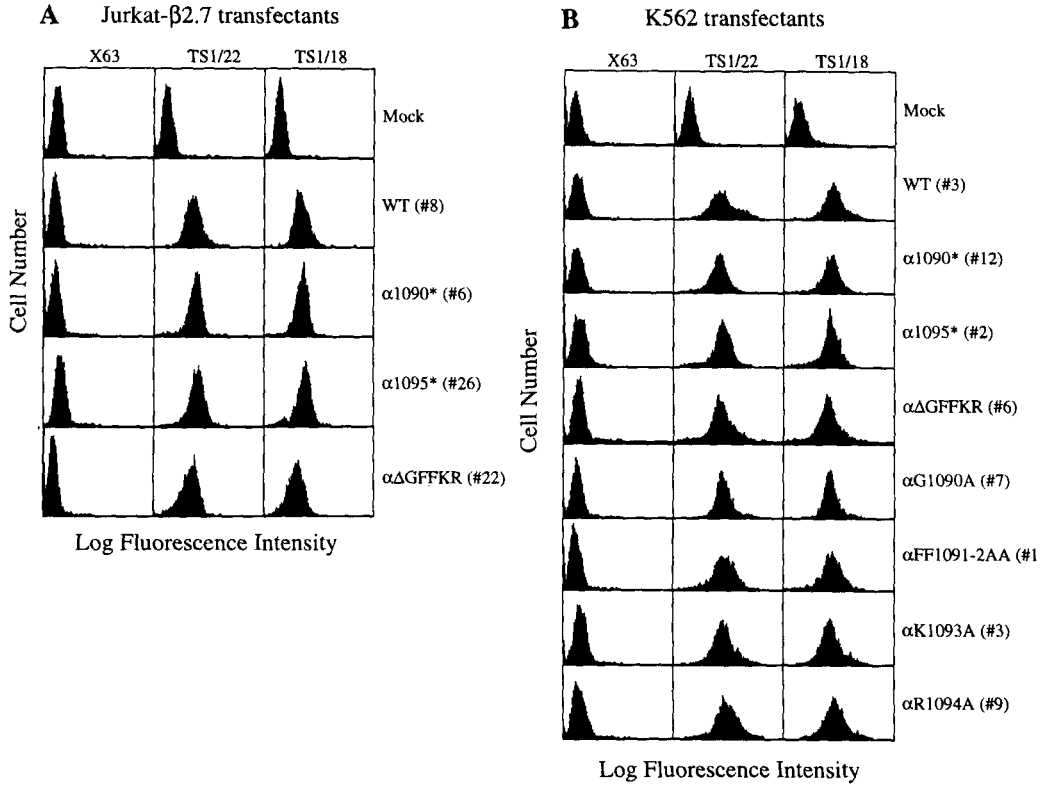

FIGURE 2. Immunofluorescence flow cytometry. Selected clones of Jurkat-$\beta_{2.7}$ (*A*) and K562 (*B*) cells stably transfected with the wild-type α or the indicated mutant α subunits or vector alone (mock) were stained with either anti-LFA-1 α (TS1/22) or anti-β (TS1/18) subunit mAbs or with a nonbinding negative control mAb (X63). Clone numbers are indicated in parentheses.

constitutive adhesiveness of LFA-1 was seen in Jurkat-$\beta_{2.7}$ transfectants, but not in K562 transfectants. K562 cells that expressed mutant α1090* or mutant αΔGFFKR showed high constitutive binding to ICAM-1. Whereas Mn^{2+} increased binding of the wild-type α and mutant α1095* transfectants to a similar level, PMA-stimulated binding of the wild-type α-transfected cells was greater than the α1095*-transfected cells. In conclusion, truncation of the α cytoplasmic domain before, but not after, the GFFKR sequence, or deletion of the GFFKR sequence, renders LFA-1 constitutively active in binding to ICAM-1 in a cell-type independent manner. However, truncation of the α cytoplasmic domain after the GFFKR motif reduces sensitivity to stimulation by PMA both in Jurkat-$\beta_{2.7}$ and K562 cells.

mAb 24 has been shown to recognize an epitope on activated LFA-1 (13, 23). Therefore, α subunit mutants that showed constitutively high binding to immobilized ICAM-1 were assessed for the expression of this epitope (Fig. 5). Jurkat-$\beta_{2.7}$ transfectants that expressed constitutively active mutants α1090* and αΔGFFKR expressed the mAb 24 epitope, whereas the wild-type α subunit transfectants did not.

The effect of the α subunit cytoplasmic domain mutations on LFA-1-adhesive activity was further examined in homotypic aggregation assays (Table II). In the control incubation, Jurkat-$\beta_{2.7}$ cells that expressed mutant αΔGFFKR were found in large aggregates after 4 h, and the formation of cell aggregates was inhibited completely by cytochalasin D or LFA-1-blocking mAb TS1/18. Jurkat-$\beta_{2.7}$ cells transfected with the wild-type α subunit, mutant α1090*, or mutant α1095* did not form cell aggregates in control incubations, and cell aggregation was induced to various degrees by addition of PMA. The PMA-stimulated cell aggregation was inhibited by cytochalasin D or mAb TS1/18. Although Jurkat-$\beta_{2.7}$ cells expressing mutant α1090* were functionally active in binding to immobilized ICAM-1, they did not aggregate without stimulation, implying that the α cytoplasmic domain is necessary for actin cytoskeleton-dependent motility or post-receptor-binding events that are required for cell aggregation.

LFA-1 constitutive ligand-binding activity is not blocked by protein phosphatase inhibitors nor by β subunit cytoplasmic domain mutations

It has been shown previously that okadaic acid, an inhibitor of protein phosphatase-1 and -2A, inhibits PMA-stimulated activation of LFA-1 (55), implicating a role for protein phosphatases in inside-out signaling. To examine whether constitutive ligand binding by GFFKR-deleted LFA-1 requires protein phosphatase activity, we treated Jurkat-$\beta_{2.7}$ cells that expressed the wild-type α, mutant α1090*, or mutant αΔGFFKR subunits with okadaic acid. Both basal and PMA-stimulated binding to ICAM-1 of the wild-type α subunit transfectants were inhibited markedly by incubation with okadaic acid. In contrast, okadaic acid treatment had no effect on binding of mutant α1090*- or mutant αΔGFFKR-transfected cells (Fig. 6). Thus, constitutive ligand binding resulting from removal of the GFFKR sequence by truncation or deletion is independent of protein phosphatase activity.

We further tested whether α subunit mutations that resulted in constitutively active LFA-1 could overcome the effect of β subunit cytoplasmic domain mutations. β subunit mutant βT758TT/AAA, which contains a substitution of alanines (A) for three contiguous threonines (T) at position 758–760, has been shown previously to abolish binding of transfected COS cells to ICAM-1 (32). We co-expressed the wild-type α or mutant α1090* subunits in association with βT758TT/AAA in COS cells. As shown in Figure 7,

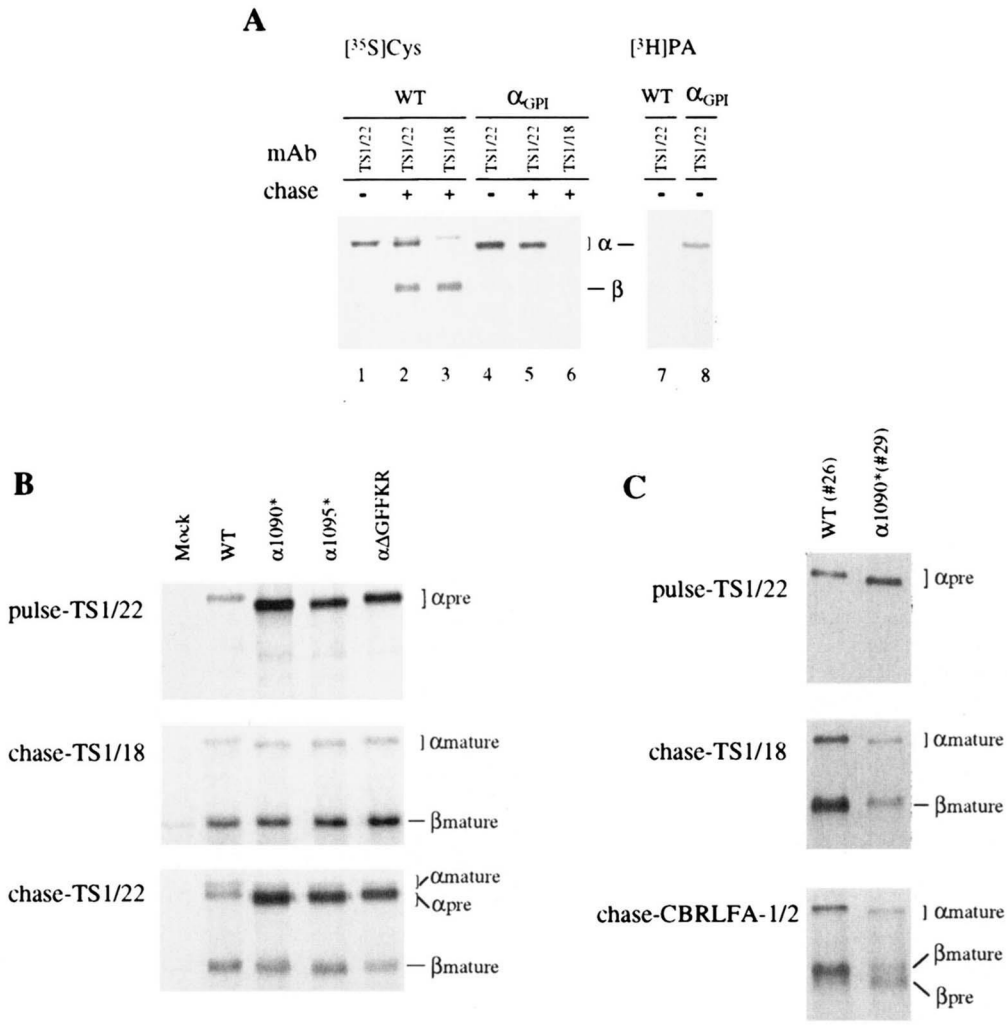

FIGURE 3. Effect of α subunit mutations on LFA-1 α and β subunit dimerization. *A*, A GPI-anchored form of the α subunit does not dimerize with the β subunit. The wild-type α- or mutant αGPI-transfected Jurkat-$\beta_{2.7}$ cells were pulse labeled with [^{35}S]cysteine for 1 h with (+) or without (−) a 16-h chase, or labeled with [^{3}H]palmitic acid for 16 h, as indicated. *B* and *C*, Truncation of the α subunit cytoplasmic domain or deletion of the conserved GFFKR sequence affects efficient dimerization of α and β subunits. *B*, Mock-transfected Jurkat-$\beta_{2.7}$ cells and clones of the wild-type α- or mutant α-transfected Jurkat-$\beta_{2.7}$ cells that expressed comparable surface LFA-1 levels, as determined by flow cytometry (Fig. 2*A*), were pulse labeled with [^{35}S]cysteine for 1 h and chased for 16 h. *C*, The wild-type α and mutant α1090* transfectants that expressed different levels of surface LFA-1 were pulsed for 1 h with [^{35}S]cysteine and chased for 16 h with unlabeled cysteine, as indicated. mAb TS1/22, TS1/18, and CBRLFA-1/2 are specific for α, β complexed with α, and β, respectively. Equal amounts of immunoprecipitates from lysates prepared from equal numbers of labeled cells were subjected to SDS-PAGE and fluorography. αpre, α precursor; βpre, β precursor.

COS cells that expressed mutant α1090* with the wild-type β subunit were able to bind to immobilized ICAM-1 to a level equivalent to COS cells expressing wild-type LFA-1. Whereas COS cells that expressed the wild-type α subunit in association with βT758TT/AAA exhibited impaired binding, as previously described (32), association of α1090* with βT758TT/AAA restored ICAM-1 binding to a level comparable with that seen with wild-type LFA-1. Thus, an activating mutation in the α subunit cytoplasmic domain of LFA-1 was dominant to an inactivating mutation in the β subunit cytoplasmic domain.

Identification of amino acids in the GFFKR motif that modulates LFA-1 adhesiveness

The results shown above demonstrate that the conserved GFFKR sequence motif within the α subunit cytoplasmic domain is critical in the regulation of LFA-1 adhesiveness. To further test which amino acids in the GFFKR motif are necessary, we mutated individual amino acids in this sequence to alanine and stably expressed the mutants with the wild-type β subunit in K562 cells. All of the α subunit point mutants, with the exception of αFF1091-2AA, were expressed on the surface of transfected K562 cells equivalently to the wild-type α subunit. Mutant αFF1091-2AA was expressed at 65% of the wild-type level. Clones of the wild-type α and mutant α transfectants that expressed similar levels of surface LFA-1, as determined by flow cytometry (Fig. 2*B*), were selected and tested for their ability to bind to purified ICAM-1 (Fig. 8). Substitution of alanines for the glycine (αG1090A), the two contiguous phenylalanines (αFF1091-2AA), or the arginine (αR1094A) in the GFFKR motif resulted in constitutively high binding of transfected K562 cells to ICAM-1. By contrast, substitution of the lysine (αK1093A) did not increase ICAM-1 binding as compared with the wild-type transfectants. The levels of ICAM-1 binding of mutants αG1090A, αFF1091-2AA, and αR1094A were similar to one another and comparable with the GFFKR deletion mutant αΔGFFKR. Thus, the glycine, at least one of the two phenylalanines, and the arginine in the GFFKR motif are necessary for maintaining LFA-1 low affinity.

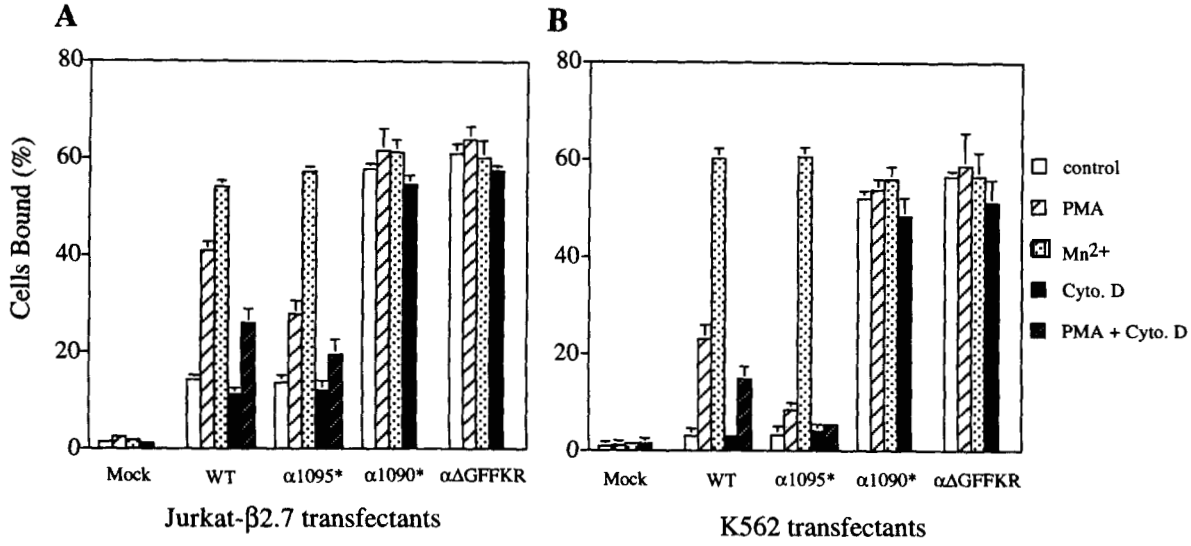

FIGURE 4. Binding of cells that stably express wild-type LFA-1 or LFA-1 with mutant α subunits to purified ICAM-1. *A*, Jurkat-$\beta_{2.7}$ transfectants. *B*, K562 transfectants. ICAM-1 was adsorbed on plastic, as described in *Materials and Methods*. Mock-transfected cells or clones of the wild-type α or mutant α subunit transfectants that expressed similar levels of surface LFA-1, as determined by flow cytometry (Fig. 2), were used for binding. Binding was performed in L15/FBS for PMA, cytochalasin D (Cyto.D), or control treatments. For Mn^{2+} stimulation, binding was conducted in HEPES/NaCl buffer supplemented with 1 mM of $MnCl_2$. For Cyto.D treatment, cells were preincubated with 10 μM of Cyto.D for 30 min at 37°C. Preincubation with Cyto.D did not affect cell viability, as determined by trypan blue staining. Binding of transfected cells was inhibited almost completely by LFA-1-blocking mAb TS1/22 or TS1/18 (>97% inhibition). Results are expressed as mean ± SD of triplicate samples and are representative of three independent experiments. At least two clones from each transfection were tested for ICAM-1 binding, and similar results were obtained as long as the clones expressed similar surface LFA-1 levels.

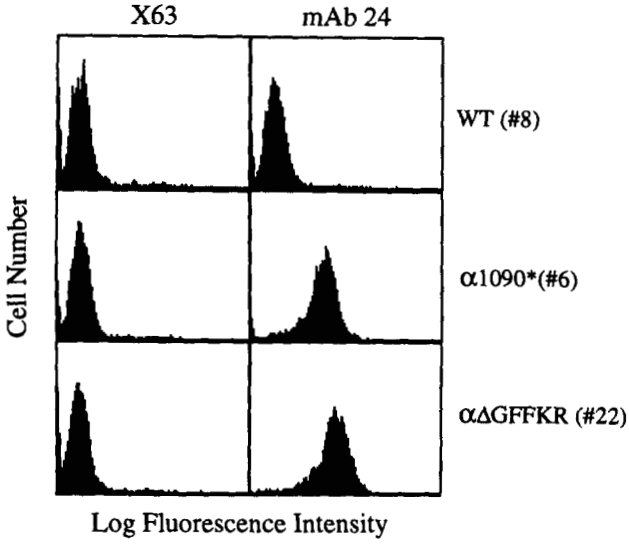

FIGURE 5. Recognition of constitutively active LFA-1 mutants by mAb 24. Jurkat-$\beta_{2.7}$ cells transfected with the wild-type α or the indicated mutant α subunits were stained with mAb 24 or a nonbinding control mAb (X63) and subjected to immunofluorescence flow cytometry. Selected clones of the wild-type α and mutant α transfectants expressed comparable levels of surface LFA-1, as stained with anti-LFA-1 mAbs TS1/22 and TS1/18 (Fig. 2*A*).

Table II. *Homotypic aggregation of Jurkat-β2.7 transfectants*[a]

α Constructs	Aggregation Score				
	Control	Cyto. D	PMA	PMA +Cyto. D	CBR LFA-1/2
Mock	0	0	0	0	0
WT	0	0	2+	0	3+
α1090*	0	0	1+	0	1+
α1095*	0	0	1+	0	1+
αΔGFFKR	4+	0	4+	0	2+

[a] The degree of cell aggregation was scored after 4 h of incubation at 37°C. Cytochalasin D (Cyto. D), PMA and CBRLFA-1/2 mAb were present in the assay medium during the incubation period. Score 0 indicates no cells were in aggregates; 1+ indicates that <10% of the cells were in aggregates; 2+ indicates that 10 to 50% of the cells were aggregated; 3+ indicates that 50 to 100% of the cells were in small loose aggregates; 4+ indicates that up to 100% of the cells were in large aggregates; and 5 indicates that all cells were in large, very compact aggregates.

Stimulatory Abs to LFA-1 inhibit adhesion of some of the constitutively active α subunit mutants

mAbs CBR LFA-1/2 and NKI-L16, which are specific for the β_2 subunit and the LFA-1 α subunit, respectively, have been shown previously to activate LFA-1-dependent cell aggregation and adhesion to ICAM-1 (12, 18). We investigated the effect of truncation of the α cytoplasmic domain and mutations in the GFFKR sequence on the ability of these stimulatory mAbs to modulate binding of transfected K562 and Jurkat-$\beta_{2.7}$ cells to ICAM-1. Clones that expressed comparable levels of wild-type and mutant LFA-1 also exhibited comparable cell surface expression of the CBR LFA-1/2 and NKI-L16 epitopes, as determined by immunofluorescence flow cytometry (data not shown). Binding to ICAM-1 of K562 cells that expressed wild-type LFA-1 or LFA-1 containing the mutant α1095* or αK1093A subunit was stimulated markedly in response to either NKI-L16 or CBR LFA-1/2 mAb (Fig. 9*A*). However, the degree of stimulation by these Abs was significantly lower for mutant α1095* transfectants than for the wild-type α subunit transfectants ($p < 0.001$), suggesting that maximal stimulation of ICAM-1 binding in response to stimulatory Abs requires an intact α subunit cytoplasmic domain. Whereas both Abs did not stimulate additional binding of constitutively active αG1090A and αR1094A mutants, these Abs significantly reduced binding of two other constitutively active mutants: αΔGFFKR and αFF1091-2AA ($p < 0.001$). The constitutively active α cytoplasmic domain truncation mutant α1090* exhibited lesser reduction than mutant

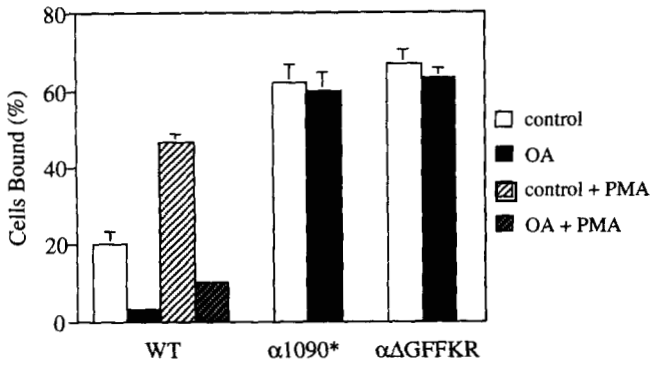

FIGURE 6. The protein phosphatase inhibitor, okadaic acid, does not inhibit the constitutive activation of LFA-1 resulting from removal of the α subunit GFFKR sequence. Jurkat-$\beta_{2.7}$ cells expressing the wild-type α or the indicated α mutant were incubated with 2 μM of okadaic acid (OA) for 15 min at 37°C before the ICAM-1-binding assay, or as control with the amount of DMSO used to solubilize okadaic acid (0.04%). ICAM-1 binding was performed in the absence or presence of PMA. Results are expressed as mean ± SD of triplicate samples and are representative of three independent experiments.

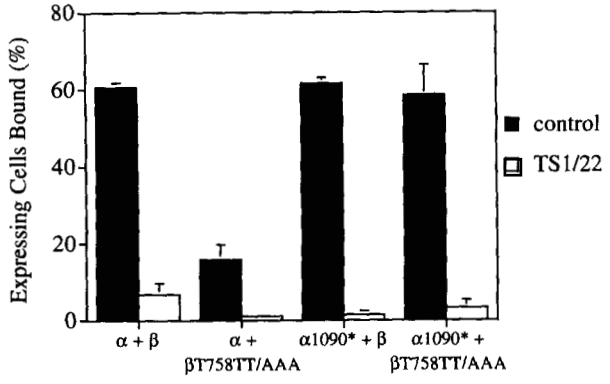

FIGURE 7. Constitutively activating α subunit cytoplasmic domain mutation is dominant over an inactivating β subunit cytoplasmic domain mutation. The wild-type α or mutant α1090* subunits were expressed transiently in COS cells in association with the wild-type β or mutant βT758TT/AAA subunits. The transfectants were analyzed for binding to purified ICAM-1 in the absence (control) or presence of LFA-1-blocking mAb TS1/22. Transfected COS cells were 53, 46, 27, and 27% LFA-1 positive, as determined by flow cytometry, for α + β, α + βT758TT/AAA, α1090* + β, and α1090* + βT758TT/AAA, respectively. The percentage of LFA-1 expressing cells bound was determined as percentage of bound cells divided by fraction of LFA-1 expressing cells. The data are the mean ± SD from a representative experiment done in triplicate.

αΔGFFKR or mutant αFF1091-2AA in response to these Abs. The inhibitory effect exerted by CBR LFA-1/2 mAb was greater than by NKI-L16 mAb (Fig. 9*A*). Similar results were obtained for Jurkat-$\beta_{2.7}$ transfectants in response to CBR LFA-1/2 mAb. Whereas CBR LFA-1/2 mAb increased binding of Jurkat-$\beta_{2.7}$ cells that expressed the wild-type α subunit or mutant α1095*, it significantly inhibited binding of constitutively active α mutants α1090*, αΔGFFKR, and αFF1091-2AA (Fig. 9*B*). Binding of the wild-type and mutant α subunit-transfected K562 and Jurkat $\beta_{2.7}$ cells to ICAM-1 was not affected by a nonstimulatory mAb CBR LFA-1/7 to the LFA-1 β subunit (Fig. 9, *A* and *B*).

To further evaluate the inhibitory effect of stimulatory Abs on adhesion of constitutively active α mutants, we examined homotypic aggregation of Jurkat-$\beta_{2.7}$ transfectants in response to

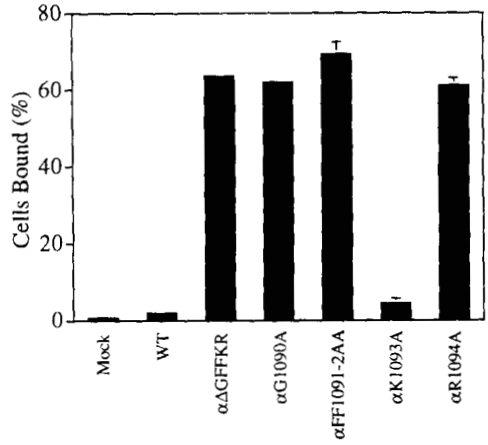

FIGURE 8. Effect of point mutations in the α subunit GFFKR sequence on binding of transfected K562 cells to ICAM-1. The wild-type α or the indicated mutant α subunit was stably expressed with the wild-type β subunit in K562 cells, and clones of transfected cells that expressed comparable levels of surface LFA-1 (Fig. 2*B*) were tested for binding to ICAM-1. The ICAM-1-binding assay was performed in L15/FBS medium in the absence of stimulation. Results are mean ± SD of triplicate samples and representative of at least three experiments.

mAb CBR LFA-1/2 (Table II). Whereas CBR LFA-1/2 stimulated cell aggregation of Jurkat-$\beta_{2.7}$ cells expressing the wild-type α subunit, mutant α1090*, or mutant α1095* to various degrees, it reduced the degree of cell aggregation of mutant αΔGFFKR transfectants. Thus, mAb CBR LFA-1/2 also inhibits cell-cell adhesion of Jurkat-$\beta_{2.7}$ cells expressing constitutively active α mutant αΔGFFKR.

Discussion

We have examined the role of the α subunit cytoplasmic domain and the highly conserved GFFKR sequence in the regulation of LFA-1 assembly and adhesion to ICAM-1. We demonstrate that the transmembrane and cytoplasmic domains of the α subunit are necessary for LFA-1 α and β subunit dimerization. Replacement of the α subunit transmembrane and cytoplasmic domains with a GPI anchor resulted in a lack of α and β subunit association and surface expression of LFA-1, although the possibility that the GPI-anchored α subunit and the transmembrane β subunit are spatially segregated en route to the cell surface cannot be ruled out (56). Truncation of the α cytoplasmic domain either before or after the GFFKR sequence greatly reduced the efficiency of α and β subunit dimerization, resulting in lower levels of surface LFA-1 on transfected cells. Our data are consistent with a previous report that cotransfection of LFA-1 β subunit with an α subunit mutant that lacked the transmembrane and cytoplasmic domains failed to result in surface LFA-1 expression on truncated COS cells, and that cotransfection with another α subunit mutant with the entire cytoplasmic domain truncated led to significantly reduced LFA-1 surface expression (57). Similar results were reported for α_2 and α_5 subunits. Deletion of the cytoplasmic domain of α_2 or α_5 led to reduced cell surface expression of $\alpha_2\beta_1$ or $\alpha_5\beta_1$ integrins (58, 59). A recent study showed that chimeric α_1 and β_1 subunits with reciprocally swapped cytoplasmic domains dimerized selectively and efficiently, whereas no dimerization occurred when the α cytoplasmic domain was replaced with the β cytoplasmic tail and vice versa (60). These observations suggest that the cytoplasmic domains of the α and β subunits associate, either directly or indirectly, and that this is important during integrin assembly and

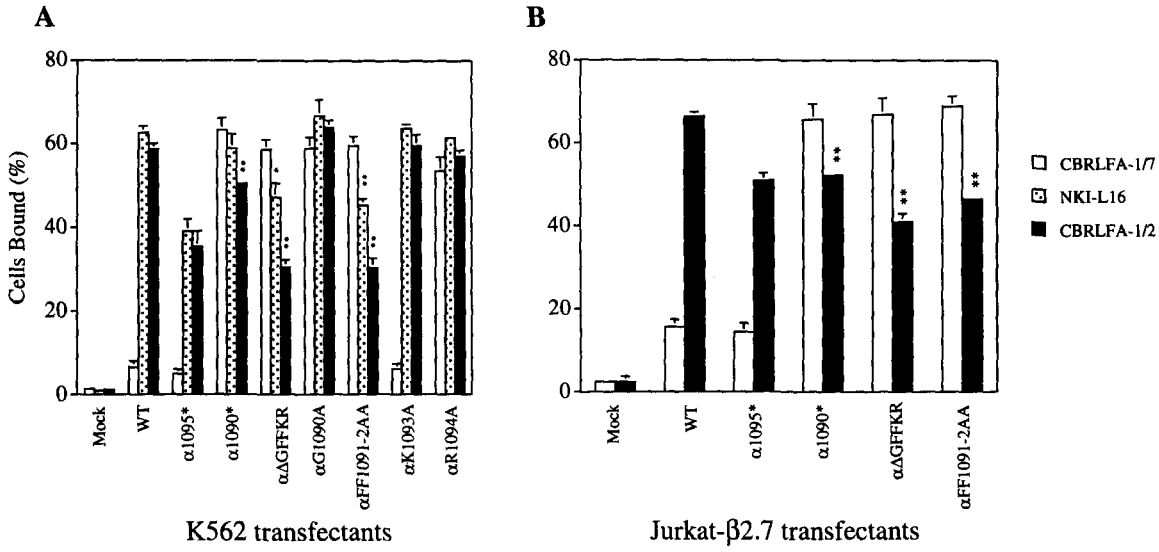

FIGURE 9. Effect of LFA-1-stimulatory Abs on binding to ICAM-1. *A*, K562 transfectants. *B*, Jurkat-$\beta_{2.7}$ transfectants. Cells transfected with the wild-type α or the mutant α subunits were incubated with NKI-L16 or CBR LFA-1/2 mAb, or as control with a nonstimulatory, noninhibitory mAb CBRLFA-1/7 to LFA-1 β subunit for 20 min on ice before the ICAM-1-binding assay. The data shown are the mean ± SD of triplicate samples and are representative of three independent experiments (Student *t* test, $*p \leq 0.05$, $**p \leq 0.001$.).

transport to the cell surface. Our finding that an internal deletion of the conserved GFFKR motif affects LFA-1 α and β dimerization directly implicates this membrane-proximal sequence motif in integrin assembly. It has been shown recently that deletion of the membrane-proximal region in the β subunit cytoplasmic tail alters the patterns of LFA-1 assembly and secretion (57). Therefore, it is possible that the membrane-proximal regions of the α and β cytoplasmic domains interact directly.

The availability of the Jurkat-$\beta_{2.7}$ cell line that lacks the endogenous LFA-1 α subunit allowed us to express α subunit mutants and study the effect of mutations on LFA-1 function in a physiologic cytoplasmic environment. We found that LFA-1 lacking the entire α subunit cytoplasmic domain (mutant α1090*) or the conserved GFFKR sequence (mutant αΔGFFKR) mediated strong binding of transfected cells to ICAM-1 in the absence of stimulation. In contrast, cells expressing wild-type LFA-1 or LFA-1 with the α subunit cytoplasmic domain truncated after the GFFKR sequence (mutant α1095*) required activation to bind to ICAM-1. Similar observations have been made with the $\alpha_{IIb}\beta_3$ integrin. Truncation that eliminates the GFFKR motif results in constitutively active $\alpha_{IIb}\beta_3$, whereas a truncation mutant that retains this motif remains inactive in the absence of stimulatory mAb (35). Moreover, deletion of the VGFFK sequence from a chimeric α subunit composed of the extracellular and transmembrane domains of α_{IIb} and cytoplasmic domain of α_L also switches $\alpha_{IIb}\beta_3$ into a high affinity state (36). Our results together with these previous observations suggest that the conserved GFFKR motif helps to maintain a low avidity (inactive) state of integrins, and further extend previous results by demonstrating this in a cell type in which the integrin is normally expressed, i.e., in a physiologic transmembrane and cytoplasmic environment. The expression of the activation reporter mAb 24 epitope on constitutively active LFA-1 mutants α1090* and αΔGFFKR suggests that removal of the conserved GFFKR sequence causes a conformational change that favors ligand binding.

We further demonstrated that mutations of certain amino acids in the GFFKR motif could activate LFA-1. We identified the Gly, at least one of the Phe, and the Arg as important residues for maintaining the low avidity state of LFA-1. The Lys was not important. A recent report showed that Ala substitution of each of the Phe and the Arg in the GFFKR motif of the α_{IIb} subunit activates $\alpha_{IIb}\beta_3$ integrin, whereas Ala substitution of the Gly and the Lys had minimal effect (61). The amino acids in the GFFKR motif that we and others identified to be important for integrin function might be required for maintaining the structural integrity of the GFFKR motif and/or participating in interactions with the membrane-proximal region in the β subunit cytoplasmic domain. It has been proposed that the Arg in the α_{IIb} subunit and the conserved Asp (D723) in the β_3 cytoplasmic domain form a salt bridge that might act as a structural constraint to prevent the activation of the $\alpha_{IIb}\beta_3$ integrin (61). A similar residue (D731) is present in the β_2 integrin cytoplasmic domain (62, 63).

We showed that high constitutive binding of transfectants to ICAM-1 resulting from removal of the GFFKR motif either by truncation or by deletion was insensitive to cytochalasin D treatment, whereas PMA-stimulated binding was partially inhibited by cytochalasin D. This suggests that the removal of the GFFKR motif may increase the affinity of LFA-1 for ICAM-1 rather than promote post-receptor-binding events dependent on the cytoskeleton. However, homotypic cell aggregation promoted by deletion of the GFFKR motif was inhibited by cytochalasin D. Cytochalasin D also blocked PMA-stimulated cell aggregation. Cytochalasin inhibits cell motility that is required for homotypic aggregation (11), and this could explain why aggregation but not adhesion to purified ICAM-1 was inhibited by cytochalasin. Peter and O'Toole recently demonstrated that cytochalasin D inhibited adhesion of Chinese hamster ovary cells bearing a chimeric integrin $\alpha_{IIb}\alpha_{L\Delta}/\beta_3\beta_2$ without affecting the high affinity state of the integrin (64). This observation led the authors to propose that post-receptor events regulate adhesiveness of the chimeric integrin. Whether the enhanced adhesion conferred by deletion of the GFFKR motif results from high affinity binding of the integrin to the ligand or post-receptor-binding events, such as cytoskeleton reorganization, is probably dependent on the individual integrin and/or the cell type in which the integrin is expressed.

We demonstrated that the high affinity state of LFA-1 induced by the removal of the GFFKR motif is independent of intracellular signaling, and resistant to a β cytoplasmic domain mutation that

inactivates the integrin. LFA-1 activation through inside-out signaling requires protein phosphatase activity (55). We confirmed that both basal and PMA-stimulated LFA-1 adhesiveness in Jurkat T lymphoblasts were diminished by the protein phosphatase inhibitor okadaic acid. By contrast, okadaic acid did not affect adhesiveness conferred by removal of the GFFKR sequence. We also demonstrated that the constitutively active state of LFA-1 could overcome a β subunit cytoplasmic mutation that abolishes ligand binding of the wild-type LFA-1. A similar observation has been made with the chimeric integrin $\alpha_{IIb}\alpha_L/\beta_3\beta_2$. High affinity state of the integrin resulting from deletion of the VGFFK sequence is independent of the β subunit cytoplasmic domain (36). Reciprocally, constitutive activity of the $\alpha_{IIb}\beta_3$ integrin conferred by complete truncation of the β_3 cytoplasmic domain does not require the α subunit cytoplasmic domain (65). Therefore, it appears that cytoplasmic domain mutations cause integrins to adopt an active conformation, reminiscent of the activated conformation induced by binding of Mn^{2+} to the extracellular domains of integrins, which is independent of active cellular processes and the cytoplasmic domains of integrins (14, 22).

The sequences on the C-terminal side of the GFFKR motif appear to mediate physiologic activation in some integrins, including VLA-2, whose α cytoplasmic domain mediates up-regulation of adhesion in response to PMA (59). The cytoplasmic tails of α_1 and α_{IIb} have been implicated in ligand-dependent focal contact localization of $\alpha_1\beta_1$ and $\alpha_{IIb}\beta_3$ integrins (60, 66, 67). We have shown that truncation of LFA-1 α cytoplasmic domain after the GFFKR sequence reduces activation of LFA-1 adhesiveness in response to PMA or stimulatory Abs. This result suggests the existence of certain element(s) in the α cytoplasmic domain that is required for full activation of LFA-1 by either PMA or stimulatory Abs.

We found that stimulatory Abs to LFA-1, CBR LFA-1/2 and NKI-L16, can inhibit adhesion of some of the constitutively active forms of LFA-1. It is unlikely that the Abs caused clearance of LFA-1 mutants from the cell surface, since the levels of cell surface LFA-1, as stained with the same Abs under the conditions used in the adhesion assays (25 min at 37°C), were comparable for wild-type LFA-1 and LFA-1 mutants (data not shown). There is evidence that suggests that stimulatory Abs directly induce a conformational change that increases integrin affinity for ligands (17, 18, 27, 68, 69). Binding of stimulatory mAb to some constitutively active forms of LFA-1 conferred by α cytoplasmic domain mutations might cause a reverse conformational change of the integrin. Alternatively, stimulatory mAb might sterically hinder ligand binding. Although mutants α1090*, $\alpha\Delta$GFFKR, αFF1091-2AA, αG1090A, and αR1094A exhibited similar ligand-binding activities, they might have other distinct structural changes that determine how stimulatory mAb binding changes the integrin affinity state.

We have demonstrated that the membrane-proximal GFFKR sequence in the α subunit cytoplasmic domain plays an important role in LFA-1 heterodimer assembly and in maintaining LFA-1 low affinity state. Although the molecular mechanism is still unclear, our data support the model that the GFFKR sequence interacts with the membrane-proximal portion of the β subunit cytoplasmic tail, and constrains the integrin into an inactive conformation. Mutations in the GFFKR sequence would remove the constraint, resulting in constitutive activation of the integrin.

Acknowledgments

We thank Dr. Chi-chi Huang for valuable discussions, Dr. Shuqi Chen for statistic analysis of the data, Mr. Mark Ryan for flow-cytometric analysis, and Dr. Nancy Hogg for providing mAb 24.

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