The Cytoplasmic Domain of the Integrin Lymphocyte Function-associated Antigen 1 β Subunit: Sites Required for Binding to Intercellular Adhesion Molecule 1 and the Phorbol Ester–stimulated Phosphorylation Site

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Summary

We have defined the regions of the cytoplasmic domain of the leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1) that are required for active binding of its extracellular domain to intercellular adhesion molecule 1 (ICAM-1). The NH$_2$-terminal 28 amino acids in the cytoplasmic domain are dispensable, but a segment of 5 amino acids including three contiguous threonines (758–760) and Phe 766 in the COOH-terminal third of the cytoplasmic domain are required for binding to ICAM-1. Mutation and phosphoamino acid analysis show that Ser 756 is the major residue phosphorylated in response to phorbol ester. Furthermore, multiple mutations demonstrate that serine phosphorylation can be dissociated from phorbol ester–stimulated binding of LFA-1 to ICAM-1. The sites we have defined are previously unremarked, are well conserved in the β1, β3, and β7 integrin subunits, and may be of broad importance in regulating adhesiveness of integrins.

Lymphocyte function-associated antigen 1 (LFA-1)[1] is a member of a family of cell-cell adhesion molecules known collectively as the leukocyte integrins. The leukocyte integrins have a common β subunit (CD18) and distinct α subunits: αL, LFA-1, CD11a; αM, Mac-1, CD11b; αX, p150,95, CD11c. LFA-1 is involved in CTL and NK cell–mediated cytolysis, antibody-dependent cytotoxicity mediated by monocytes and granulocytes, helper T lymphocyte responses to antigen presentation, and leukocyte adhesion to endothelial cells, fibroblasts, and epithelial cells (1). Two counter-receptors for LFA-1, intercellular adhesion molecules 1 and 2 (ICAM-1 and -2), have thus far been identified, and recent evidence suggests a third (2–5). Interaction with the ICAMs requires activation of LFA-1 (6, 7). This activation event can be triggered by engagement of the TCR complex, or stimulation with phorbol esters. Activation results in the ability of LFA-1 to bind stably to ICAM-1 without any change in the quantity of LFA-1 per cell. Since this is measured as cellular binding to purified ICAM-1 on a substrate and involves multivalent LFA-1/ICAM interactions, it is referred to as a change in avidity; it may reflect conformational changes in the extracel-

lular domain of LFA-1. PMA induces a sustained increase in LFA-1 avidity, whereas T cell receptor crosslinking induces a transient increase in avidity that peaks 10 min after crosslinking and declines by 30 min (6). This may provide a mechanism for regulating T cell adhesion and de-adhesion with antigen-presenting and target cells. Pharmacologic studies suggest that the high avidity state of LFA-1 is stimulated by protein kinase C, and inhibited by protein kinase A. By contrast to this regulatability, the ICAMs are constitutively avid for LFA-1.

Although it has been suggested that phosphorylation of the β subunit may activate the high avidity state of leukocyte integrins (8, 9), this requires further study. The α subunit of LFA-1 is constitutively phosphorylated, whereas the β subunit is phosphorylated in response to phorbol esters (10), correlating with induction of adhesiveness through LFA-1 (6). The findings that the induction of the high avidity state of LFA-1 is not due to quantitative changes in density and that LFA-1 can go from low to high avidity after antigen receptor crosslinking, return to the low avidity state, and then after restimulation with phorbol ester can return to the high avidity state all within 40 min (6) suggest that modifications of a reversible nature, such as phosphorylation, are good candidates for the regulatory mechanism. Phorbol esters stimu-

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[1] Abbreviations used in this paper: ICAM, intercellular adhesion molecule; LAD, leukocyte adhesion deficiency; LFA, lymphocyte function-associated.

late phosphorylation of the β subunit associated with the α subunits of Mac-1, LFA-1, and p150,95 on neutrophils and mononuclear cells, correlating with induction of prolonged adhesiveness; the chemoattractant fMLP stimulates more transient adhesiveness and has been reported either to induce transient phosphorylation (8) or not to stimulate phosphorylation (11) of the CD18 β subunit. The phorbol ester–stimulated phosphorylation of the β subunit for both LFA-1, Mac-1, and p150,95 has been reported to be predominantly on serine residues (8, 10). Studies to date have provided correlative rather than direct evidence that phosphorylation of the β subunit regulates adhesiveness. An alternative interpretation is that phosphorylation activates a distinct protein that promotes transition of LFA-1 to a high avidity state, perhaps by binding to a critical region of LFA-1, or that phosphorylation is not critical to avidity regulation at all.

Establishing the contact sites on leukocyte integrins that are important to avidity regulation is key to unraveling the nature of the covalent modifications or noncovalent interactions with other molecules that provide the regulatory signals. Studies to date have shown that the β subunit cytoplasmic domain is required (12), but specific sequence motifs within the cytoplasmic domain have not been defined. Deletion of the cytoplasmic domain of the LFA-1 β subunit, but not the α subunit, eliminates binding to ICAM-1 and prevents stimulation of LFA-1 by PMA. The importance of the β subunit cytoplasmic domain for integrin function is also suggested from cDNA cloning studies that show very strong evolutionary conservation of the leukocyte integrin β but not α subunit cytoplasmic domain sequences (reviewed in reference 12). Although the COOH-terminal five amino acids of the β subunit were shown to be crucial for retention of the adhesive properties of LFA-1, other important regions in the cytoplasmic domain were not identified due to the deleterious effect of truncating this region on ICAM-1 binding. Similar truncation studies on the integrin β1 or VLA subfamily show that cytoplasmic domain truncation abolishes ability to localize at focal contacts where both the extracellular matrix and the cytoskeleton are closely associated with the plasma membrane (13–15), but that solubilized truncated receptors bind to fibronectin (13). In this study, we define a cluster of three threonine residues and a single phenylalanine, located in the COOH-terminal third of the β subunit cytoplasmic domain, that are necessary for ligand binding. We demonstrate that Ser 756 in the β subunit cytoplasmic domain is the major site phosphorylated in response to PMA, but that phosphorylation of this residue can be dissociated from regulation of LFA-1 avidity.

Materials and Methods

Monoclonal Antibodies. All mAbs have been described previously. W6/32 is a mouse mAb specific for human HLA-A,B and was used as a control in these studies (16). RR1/1 is a mAb against ICAM-1 (2). TS1/22 is a mAb directed against the LFA-1 α subunit, and TS1/18 and R15.7 are mAbs that recognize the β subunit (17, 18).

Cell Lines and cDNA Transfection. COS cells are SV40-transformed monkey kidney fibroblasts (19) and were used for transient expression of wild-type and mutant LFA-1 molecules. COS cells

were cotransfected with purified LFA-1 α and β subunit cDNAs contained in the transient expression vector CDM8 (20, 21) as described (22). An EBV-transformed B lymphoblastoid cell line derived from a patient with severe leukocyte adhesion deficiency (LAD) (patient 2, SLA) (23) was used to generate stable cell lines expressing mutant LFA-1 molecules. β subunit mutants were subcloned into the EBV-based vector p205L18a and transfected into the LAD patient B lymphoblastoid cells as described (20). An LAD patient cell line derived from patient 2 that expresses the wild-type β subunit has been described (20). Flow cytometric analysis was used to quantitate cell surface expression of the transfected COS and LAD B lymphoblastoid cells (20).

Construction of Mutant β Subunits. β subunit mutants were generated by oligonucleotide-directed mutagenesis in CDM8, using second-strand synthesis on uracil-containing templates made in a dut^- ung^- mutant host followed by selection against the wild-type, uracil-containing template in a dut^+ ung^+ host (24). All mutations were verified by nucleotide sequencing of the region encoding the cytoplasmic domain.

Purification of ICAM-1 and Binding of Transfected Cells to Purified ICAM-1. ICAM-1 was purified from lysates of splenocytes from patients with hairy cell leukemia and absorbed to the wells of a microtiter plate as described (20). Binding of patient LAD transfectants to purified, plastic-bound ICAM-1 was performed as described (20). Binding of COS cell transfectants to ICAM-1 was as described (20), except that 2 × 10^4 cells were added to each well and incubation was carried out at room temperature for 1 h.

Radiolabeling, Immunoprecipitation, and Electrophoresis. COS cell transfectants and transfected B lymphoblastoid cells were harvested and washed four times with phosphate-free medium (30 mM Hepes, pH 7.4, 110 mM glucose, 110 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 2 mg/ml BSA). The cells were resuspended in 1 ml phosphate-free medium containing 2 mCi ^{32}P (ICN Biomedical, Costa Mesa, CA) and incubated at 37°C for 2 h. After washing once, each cell type was divided into two and either incubated for 10 min at 37°C with medium alone or with 125 ng/ml PMA. Labeled cells were solubilized in 1 ml 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 50 mM NaH$_2$PO$_4$, 50 mM NaF, 25 mM Na$_4$P$_2$O$_7$, 10 mM iodoacetic acid, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 1% NP-40, 1 μg/ml pepstatin, 1 μg/ml antipain, and 5 μg/ml leupeptin. The extract was centrifuged at 16,000 g for 15 min at 4°C, and hemoglobin was added to the supernatant to 10 mg/ml. LFA-1 was immunoprecipitated with an anti-β subunit mAb directly conjugated to Sepharose (R15.7, a generous gift of Dr. R. Rothlein, Boehringer-Ingelheim, Danbury, CT) for 1 h at 4°C. Immunoprecipitates were washed twice with lysis buffer containing 1 mg/ml hemoglobin, twice with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and once with 20 mM Tris-HCl, pH 6.8, then boiled and run on reducing 7.5% SDS-polyacrylamide mini-gels (25). The coomassie-stained gels were dried and autoradiographed using Kodak XAR film with Kodak X-Omatic intensifying screens. Both the coomassie-stained gels and autoradiographs were scanned using an Ultroscan XL densitometer (LKB Instruments, Gaithersburg, MD).

Phosphoamino Acid Analysis. The β subunit band was excised from the dried gel and digested with 10 μg trypsin in 200 μl 50 mM ammonium bicarbonate overnight at 30°C. The supernatant was removed and the gel slice lyophilized. The trypsin digestion was repeated using 5 μg trypsin in 100 μl 50 mM ammonium bicarbonate for 6 h. The supernatants were pooled and lyophilized. The pellet was resuspended in 300 μl 6 N HCl and heated to 110°C under vacuum for 90 min in an hydrolysis tube (Pierce Chemical Co., Rockford, IL). The hydrolysate was lyophilized and resuspended

in 3 μl of pH 1.9 buffer (7.8% acetic acid, 2.2% formic acid) containing 1 μg each of phosphoserine, phosphothreonine, and phosphotyrosine standards. The sample was spotted on a TLC plate (T-6890; Sigma Chemical Co., St. Louis, MO) and subjected to electrophoresis in pH 1.9 buffer at 1,500 V for 45 min using an electrophoresis unit (2117; LKB Instruments). The plate was then dried and rewet with pH 3.5 buffer (5% acetic acid, 0.5% pyridine). After a 90° rotation, the sample was subjected to electrophoresis in the second dimension in pH 3.5 buffer at 1,000 V for 35 min. The plate was then dried and sprayed with 1% ninhydrin to reveal the position of the phosphoamino acid standards. The radiolabeled phosphoamino acids were visualized by autoradiography as above.

Results

Identification of Important Amino Acids at the COOH Terminus of the β Subunit. Mutagenesis studies have demonstrated that the cytoplasmic domain of the LFA-1 β subunit is crucial for regulation of adhesion of LFA-1 to ICAM-1 (12). More specifically, these studies have shown that the COOH-terminal five amino acids of the β subunit are required. To determine which amino acid(s) in this COOH-terminal region were necessary for adhesion regulation, a series of β subunit point mutants were constructed (Fig. 1). The β subunit point mutants were cotransfected into COS cells with the wild-type LFA-1 α subunit, and their ability to bind to ICAM-1 was examined (Fig. 2). All of these β subunit point mutants could be expressed on the surface of COS cells to essentially the same degree as wild-type LFA-1 (not shown). Removal of the COOH-terminal five amino acids of the β subunit (mutant BK765*) severely diminished ICAM-1 binding as previously demonstrated; however, deletion of the COOH-terminal two amino acids including the COOH-terminal serine (mutant βE768*) had no effect (Fig. 2). Mutation of the phenylalanine at position 766 to either an alanine or a leucine (βF766A or βF766L) reduced ICAM-1 binding to the levels observed when the COOH-terminal five amino acids were removed. With the conservative substitution of tyrosine for phenylalanine (βF766Y), LFA-1 function was retained. Substitution of the remaining amino acids in this COOH-terminal five amino acid segment, K765 and A767, had no effect on ICAM-1 binding. Thus, a single amino acid in this region of the β subunit, F766, is required for LFA-1 function.

Identification of Other Important Functional Regions in the β Subunit Cytoplasmic Domain. To determine whether there are other regions within the β subunit cytoplasmic domain that are essential for adhesion to ICAM-1, several internal deletion mutants were constructed (Fig. 1). These deletion mutants, when cotransfected with the wild-type LFA-1 α subunit cDNA, were expressed on the surface of COS cells to the same level as wild-type LFA-1 (not shown). Deletion of either 17 or 28 amino acids from the NH₂ terminus of the β subunit cytoplasmic domain had no effect on binding of LFA-1 to ICAM-1, demonstrating that the first 28 amino acids in the β subunit cytoplasmic domain are not required for adhesion (Fig. 3). By contrast, removal of the five amino acids between S756 and M762 (mutant βS756ΔM762)
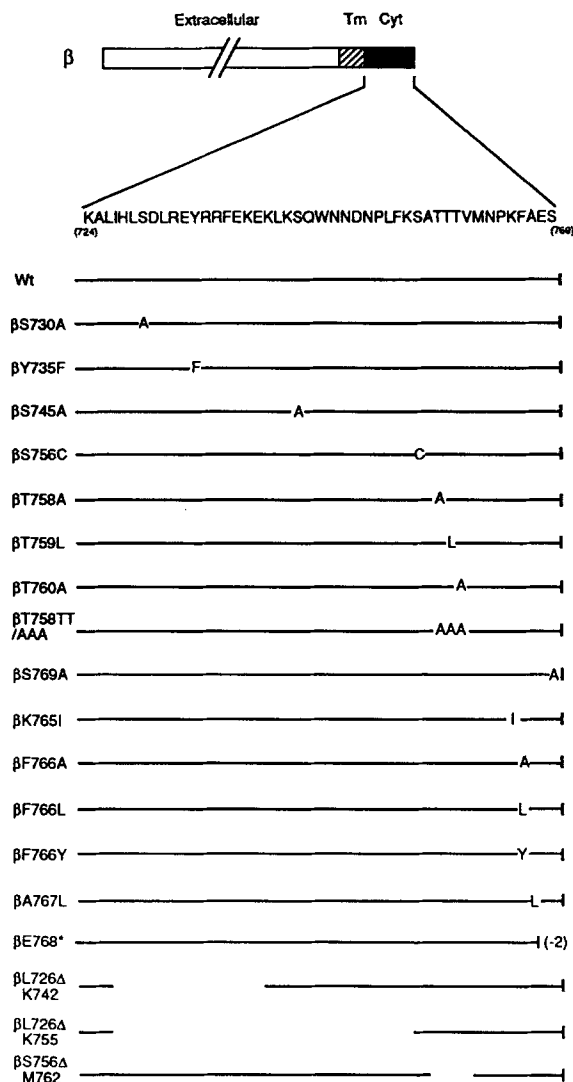

Figure 1. Schematic representation of the wild-type β subunit showing extracellular, transmembrane (Tm), and cytoplasmic (Cyt) domains, and β subunit point and deletion mutants. The sequence of the β subunit cytoplasmic domain beginning at K724 is shown. Nomenclature for the point mutations: βS730A indicates that S730 in the unprocessed β subunit has been mutated to an alanine. Nomenclature for the deletion mutants: βL726ΔK742 indicates a deletion that joins L726 to K742.

significantly reduced ICAM-1 binding, indicating that either the amino acids in this region were important for adhesion or that the structural integrity of the cytoplasmic domain had been disturbed in this mutant. Additional mutagenesis studies of amino acids in this region (see below) support the first hypothesis.

Functional Analysis of Potential Phosphorylation Sites in the Cytoplasmic Domain of the β Subunit. To determine whether direct phosphorylation of the β subunit of LFA-1 is responsible for the LFA-1 avidity change, point mutants to all potential phosphorylation sites in the cytoplasmic domain of the β subunit were made (Fig. 1). These mutants were first tested for their ability to bind to ICAM-1 (Fig. 4), and then their
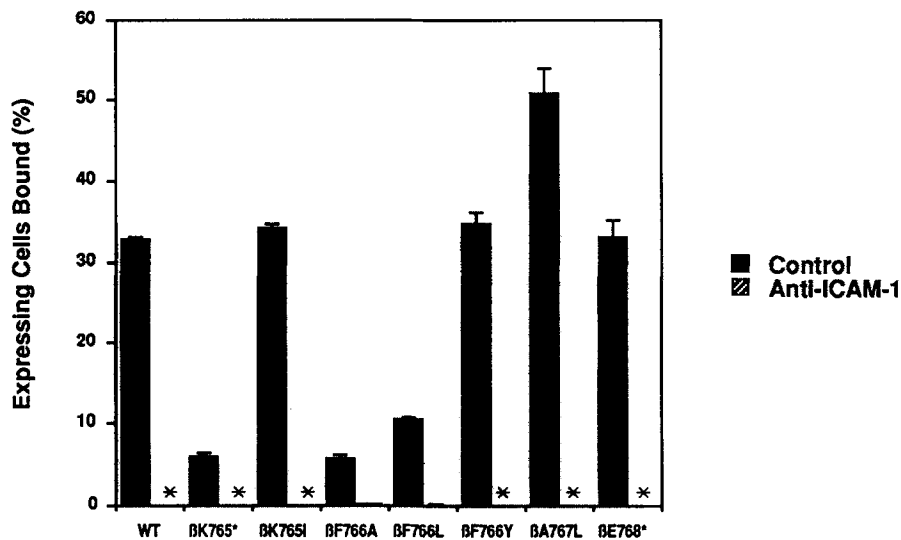
Figure 2. Binding of COS cells expressing LFA-1 with mutations in the COOH-terminal five amino acids of the β subunit to purified ICAM-1. Binding was performed in the presence of anti-HLA-A,B (control; one-fourth dilution of supernatant, *solid bars*) or anti-ICAM-1 (RR1/1; one-fourth dilution of supernatant, *hatched bars*; binding was 0 in most cases so bars are not visible as denoted by asterisks). Cell surface expression of LFA-1 was determined by flow cytometry using an anti-LFA-1 α subunit mAb (TS1/22) before each binding assay. Cell binding was corrected for the levels of LFA-1 expressed by the different transfectants. Results are expressed as mean ± SD. This experiment is representative of four independent trials.

phosphorylation status was determined (see below). The cytoplasmic domain of the β subunit contains eight potential sites of phosphorylation (Fig. 1), and previous studies have suggested that phosphorylation is on serine residues (8, 10). Substitution of the four serine residues in the cytoplasmic domain individually to amino acids incapable of being phosphorylated had little, if any, effect on ICAM-1 binding (Fig. 4 *A*). In addition, mutation of the four serine residues in combination was not deleterious to ICAM-1 binding and, in fact, surface expression and ICAM-1 binding by this mutant appeared to be marginally enhanced (not shown). Similarly, mutation of the single tyrosine residue in the β subunit cytoplasmic domain to a phenylalanine had no influence (Fig. 4 *A*). In contrast, alteration of each of the three contiguous threonine residues in the β subunit cytoplasmic domain (T758, T759, T760; Fig. 1), had a significant effect on the adhesive capacity of LFA-1 (Fig. 4 *B*). Mutation of each of the threonine residues individually resulted in a 50% reduction in LFA-1

function, and when the three threonines were mutated in combination, there was almost complete loss of function (Fig. 4 *B*). These results parallel those seen with the nonfunctional deletion mutant βS756ΔM762 in which all threonine residues were deleted (Figs. 1 and 3), and indicate that the three threonine residues in the β subunit cytoplasmic domain are essential requirements for normal LFA-1 adhesion.

Phosphorylation of the LFA-1 β Subunit. Phosphorylation was initially examined using COS cells transiently expressing mutant β subunits. LFA-1 expressed in COS cells is believed to be in a constitutively active state as no stimulation is required for adhesion to ICAM-1 and phorbol ester treatment has little effect on adhesion (21). The β subunit is constitutively phosphorylated to a low level in COS cells transiently expressing wild-type LFA-1, and, in contrast to the lack of effect on adhesion, a three- to five-fold increase in phosphorylation of the β subunit is observed after treatment with PMA (Fig. 5, *A* and *B*). The phosphoamino analysis of the wild-
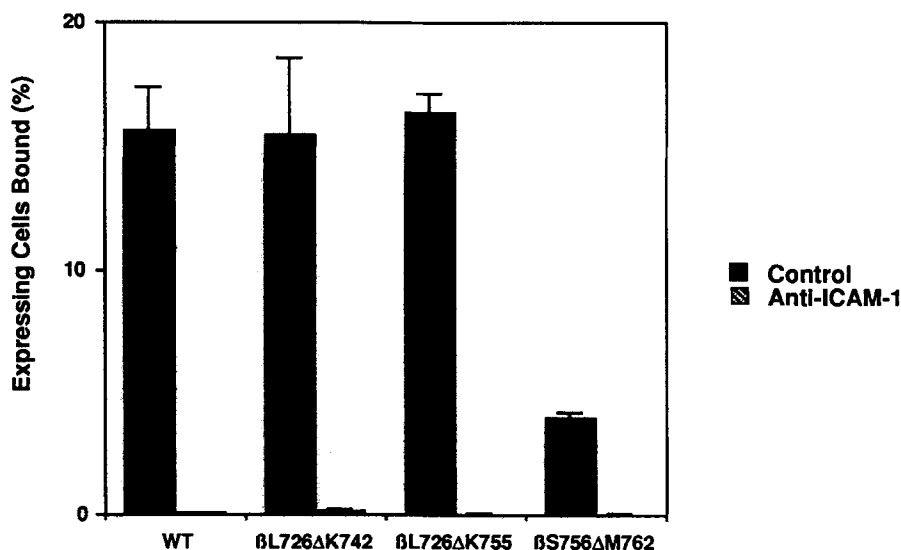

Figure 3. Binding of COS cells expressing LFA-1 with internal deletions in the β subunit cytoplasmic domain to purified ICAM-1. Binding was performed in the presence of anti-HLA-A,B (control) or anti-ICAM-1 (RR1/1) and as described in the legend to Fig. 2.

A

Expressing Cells Bound (%)

Control
Anti-ICAM-1

WT βS730A βY735F βS745A βS756C βS769A

B

Expressing Cells Bound (%)

Control
Anti-ICAM-1
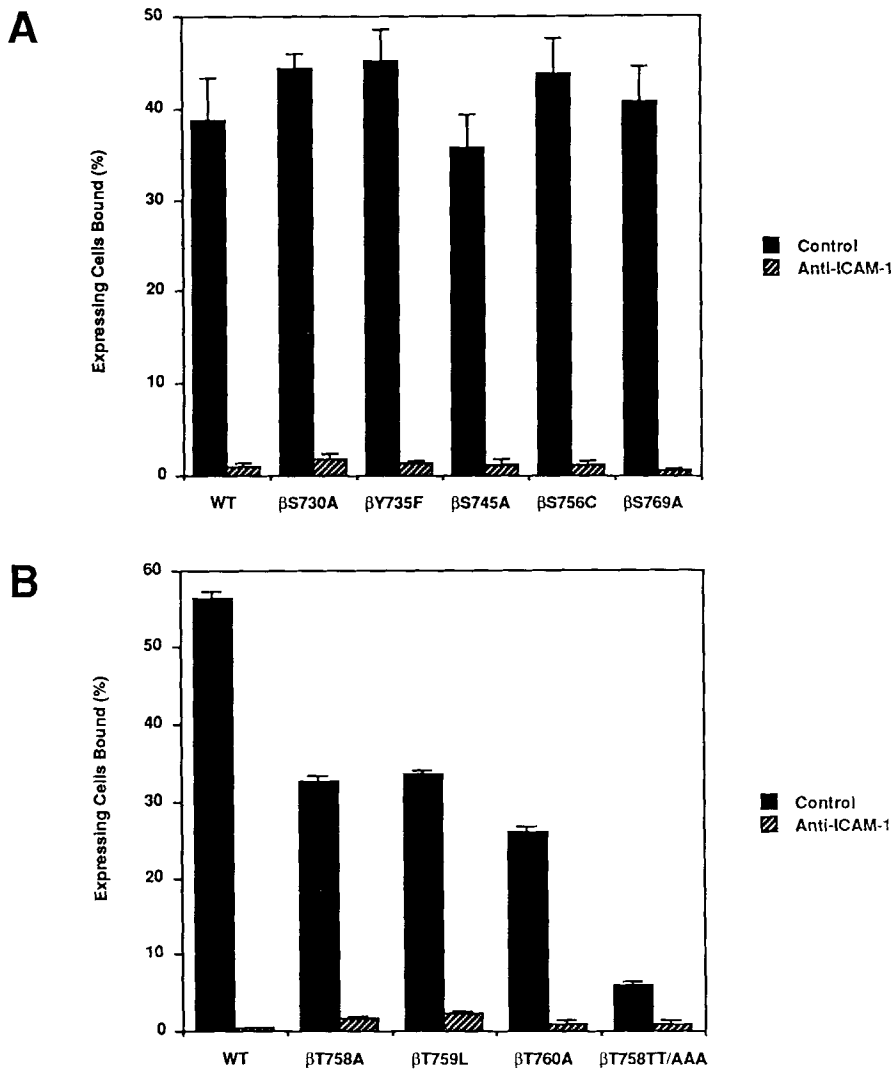
WT βT758A βT759L βT760A βT758TT/AAA

Figure 4. Effect of mutation of potential phosphorylation sites in the cytoplasmic domain of the β subunit on transfected COS cell binding to ICAM-1. (*A*) Serine and tyrosine mutations; (*B*) threonine mutations. Binding was performed in the presence of anti-HLA-A,B (control) or anti-ICAM-1 (RR1/1) as described in Fig. 2.

type β subunit from PMA-stimulated cells established that the phosphorylation occurs on serine with a much smaller amount of threonine phosphorylation (Fig. 5 *C*). To determine if there was a direct correlation between phosphorylation of the LFA-1 β subunit and the change in LFA-1 avidity, the phosphorylation status of all β subunit serine, tyrosine, and threonine mutants was analyzed. LFA-1 was isolated by immunoprecipitation and SDS-PAGE gels were subjected to coomassie blue staining and then autoradiography. Both the stained gel and the autoradiogram were subjected densitometry, which confirmed that: (*a*) similar but slightly varying amounts of mutant and wild-type LFA-1 were precipitated (Fig. 5 *A*); (*b*) the ratio of ^{32}P to protein in the α subunit was the same in all precipitates; and (*c*) this ratio of ^{32}P to protein was the same for the α subunit with and without PMA. Therefore, to correct for small variations in the efficiency of mutant LFA-1 expression or immunoprecipitation, β subunit phosphorylation is expressed as the percent of the phosphorylation of the α subunit in the same lane. The elimination of PMA-stimulated phosphorylation in the mutant

βS756C identifies serine 756 as the major phosphorylation site. Little, if any, of the PMA-stimulated phosphorylation could be attributed to the other three potential serine phosphorylation sites (Fig. 5, *A* and *B*) or the potential tyrosine phosphorylation site. Analysis of β subunit mutants that had truncated cytoplasmic domains (Hibbs et al.) and lacked S756 demonstrated very little phosphorylation and no enhancement with PMA (not shown). Of the three contiguous threonine residues, mutation of T758 abolished PMA-stimulated phosphorylation, whereas mutation of T759 and T760 had little effect (Fig. 5, *A* and *B*). The reduction of phosphorylation caused by the mutant βT758A could not be accounted for by an effect on phosphothreonine; phosphothreonine is present in lower amount than phosphoserine after PMA stimulation, suggesting that the replacement of threonine to alanine at 756 affects phosphorylation of the nearby serine 756 residue. Further evidence for an effect on phosphorylation by T758 was obtained from mutant βT758TT/AAA, in which all three threonines had been mutated in combination. β subunits immunoprecipitated from PMA-treated cells ex-
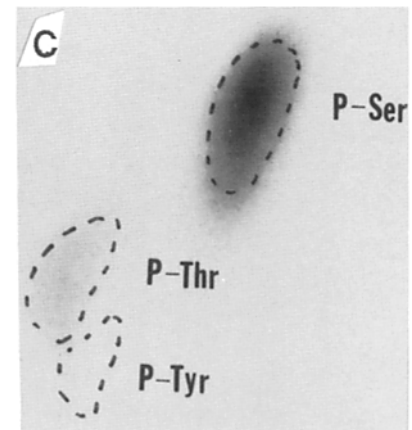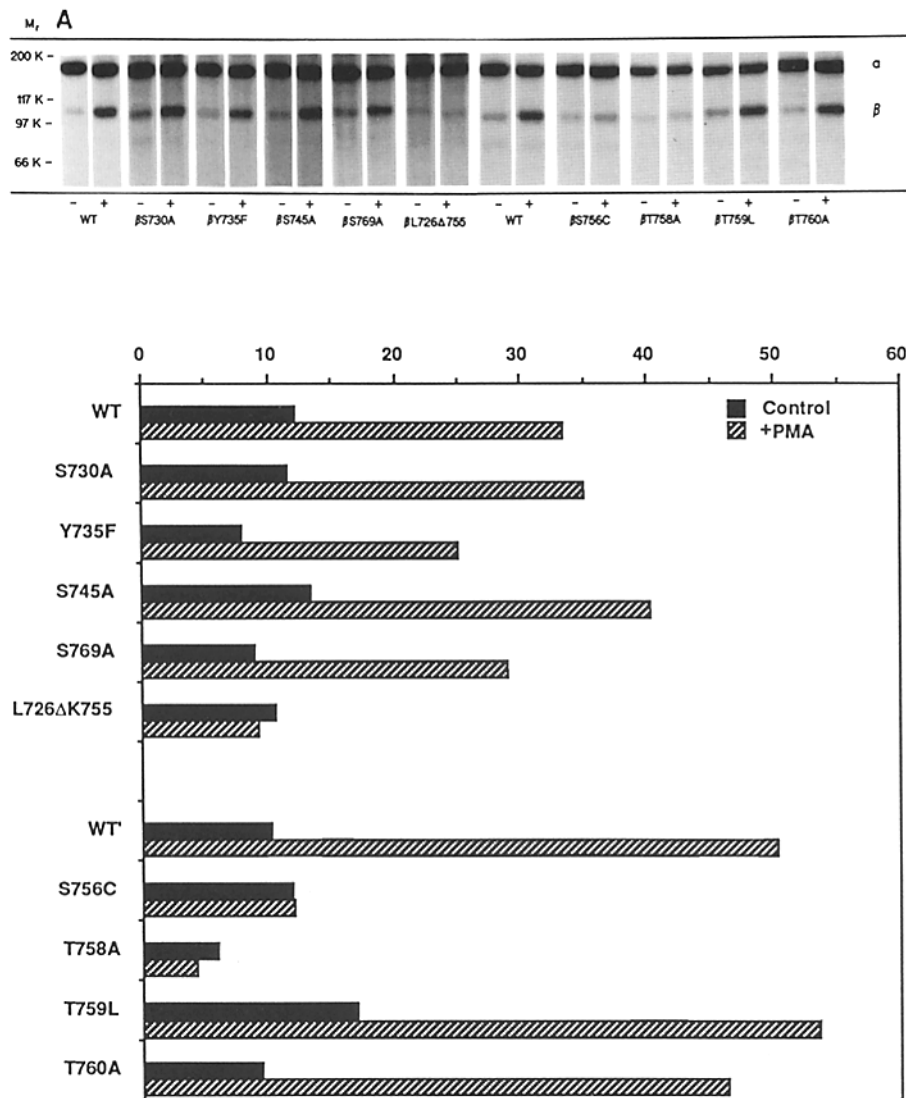
Figure 5. Identification of the phosphorylation sites in the β subunit cytoplasmic domain using COS cells expressing mutant β subunits. (*A*) Autoradiograph of ^{32}P-labeled LFA-1 immunoprecipitated from COS cells cotransfected with the wild-type LFA-1α subunit and either the wild-type β subunit, or the designated mutant β subunit. Cells were either treated with medium alone "–" or with 125 ng/ml PMA "+" before lysis and immunoprecipitation. Two separate, representative experiments are shown, each with a wild-type control. (*B*) Scanning densitometry of the β subunit bands shown in *A*. β subunit phosphorylation is expressed as a percentage of the phosphorylation of the α subunit in the same immunoprecipitate, as described in the text. (*C*) Phosphoamino acid analysis of the β subunit immunoprecipitated from COS cells transfected with wild-type LFA-1 and treated with PMA.

pressing this mutant showed baseline levels of phosphorylation (not shown). Upstream residues also were important for kinase recognition, because a deletion terminating at the lysine just NH$_2$ terminal to S756 (L726ΔK755) abolished PMA-stimulated phosphorylation (Fig. 5, *A* and *B*).

To examine β subunit phosphorylation sites in a more physiological cell type, each of the phosphorylation site point mutants was expressed in an EBV-transformed B lymphoblastoid cell line derived from a patient with LAD. This cell line lacks expression of LFA-1 due to a single point mutation in the extracellular domain of its β subunit, which presumably affects α/β association (26), and has been used successfully before for transfection studies (12, 20). Flow cytometric analysis of hygromycin-resistant cell lines transfected with the wild-type β subunit or the β subunit point mutants demonstrates that expression of LFA-1 α and β subunits has been reconstituted on the surface of these cells (Fig. 6). Equivalent levels of LFA-1 were expressed by the different transfectants. Untransfected

cells show very low levels of expression of LFA-1 (Fig. 6; reference 12).

Phosphoamino acid analysis of the β subunit immunoprecipitated from PMA-stimulated LAD B lymphoblastoid cells expressing wild-type LFA-1 demonstrated that the major phosphorylation site occurs on serine and that there is a small amount of threonine phosphorylation (Fig. 7 *A*), corroborating the results in COS cells.

Phosphorylation studies using the LAD patient B lymphoblastoid cells expressing each of the phosphorylation site point mutants confirmed the results with the COS cell transfectants (Fig. 7, *B* and *C*). β subunits immunoprecipitated from cell lines expressing either βS756C or βT758A showed a lack of PMA-stimulated phosphorylation compared with β subunits immunoprecipitated from cells expressing wild-type LFA-1 or the other six β subunit phosphorylation site point mutants. The phosphoamino acid analysis coupled with the point mutation phosphorylation studies unequivocally
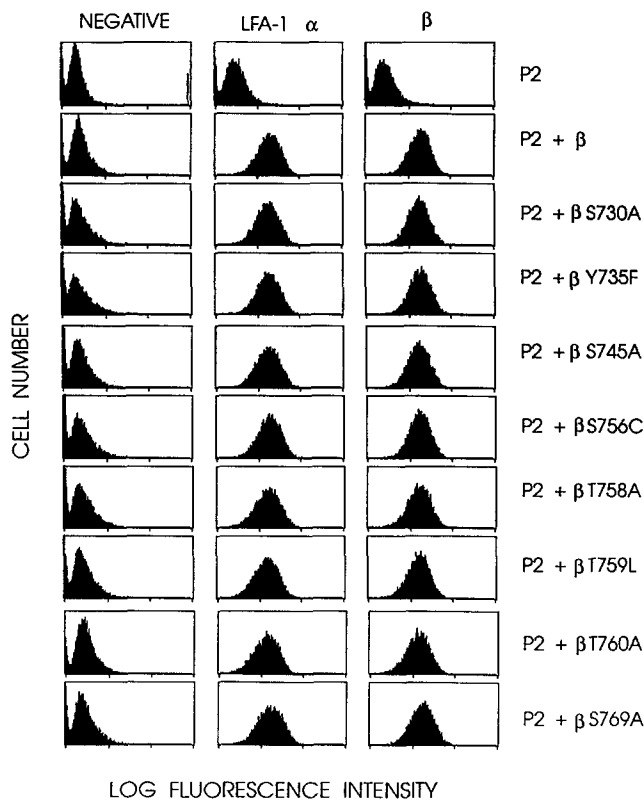
Figure 6. Cell surface expression of β subunit point mutants following transfection of B lymphoblastoid cells derived from an LAD patient (P2). The indicated transfectants were stained with either anti-LFA-1α subunit (TS1/22), or anti-β subunit mAbs (TS1/18), or a nonbinding negative control mAb (×63).

demonstrate Ser 756 is phosphorylated in response to PMA. The studies further demonstrate that phosphorylation of Ser 756 is affected by mutation of Thr 758.

Lack of Correlation between β Subunit Phosphorylation and En-hancement of LFA-1 Avidity. In an attempt to directly corre-late β subunit phosphorylation with the LFA-1 avidity change, transfected EBV-transformed B lymphoblastoid cells were tested for their ability to bind to ICAM-1 in the presence and absence of PMA (Fig. 8). A transfectant with a cyto-plasmic domain truncation mutation which has previously been shown to be defective in ICAM-1 binding (P2+βD731*; reference 12), was used as a negative control. It shows no basal or stimulated binding to ICAM-1. B cells stably ex-pressing the point mutant βS756C, which was not phos-phorylated in response to PMA, were both able to bind to ICAM-1 and to be stimulated in the presence of PMA (Fig. 8). The amount of stimulated binding was equivalent to wild type. Unstimulated B cells expressing mutants to each of the three Thr residues consistently bound to ICAM-1 to lower levels than cells expressing wild-type LFA-1. ICAM-1 binding in the absence of PMA was reduced to ∼20% of normal. However, binding to ICAM-1 was still greatly enhanced by PMA in each of the three mutants. Thus, although phos-phorylation of β subunit threonine is found after PMA stim-

ulation, phosphorylation of a specific threonine residue is not required for enhanced avidity of LFA-1. Furthermore, although mutants βS756C and βT758A are incapable of being phos-phorylated in response to PMA, their binding to ICAM-1 is still stimulated by PMA. This demonstrates that phosphory-lation of the β subunit can be dissociated from the change in avidity of LFA-1 for ICAM-1 in response to PMA. B cells stably expressing β subunit mutations in the other potential phosphorylation sites in the cytoplasmic domain behaved like cells transfected with the wild-type β subunit; the cells could bind to ICAM-1, and their adhesive capacity was enhanced in the presence of PMA.

Discussion

We have identified key amino acids in two COOH-terminal regions of the cytoplasmic domain of the β subunit of the β2 integrin LFA-1 that are important for adhesiveness. These residues are F766, which occurs four amino acids from the COOH terminus of the β subunit, and a string of three threo-nines, T758–760. Since activation of adhesiveness is not a unique property of the β2 integrins, and has been reported for the β1 and β3 integrins as well (reviewed in reference 1), the structural features we have defined may have broader relevance. The cytoplasmic domains of the β1, β2, and β3 integrin subunits are highly homologous in the regions we have defined as important in avidity regulation (Fig. 9). The β6 and β7 subunits, and less so the β5 subunit, and not the β4 subunit, are also closely related in this region. Phenylala-nine 766 in the integrin β2 cytoplasmic domain is conserved as phenylalanine or tyrosine in β1, β3, β6, and β7. This corre-lates with our observation that substitution with tyrosine resulted in retention of binding to ICAM-1, whereas non-conservative substitutions were deleterious. The other four residues in the COOH-terminal segment could be deleted or substituted without affecting binding to ICAM-1; these are not conserved in the two other integrins that are known to undergo avidity regulation, β1 and β3. The three contig-uous threonines required for binding to ICAM-1 show similarity to motifs in other integrin β subunits. They align with two contiguous threonines in β1, threonine-serine-threonine in β3 and β5, three contiguous threonines in β7 that are displaced one residue COOH terminally, and serine-threonine in β5. Thus, the sequence motifs we have defined that are important for the adhesiveness of the β2 integrins may also participate in regulation of adhesiveness of the β1, β3, β6, and β7 integrins. The β4 cytoplasmic domain is most distinctive in sequence and also in cytoplasmic associations; it localizes to hemidesmosomes and thus associates with the keratin cytoskeleton (27, 28).

Observations that the β subunit of LFA-1, Mac-1, and p150,95 is phosphorylated in response to PMA, whereas the LFA-1, Mac-1, and p150,95 α subunits are constitutively phos-phorylated (8, 10, 11), has led to speculation (8, 9), which we have tested, that phosphorylation of the β subunit is respon-sible for the increased avidity of integrins on stimulated cells. The first step was to identify the phosphorylation site(s). Mu-tation of all the potential phosphorylation sites in the cyto-
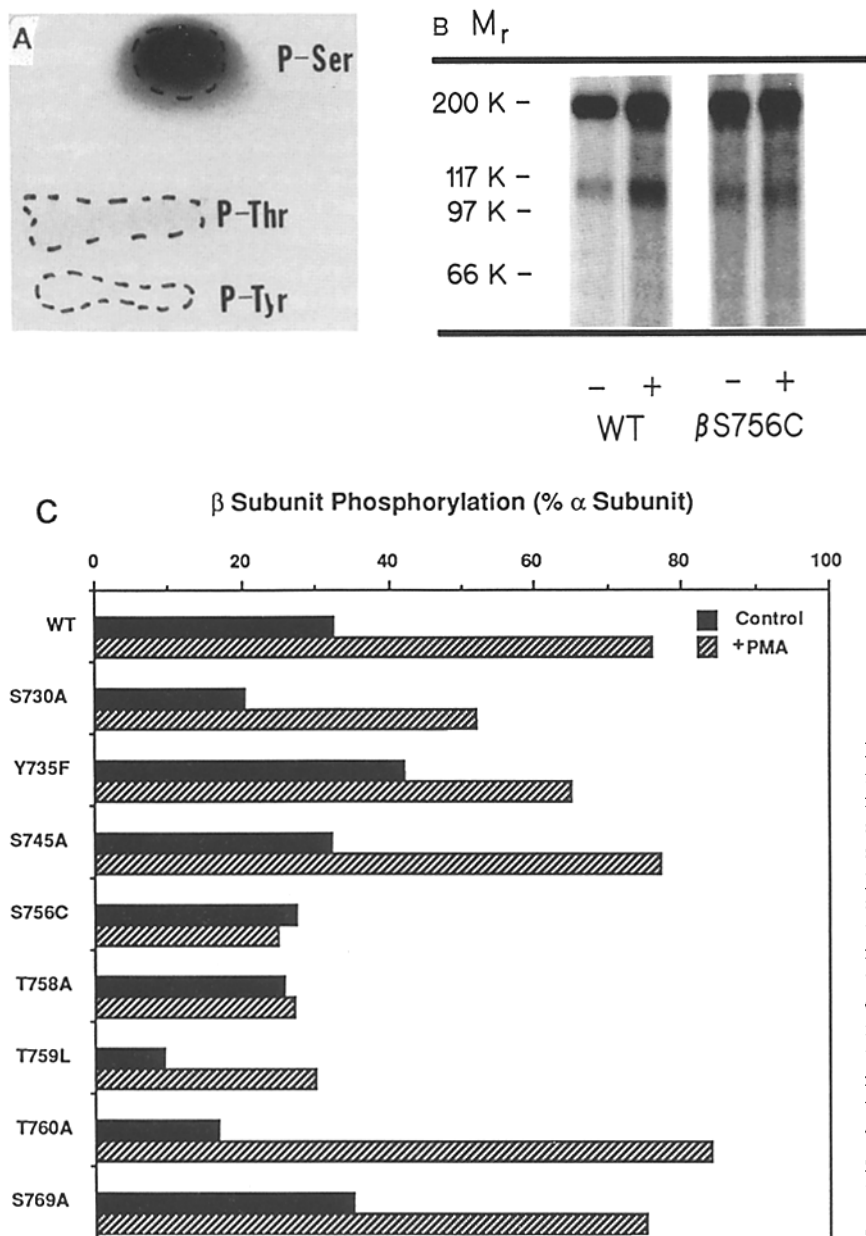
Figure 7. Identification of the phosphorylation sites in the β subunit cytoplasmic domain using stably transfected B lymphoblastoid cells expressing mutant β subunits. (A) Phosphoamino acid analysis of the β subunit immunoprecipitated from LAD patient B lymphoblastoid cells transfected with the wild-type β subunit and treated with 125 ng/ml PMA. (B) Autoradiograph of ³²P-labeled LFA-1 immunoprecipitated from LAD B lymphoblastoid cells transfected with either the wild-type β subunit, or βS756C. Cells were either treated with medium alone "−" or with 125 ng/ml PMA "+" before lysis and immunoprecipitation. (C) Scanning densitometry of the β subunit immunoprecipitated from LAD B lymphoblastoid cells transfected with either the wild-type β subunit or the designated mutant β subunit. Phosphorylation of the β subunit is presented as a percent of the phosphorylation of the α subunit in the same immunoprecipitate; phosphorylation of α did not vary with PMA or β subunit mutation, as described for COS cells.

plasmic domain of the β subunit together with the phosphoamino acid analysis of the β subunit from wild-type PMA-stimulated cells allowed us to identify serine 756 as the major residue in the β subunit that is phosphorylated in response to PMA. Our phosphoamino acid analysis agrees with previous studies that have shown that serine is the principal phosphorylated residue (8, 10, 11). While the most obvious protein kinase responsible for PMA-induced phosphorylation is protein kinase C, the sequence surrounding serine 756 would not be predicted to be a good potential protein kinase C phosphorylation site (29). This site may be an exceedingly poor substrate for protein kinase C, phosphorylated only under the influence of the potent activator PMA, or it may be phosphorylated by a second protein kinase that is activated by pro-

tein kinase C. Phosphorylation on serines homologous with serine 756 is predicted in β1 and β7 based on conservation of this serine and flanking sequences (Fig. 9). Indeed, this serine must be phosphorylated in β1 because phosphoserine is present on β1 isolated from transformed cells (30) and there are no other serines in the β1 cytoplasmic domain (31). The β1 subunit tyrosine that is phosphorylated in cells transformed by tyrosine kinase oncogenes is just two residues NH₂ terminal to the serine phosphorylation site (Y788; Fig. 9); it is conserved as a tyrosine in most β subunits but is a phenylalanine in β2. β5 has been shown to be phosphorylated in response to phorbol ester in vivo and by protein kinase C in vitro (32). This must be at a different site because serine 756 is not conserved in β5; two serines flanked with basic residues
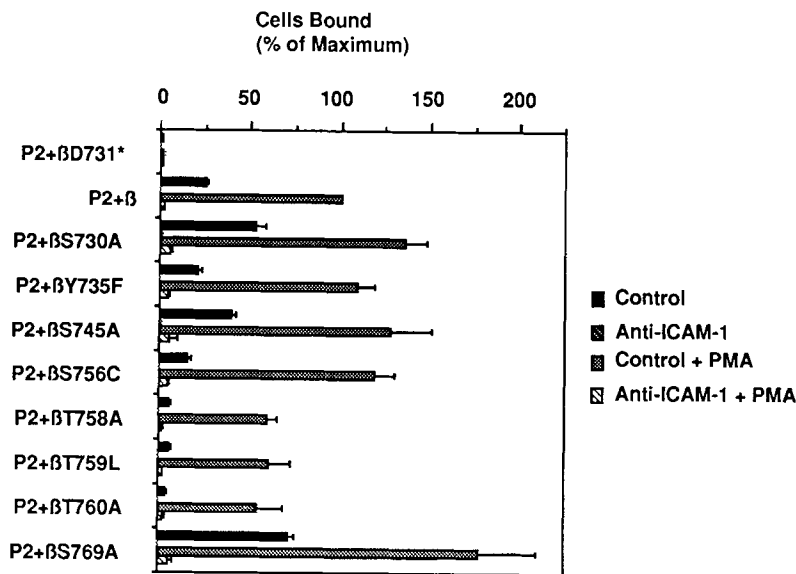
**Cells Bound
(% of Maximum)**

Figure 8. Binding of B lymphoblastoid cells derived from patient 2 (P2) stably transfected with the indicated β subunit mutants in the absence or presence of PMA to ICAM-1-coated plastic. Binding was performed in the presence of anti-HLA-A, B (control) or anti-ICAM-1 (RR1/1). Results are expressed as mean ± SE. This experiment represents an average of two independent trials. Binding was normalized in the two experiments as a percent of P2 + B binding after PMA stimulation, which was 27% and 91% of input cells, respectively.

in protein kinase C recognition motifs (29, 33) are found 14 and 17 residues in the NH₂-terminal direction in β5 (34, 35) and are not present in other integrin β subunits.

We have identified residues that influence phosphorylation of serine 756, presumably by forming part of the kinase recognition site or contributing to its conformation. The deletion L726ΔK755 abolishes PMA-stimulated phosphorylation, showing that residues NH₂ terminal to lysine 755 are important for phosphorylation of serine 756. On the COOH-terminal side, threonine 758 but not threonine 760 contributed to PMA-stimulated phosphorylation. Although threonine was identified as a substrate in the phosphoamino acid analysis, the reduction in phosphorylation in mutant βT758A cannot be accounted for by the loss of this minor amount of phosphothreonine. The loss of phosphorylation in this mutant is most easily explained by a change in the kinase binding site that affects phosphorylation at Ser756.
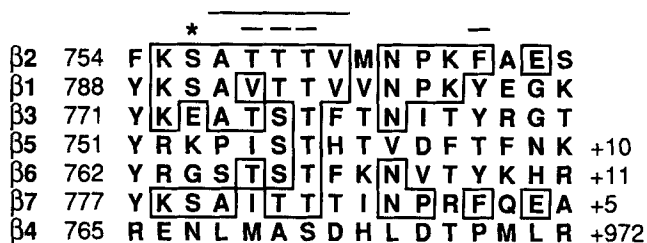

Figure 9. Alignment of the COOH-terminal region of the β2 integrin cytoplasmic domain with other integrin β subunits. Amino acid substitution or deletion mutations that reduce binding to ICAM-1 are overlined. The PMA-stimulated phosphorylation site is asterisked. For β5, the spacing between the threonine-rich sequence and the phenylalanine is the same as for β1-3 and β6-7, but is shifted +1. The number of the beginning residue, and number of COOH-terminal residues not shown, are indicated. Numbering for β1 is for the chicken sequence; the human sequence is identical in the region shown but the corresponding number is 783. Sequence references: β2 (43), β1 (31, 44), β3 (45), β5 (34, 35), β6 (46), β7 (47), and β4 (48, 49).

If the LFA-1 avidity change were regulated through β subunit phosphorylation, then no increase in LFA-1 adhesiveness should be observed by a mutant that is incapable of being phosphorylated in response to PMA. However, mutants, in which the PMA-stimulated phosphorylation site at serine 756 as well as all other potential phosphorylation sites were substituted, were capable of a dramatic increase in ICAM-1 binding in response to PMA. These findings demonstrate that phosphorylation of the β subunit on the major site at serine 756 can be dissociated from the increase in the avidity of LFA-1 for ICAM-1. Further evidence against regulation of avidity by phosphorylation of S756 comes from studies using LFA-1 expressed in COS cells. LFA-1 expressed in COS cells is in a constitutively active state as PMA cannot stimulate additional ICAM-1 binding (21); however, we have demonstrated that PMA can induce a substantial increase in β subunit phosphorylation in COS cells. Furthermore, the deletion mutant BL726ΔK755 binds to ICAM-1 identically to wild type, yet shows no PMA-stimulated phosphorylation. The three contiguous threonines are the only threonines present in the cytoplasmic domain, and it is intriguing that at least one of them is phosphorylated and that the threonines constitute a site important for activity of the extracellular domain in binding to ICAM-1. Although mutation of any one of these residues permitted increased avidity stimulated by PMA, both the basal- and PMA-stimulated binding were lower than for the wild-type β subunit, and it remains possible that phosphorylation of these residues is important in the function of LFA-1.

We have identified two sites located close to the COOH-terminus of the β subunit that are important for maintaining the adhesive nature of LFA-1. The role of the amino acids in these regions in maintenance and regulation of the adhesive states of LFA-1 is not yet understood. These sites may form part of a contact site for another protein, possibly a cytoskeletal protein, that is involved in maintaining or

regulating LFA-1 avidity. Alternatively, if aggregation of LFA-1 molecules induces the increase in avidity, these regions may be important for this self-association. We have thus far been unable to identify other proteins that coprecipitate with LFA-1. However, peptides based on the regions we have defined can now be synthesized to search for lower affinity interactions that have the same structure-activity relationships as defined by our mutational analysis. Cytoplasmic proteins that interact with the cadherin family of adhesion receptors have recently been defined. Cadherin adhesiveness is regulated through the cytoplasmic domain (36). Cytoplasmic proteins termed catenins have been shown to coprecipitate with the detergent soluble fraction of cadherins. Deletions in the COOH-terminal half of the cytoplasmic domain of cadherins not only abolish adhesiveness, but association with the catenins (37, 38). Truncation of the cytoplasmic domain of the chicken β1 integrin abrogates its ability to concentrate to focal contacts, areas of close apposition between cells and substrates (13–15). The cytoskeletal proteins talin, vinculin, and α-actinin localize in close association with integrins in focal contacts

(39). None of these proteins have been demonstrated to associate with integrins in vivo by techniques such as immunoprecipitation, but evidence for an association between the cytoplasmic domain of the integrin β1 subunit and both talin (40) and α-actinin (41) has been obtained using purified proteins in vitro. Phosphorylation of Tyr788 of the B1 subunit has been suggested to decrease interaction with talin (42), although subsequent mutagenesis of this residue has shown no effect on association with focal contacts (14). This residue is homologous to Phe754 of B2, which can be deleted without effect on adhesion to ICAM-1. A peptide found to compete integrin B1 binding to talin (42) is homologous to residues 747 to 756 of B2, which is NH2 terminal to the functionally important region we have defined. Thus, the present study focuses attention on two previously unappreciated sites in the COOH-terminal one-third of integrin B subunits that are important for ligand binding by the extracellular domain of integrins. It will be interesting to determine whether previously defined cytoskeletal components, or novel, as yet undefined cytoplasmic proteins interact with these sites.

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