Differential Requirements for LFA-1 Binding to ICAM-1 and LFA-1-Mediated Cell Aggregation[1]

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Cellular adhesion through the β_2 integrin lymphocyte function-associated Ag (LFA)-1 is a complex event involving activation, ligand binding, and cell shape changes that ultimately result in enhanced adhesion. In this report we define requirements for ligand binding and post receptor signaling by comparing two mechanisms of activation of LFA-1: 1) inside-out signaling and 2) direct activation by the β_2 Ab, CBR LFA-1/2. Our results demonstrate that activation of LFA-1 binding to ICAM-1 by CBR LFA-1/2, in contrast to inside-out signaling mechanisms, does not require protein kinase C activation or protein phosphatase 2A activity nor is it affected by agents that interfere with reorganization of the cytoskeleton. Inhibition of protein tyrosine kinase activity does not affect ICAM-1 binding by either mechanism of activation. However, activation by either mode does require the presence of the β cytoplasmic domain; deletion of the C-terminal phenylalanine or the five amino acid stretch between 756–762 abolished activation of LFA-1. This, combined with the observation that intracellular energy pools must be preserved, implicates the β cytoplasmic domain in a key energy-dependent conformational change in LFA-1 that is required to achieve enhanced ligand binding. Post ligand binding events induced by both PMA and Ab stimulation, as measured by homotypic aggregation, require protein tyrosine kinase, phosphatase, and RhoA activities. By examining both ligand binding and aggregation, we have been able to dissect the signaling components critical in the multistep process of LFA-1-mediated cellular adhesion. ***The Journal of Immunology*****, 1998, 160: 4208–4216.**

Cell-cell and cell-extracellular matrix interactions play a central role in lymphocyte activation, migration of cells during the inflammatory response, and the localization of specific cells to lymphatic organs (1). The leukocyte integrins form a subgroup of adhesive molecules in these processes. They share a common β_2 (CD18) subunit noncovalently associated with at least four α subunits, α_L (CD11a), α_M (CD11b), α_X (CD11c), and α_D, to form lymphocyte function-associated Ag (LFA-1)[3], Mac-1, p150,95, and $\alpha_D\beta_2$, respectively (1, 2). LFA-1 is a well-characterized member of this family, and Abs directed against LFA-1 have been used to demonstrate its role in T cell- and NK cell-mediated killing, T and B cell interactions, and leukocyte-endothelial cell adhesion (3). LFA-1 interacts with at least three members of the Ig-like supergene family of proteins, ICAM-1, ICAM-2, and ICAM-3 (4–7). Ligand binding is divalent cation dependent and requires energy but is not affected by disruption of the actin cytoskeleton by cytochalasin B (4, 8, 9). Structurally, LFA-1 is comprised of an α subunit that contains, in its external region, a putative β-propeller domain within which an I domain is inserted that is involved in ligand recognition (10–12). The α subunit has a short cytoplasmic tail of 58 amino acids that contains the membrane-proximal GFFKR amino acid motif that is conserved among all α subunits (13, 14). The β_2 subunit contains highly conserved and cysteine-rich domains in its extracellular region and a short cytoplasmic tail (15).

Cellular adhesion through integrin molecules involves a complex set of events that include activation, ligand binding, and reorganization of the cytoskeleton (16, 17). To mediate cell-cell adhesion, LFA-1 undergoes activation through an array of known signal transduction pathways that include cross-linking of the T cell receptor/CD3, CD2, cell surface Ig, or MHC Class II molecules, stimulation of protein kinase C activity by phorbol esters, and activation of chemokine receptors (17–22). Ligand binding can also be induced by Abs against integrin family members (23–26). A panel of Abs has emerged that is directed against both the α_L and β subunits and that is able to either tag the activation state of LFA-1 or enhance binding to ligand (8, 11, 27–30). Specifically, we have described an Ab to the β_2 subunit that enhances binding of LFA-1-bearing cells to ICAM-1 and ICAM-3, independently of Fc interactions or whether dimeric or monomeric Fab preparations are used (30).

Divalent cations are critical for ligand binding and can directly modulate binding of LFA-1 to ligand (4, 8, 30). There are metal binding domains in both the α and β subunits of integrin family members, and divalent cations, in particular Mg^{2+}, are required for ligand binding (4, 10, 13, 31–33). In contrast to Mg^{++}, whose presence is required but which only enhances binding in response to activation of intracellular signaling or mAb, Mn^{2+} alone is able to stimulate binding of LFA-1-bearing cells to ligand (8, 30).

Although a broad range of tools can be used to activate LFA-1, an exact understanding of the molecular steps that mediate activation has remained elusive. Protein kinase C can serve as a common second messenger for several of these stimuli; however, the use of inhibitors suggests that more than one signaling pathway may play a role in reaching the common goal of activation of LFA-1 through inside-out signaling (18). Both protein kinase and protein phosphatase activities have been implicated in the activation of integrin family members (34, 35). Activation is transient

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[3] Abbreviations used in this paper: LFA-1, lymphocyte function-associated Ag; LAD, leukocyte adhesion deficiency; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and -6) carboxy-fluorescein acetoxymethyl ester.

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and is independent of a change in cell surface expression of LFA-1 (18). The increase in avidity of LFA-1-bearing cells for ICAM-1 may in part be due to enhanced affinity of LFA-1 for ligand (36). Detailed mutagenesis of the β subunit has shown that activation of lCAM-1 binding through signal transduction pathways requires the presence of amino acids between residues 756 and 762 and the phenylalanine at position 766 within the β_2 cytoplasmic domain (37). Although protein kinase and phosphatase activities have been implicated in the activation of LFA-1, phosphorylation of the molecule on either serine or tyrosine residues does not appear to play a role in avidity regulation (37–39). To examine minimally required events involved in the mechanism of activation of integrin activity, we have employed the Ab CBR LFA-1/2, known to induce binding of LFA-1 to ICAM-1 and to lower the Mg^{2+} concentration required to form the LFA-1/ICAM-1 complex (30). We use this Ab to compare the intracellular pathways that are essential for activation of LFA-1 with those events needed for cell aggregation and to determine the structural features of LFA-1 that are required in this process.

Materials and Methods

Antibodies

The Ab CBR LFA-1/2 was prepared, isolated, subcloned, and purified as described (30). Abs to the LFA-1 α_L subunit, TS 1/22 (40) and TS 2/4 (40), to the β_2 subunit, TS1/18 (40), to ICAM-1, RR1/1 (41) and R6.5 (41), and to Mac-1, CBRM1/29, have previously been described (42).

Cell lines

The B lymphoblastoid cell line JY was grown in RPMI 1640 medium supplemented with 5% FCS. The EBV-transformed cell line derived from patient 2 (P2) with leukocyte adhesion deficiency (LAD) (43) was maintained in RPMI 1640 medium containing 20% FCS. Patient 2 transfected cell lines were maintained in RPMI 1640 medium, 20% FCS, and hygromycin (200 μg/ml).

Cell binding to immobilized ICAM-1

ICAM-1 was purified and adsorbed to plastic (Linbro Titertek 96-well plates, ICN/Flow, Aurora, OH) as described (18). Cells (2 ml, 2×10^7/ml in L15 medium with 2.5% FCS) were labeled with 3 μg of 2′, 7′-bis-(2-carboxyethyl)-5-(and -6) carboxy-fluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, Oregon) for 20 min at 37°C in the presence of 4 μg/ml of the anti-ICAM-1 mAb R6.5 to block cell aggregation. Cells were washed three times in buffer A (L15 medium, 2.5% FCS) and resuspended at 2×10^6 cells/ml in buffer A. Cells (50 μl, 2×10^6/ml) were applied to wells containing buffer A (50 μl) alone, or buffer A with control Ab (5 μg/ml), CBR LFA-1/2 (5 μg/ml), or PMA (50 ng/ml). Nonspecific binding was assessed by comparing binding in the presence or absence of the blocking mAb to LFA-1, TS1/22 (5 μg/ml). The total fluorescent content of the cells was quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, Maine), after which the plate was centrifuged at $200 \times g$ for 2 min at 4°C. After incubation at 4°C for 20 min, the plate was transferred to 37°C for the indicated time. Unbound cells were removed by aspiration in buffer A six times with a 20-gauge needle. Bound cells were quantitated in the Fluorescent Concentration Analyzer (IDEXX) and expressed as a percentage of total input cells per sample well.

A tip plate assay was performed as described (42). Briefly, 25 μl of ICAM-1 (200 μg/ml) or BSA (200 μg/ml) in PBS were spotted on a 5-mm circle in a 60-mm polystyrene petri dish (Falcon 1007, Becton Dickinson, Lincoln Park, NJ) for 1.5 h. The plates were washed four times in buffer A and allowed to incubate in buffer A for 30 min at room temperature. Cells (1 ml, 2×10^6/ml) were then allowed to adhere in the presence of PMA or CBR LFA-1/2 mAb as described above for 10 min at 37°C. Unbound cells were removed by transfer pipet and gentle replacement with buffer A. Bound cells were quantitated by counting four to five high-powered fields/protein circle after 10 to 12 washes. Nonspecific binding was determined for each transfected cell line in the presence of the blocking Ab TS1/22 to LFA-1 and was less than five cells per high-powered field for each cell line.

Table I. *Aggregation in response to CBR LFA-1/2 and PMA*[a]

	Control		PMA		CBR LFA-1/2	
	None	RR1/1	None	RR1/1	None	RR1/1
No treatment	1	0	4	1	4	0
Staurosporine	1	1	1	1	4	0
Okadaic acid	0	0	0	0	0	0
Genistein	0	0	0	0	0	0
C3 exoenzyme	0	0	0	0	0	0

[a] JY cells were preincubated with DMSO (1%), genistein (50 ng/ml), staurosporine (5 μg/ml), okadaic acid (0.75M), or C3 exoenzyme (20 μg/ml). After 30 min, cells were treated with control Ab, CBR LFA-1/2, or PMA in the presence or absence of the blocking Ab TS 1/22. Aggregation was scored as described (45). 0 indicates no aggregates; 1, <10% of cells aggregated; 2, <50% of cells aggregated; 3, up to 100% of cells in small loose clusters; 4, up to 100% of the cells in large clusters. The experiment is representative of three separate studies.

Cell binding to ICAM-1 in the presence of protein kinase inhibitors, phosphatase inhibitors, or the C3 exoenzyme

The optimal concentration of inhibitors was determined by titration of ICAM-1 binding at increasing concentration of inhibitors; cell viability was >90% as assessed by trypan blue exclusion at each concentration. DMSO, used as a solvent for each inhibitor, was included in controls and was at a final concentration less than or equal to 1% v/v. Specifically, cells were incubated with BCECF and the anti-ICAM-1 Ab R6.5, as described above, and then incubated with or without staurosporine (5 μg/ml), genistein (50 μg/ml), and okadaic acid (0.75 μM), in buffer A for 30 min at room temperature. They were transferred to wells containing the respective inhibitors, and binding to ICAM-1 was assessed as above. Cells were incubated in growth medium with C_3 exoenzyme for 16 h at 5, 10, 20, and 30 μg/ml. They were washed into L15 2.5% FCS, incubated with BCECF and the blocking Ab R6.5, and ICAM-1 binding was assessed as above.

Depletion of intracellular energy pools was as described (4). Cells were incubated with 2-deoxyglucose (50 mM) and sodium azide (10 mM) for 15 min at room temperature. Binding to ICAM-1 in response to phorbol esters, CBR LFA-1/2 mAb, or control Ab was determined as described above.

Expression of β_2 subunit cytoplasmic truncation mutants in an LAD cell line

The β_2 subunit cytoplasmic domain mutant cDNAs in an episomal vector with an EBV origin of replication were prepared as described (43). EBV-transformed B cells derived from LAD patient 2, P2, deficient in expression of the β subunit (43), were transfected with 20 μg of plasmid DNA by electroporation of 2×10^7 cells in 0.8 ml PBS at 960 μF and 200 V. The cells were placed in RPMI 1640 medium containing 20% FCS for 48 h and then transferred to selection in hygromycin (200 μg/ml). After 2 weeks in culture, cells expressing LFA-1 were enriched by adherence to the anti-CD18 Ab CBR LFA-1/7. Briefly, CBR LFA-1/7 (40 μg/ml) was bound to polystyrene plates (NUNC 10, Nunc, Naperville, IL) for 30 min at room temperature. The unbound Ab was removed by washing in RPMI 1640 medium with 5% FCS, and nonspecific sites were blocked with RPMI 1640/5% FCS for 30 min at room temperature. The transfected cell lines were added to plates in 12 ml. After 5 min, nonadherent cells were removed by washing in RPMI 1640/5% FCS ten times. Adherent cells were removed with trypsin/EDTA and placed in RPMI 1640 medium containing 20% FCS and 200 μg/ml hygromycin. Continued antibiotic selection was necessary for maintenance of expression of LFA-1.

Flow cytometry was performed as described (44).

Aggregation assays were performed as described (45). Cells cultured to a density of 5×10^5 cells/ml were collected and resuspended in buffer A (2×10^6 cells/ml) containing staurosporine, genistein, or okadaic acid for 30 min. Cells were also treated with C3 exoenzyme or DMSO for 16 h. Cells were then added to the 96-well plate (Falcon 3072, Becton Dickinson) with or without 4 μg/ml of RR1/1 mAb. After 10 min, cells were then stimulated with control Ab (10 μg/ml), CBR LFA-1/2 (10 mg/ml), or PMA (50 ng/ml). After 15 min, aggregation was scored as described in Table I.

Results

Characterization of binding of JY cells to ICAM-1 stimulated by CBR LFA-1/2 mAb

We have previously described an Ab, CBR LFA-1/2, that modulates increased binding of LFA-1 bearing cells to ligands through

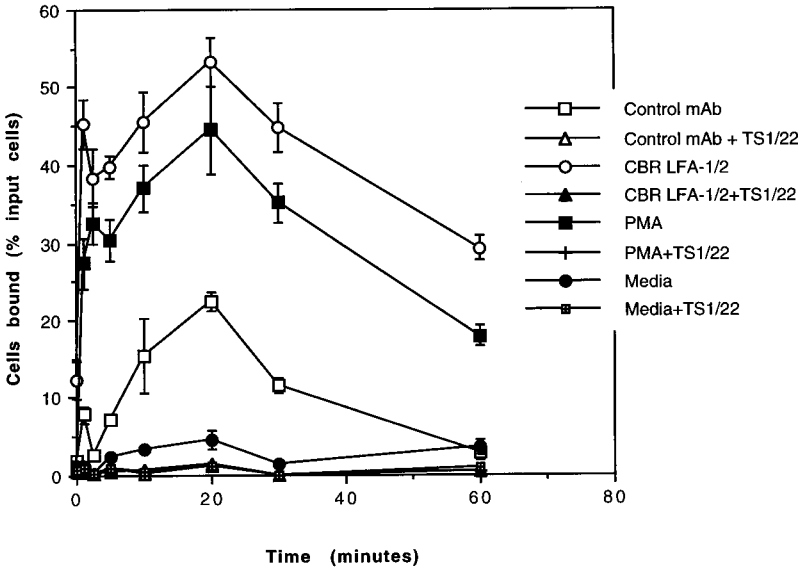

FIGURE 1. Time course of activation of cell binding to ICAM-1. JY cells were fluorescently labeled with BCECF and incubated with phorbol esters, CBR LFA-1/2 mAb, a control Ab, or media, and with or without a blocking mAb to LFA-1/TS1/22. Binding to purified ICAM-1 absorbed to microtiter wells was performed at 37°C and was assessed at 2.5, 5, 10, 20, 30, and 60 min of stimulation. Data are the average of quadruplicate samples and SE is reported.

a specific interaction with the β subunit of LFA-1 and mimics the effect seen by activation of intracellular signal transduction pathways (30). To define the molecular steps required for activation of LFA-1, we have compared the mode of activation by CBR LFA-1/2 to that produced by phorbol ester stimulation. The rate of activation of binding to immobilized ICAM-1 of LFA-1-bearing cells by CBR LFA-1/2 mAb was compared with that induced by phorbol esters. CBR LFA-1/2 mAb induced binding of JY cells to ICAM-1 at a rate comparable to that seen with phorbol ester stimulation (Fig. 1). As demonstrated previously with cross linking of CD3 or phorbol ester treatment of T cells (18), maximal levels of binding with both stimuli were seen by 10 min. Binding to ICAM-1 was specific under both conditions of stimulation as shown by inhibition with mAb TS1/22 to LFA-1 (Fig. 1).

Effect of the protein kinase inhibitor staurosporine on activation of LFA-1

We compared activation of LFA-1 adhesiveness by intracellular signaling pathways to activation by CBR LFA-1/2 mAb. Two assays were used, binding of JY cells to ICAM-1 adsorbed to plastic substrates and ICAM-1-dependent homotypic aggregation of JY cells. Since protein kinase activity has been implicated in the activation of LFA-1, we examined whether inhibition of its activity by staurosporine affected binding to ICAM-1. Pretreatment with staurosporine of the B cell line JY abolished PMA-stimulated binding to ICAM-1 (Fig. 2). In contrast, staurosporine did not inhibit CBR LFA-1/2-stimulated binding to ICAM-1 substrates. Similar results were obtained in homotypic aggregation of JY cells,

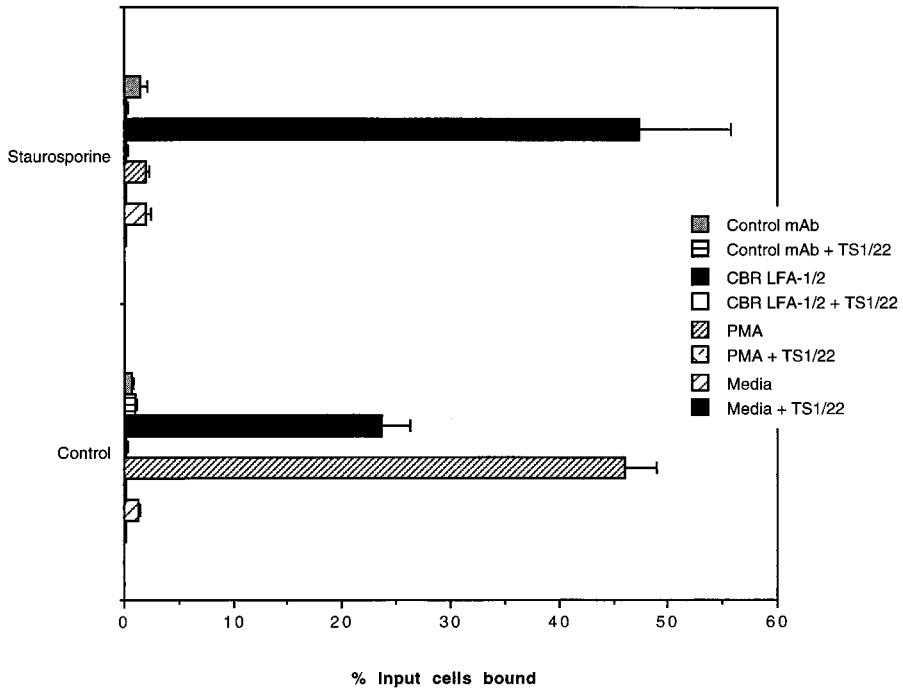

FIGURE 2. Effect of the protein kinase inhibitor staurosporine on cell binding to ICAM-1. Cells were treated with or without staurosporine (5 μg/ml) for 30 min at room temperature after labeling with BCECF as described in *Materials and Methods*. Cells were then stimulated with CBR LFA-1/2, control Ab, phorbol esters, or media alone for 10 min at 37°C. Binding to ICAM-1 was assessed as described in *Materials and Methods*. Specific binding was determined in the presence of the blocking Ab TS1/22. Each data point was performed in quadruplicate and SE is reported.

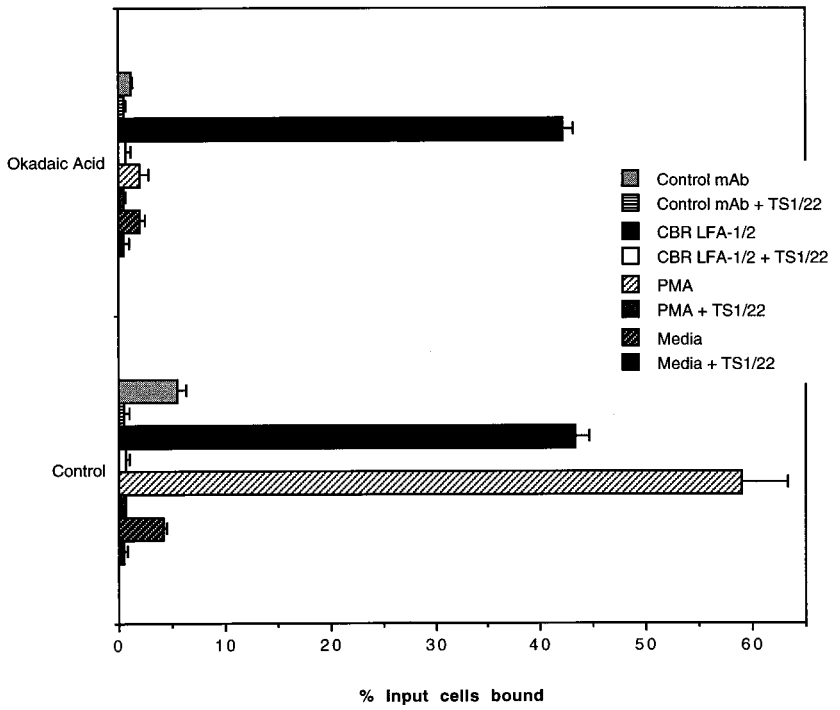

FIGURE 3. Effect of the phosphatase inhibitor, okadaic acid, on cell binding to ICAM-1. JY cells were treated with or without okadaic acid (0.75 μM) for 30 min at room temperature after labeling with BCECF as described in *Materials and Methods*. They were then incubated with media, PMA, CBR LFA-1/2, or control Ab with or without the blocking Ab, TS1/22. Binding to ICAM-1 was assessed after 10 min as described in *Materials and Methods*. Each point was performed in quadruplicate and SE is reported.

which is dependent on interaction of LFA-1 with ICAM-1 (46). PMA-stimulated, but not CBR LFA-1/2 mAb-stimulated aggregation, was inhibited by staurosporine (Table I). Aggregation was dependent on ICAM-1 as shown by inhibition with RR1/1 mAb. Since staurosporine has been reported to have an effect on a broad range of protein kinases, the effect on PMA-induced binding to ICAM-1 and aggregation may be due to inhibition of not only protein kinase C but other kinases in the pathway required for inside-out activation of LFA-1. However, its activity is not required for mAb-induced binding to ICAM-1 or homotypic aggregation.

The role of protein phosphatases in stimulation of cell binding

Protein phosphatases play a critical role in the modulation of signal transduction events (47). To determine whether preservation of phosphatase activity was essential for activation of LFA-1 adhesiveness by CBR LFA-1/2 or protein kinase C, we examined the effect of treatment of cells with the protein phosphatase inhibitor, okadaic acid. Okadaic acid markedly diminished activation by phorbol esters, but not by CBR LFA-1/2 mAb (Fig. 3). Titration revealed that half maximal inhibition occurred between 0.4 and 1 μM of okadaic acid (not shown). Concentrations of less than 0.1 μM okadaic acid had no effect on control mAb-, PMA-, or mAb CBR LFA-1/2-stimulated binding to ICAM-1 (data not shown). Studies on homotypic adhesion revealed an interesting contrast. Okadaic acid completely inhibited both CBR LFA-1/2 mAb-stimulated and PMA-stimulated homotypic aggregation. These results suggest that CBR LFA-1/2 mAb is able to activate binding of LFA-1 independently of phosphatase 1 and/or 2A activity, whereas protein kinase C-mediated activation requires preservation of this activity and implicates phosphatase activity in the pathway of activation by protein kinase C. However, LFA-1-dependent aggregation is more complex, and an additional step distal to CBR LFA-1/2 mAb-stimulated activation that is phosphatase dependent appears required for aggregation.

Effect of the protein tyrosine kinase inhibitor genistein on cell binding

Protein tyrosine kinase activity has been shown to play a role in intracellular signaling following binding of integrin family members to ligand, and stimulation of a protein tyrosine kinase cascade has been demonstrated upon activation of both β_1 and β_2 integrin family members. Since activation of LFA-1 by PMA stimulation does not occur through direct phosphorylation, we examined whether tyrosine kinase activity was a component of the pathway to activation or whether it was a component of the signaling cascade required to induce cell-cell aggregation. We used the protein tyrosine kinase inhibitor genistein to examine whether this activity was required to mediate both LFA-1 binding to ICAM-1 and cell aggregation. Genistein had little effect in JY cells on CBR LFA-1/2 mAb or PMA-induced adhesiveness to ICAM-1 (Fig. 4). In contrast, aggregation by both stimuli was completely blocked by treatment of the cells with the inhibitor (Table I).

Effect of C3 exoenzyme on ICAM-1 binding

C3 exoenzyme, which covalently modifies and inactivates the small G protein RhoA, has previously been shown to inhibit aggregation of JY cells (9). We examined the effect of this agent on ICAM-1 binding to determine whether RhoA-stimulated functions, such as cytoskeletal reorganization, are critical for activation of LFA-1 by either PMA or direct activation with mAb (Fig. 5). After 16 h of treatment with the C3 exoenzyme, binding to ICAM-1 in response to both phorbol esters or CBR LFA-1/2 was preserved (Fig. 5). These conditions impaired the ability of cells to aggregate as previously reported (9). C3 exoenzyme inhibited both PMA- and CBR LFA-1/2 mAb-stimulated homotypic aggregation (Table I). The lack of effect on activation of LFA-1 binding to ICAM-1 on substrates suggests that the activity of the Rho family of GTP-binding proteins is not required for the formation of a stable LFA-1/ICAM complex, but for a later step. This was consistent with the observation that the cells, once bound to ICAM-1 substrates, failed to spread (data not shown).

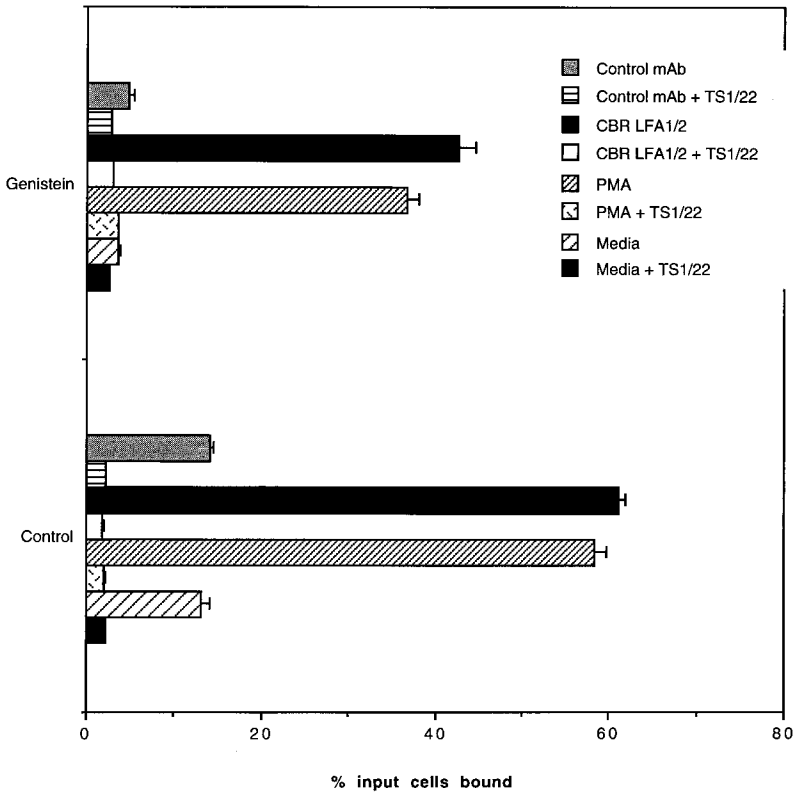

FIGURE 4. Effect of the protein tyrosine kinase inhibitor genistein of binding to ICAM-1. JY cells were incubated with genistein or solvent (50 ng/ml; 1% DMSO) for 30 min at room temperature after labeling with BCECF as described in *Materials and Methods*. Cells were stimulated with CBR LFA-1/2, PMA, or control Ab. Binding to ICAM-1 was assessed after incubation for 10 min at 37°C. Specific binding was determined in the presence of the blocking Ab, TS1/22.

Effect of depletion of intracellular energy stores on ICAM-1 binding

It has previously been shown that binding of T lymphoblasts to ICAM-1 in response to phorbol ester stimulation can be inhibited by depletion of intracellular energy pools (4). We compared whether activation of binding by CBR LFA-1/2 or PMA-mediated binding to purified ligand requires intact energy pools (Fig. 6). Cells that were pretreated with 2-deoxyglucose and sodium azide exhibited little binding to ICAM-1 in response to CBR LFA-1/2 or phorbol esters. Cell-cell aggregation is inhibited by treatment of cells with sodium azide (29). Thus, although activation through CBR LFA-1/2 appears to occur independently of several known

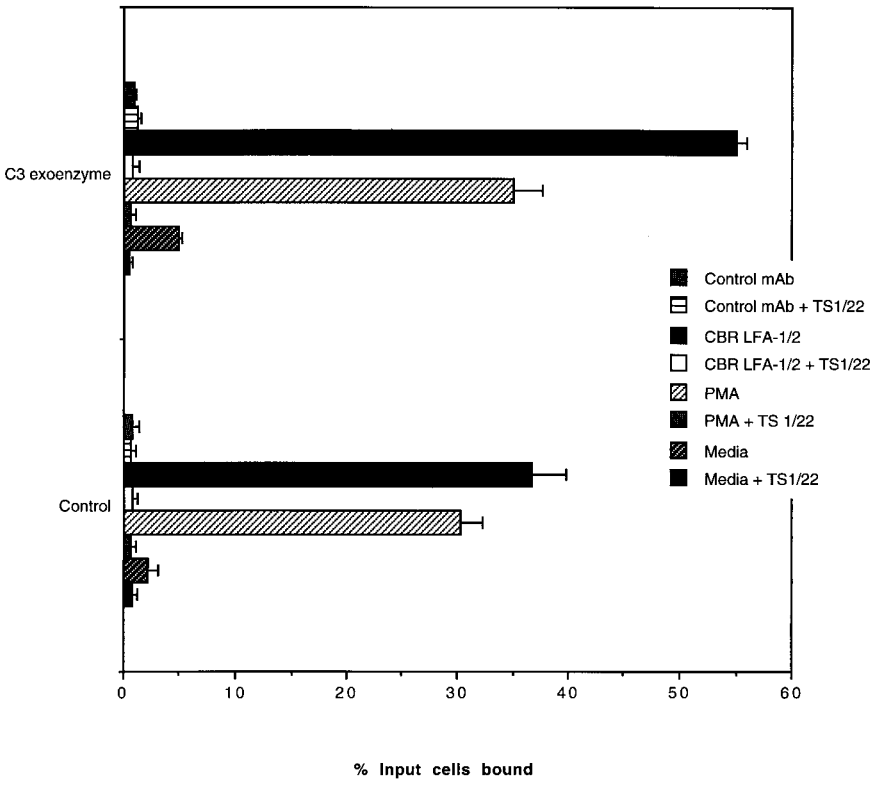

FIGURE 5. Effect of the C3 exoenzyme on ICAM-1 binding. JY cells were cultured in presence of the C3 exoenzyme for 16 h, and ICAM-1 binding was assessed in response to phorbol ester stimulation, CBR LFA-1/2, control Ab (TS 2/4), and media. Values are reported in triplicate with SE.

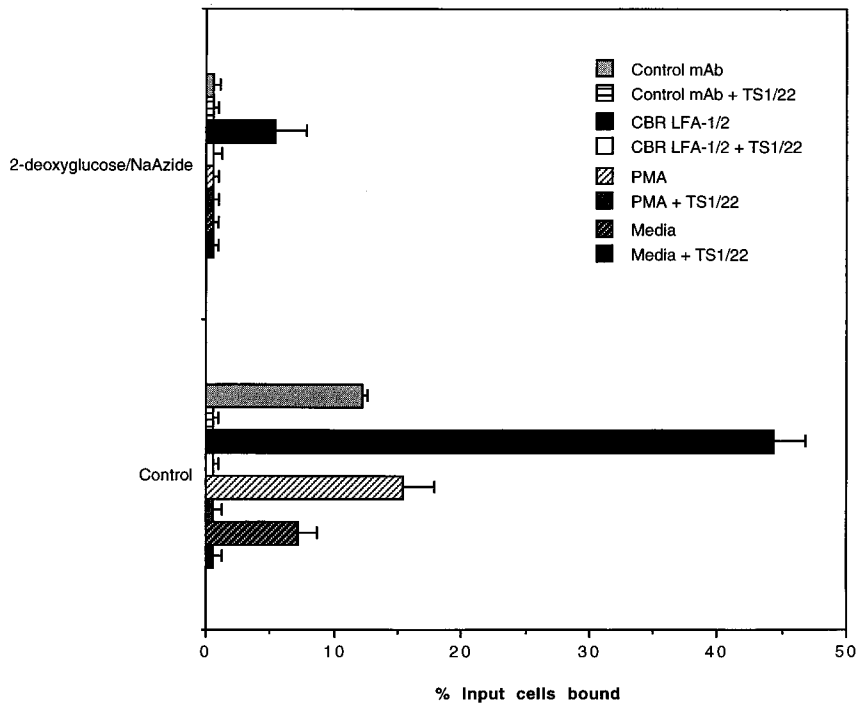

FIGURE 6. Effect of depletion of intracellular energy on stimulation of binding through CBR LFA-1/2. Cells were incubated in the presence or absence 2-deoxyglucose and sodium azide as described in *Materials and Methods*. After 15 min at room temperature, cells were treated with CBR LFA-1/2, PMA, or control Ab at 37°C for 10 min. Each point was performed in quadruplicate and SE is reported.

signal transduction pathways, it still requires cellular energy to achieve the high avidity conformation.

Effect of truncation of the cytoplasmic domain of the β_2 subunit on the ability of CBR LFA-1/2 to modulate binding to ICAM-1

The cytoplasmic domain of the LFA-1 β subunit is critical for avidity regulation through intracellular signaling pathways. Since CBR LFA-1/2 is able to modulate binding to ICAM-1 despite inhibition of various signal transduction pathways, we examined whether the Ab was able to induce binding to ICAM-1 through the extracellular domain, independently of an intact β cytoplasmic domain. We first examined the effect of truncation of the cytoplasmic domain of the β subunit of LFA-1 on binding to ICAM-1 under our standard assay conditions. Deletion of the β_2 cytoplasmic tail of LFA-1 (731*) resulted in loss of binding to ICAM-1 in response to activation of intracellular signaling pathways by PMA or through CBR LFA-1/2 (Fig. 7*A*).

To probe this in greater detail, we examined a series of deletion and point mutants that have previously been shown to be deficient in activation in response to phorbol esters (48). The mutants exhibited comparable cell surface expression of LFA-1 (Fig. 7*B*). We examined binding to ICAM-1 under conditions of low-stringency washing to accentuate any subtle differences in the activation pattern. When cells were activated by phorbol ester stimulation, ICAM-1 binding was markedly diminished if the critical region between amino acids 756–762 was deleted or the C-terminal phenylalanine (766A) was changed to alanine (Fig. 7*C*). In parallel, activation by CBR LFA-1/2 was also diminished. Mutation of Phe 766 to tyrosine had no effect, as previously shown (37).

Effect of Mn^{2+} on β cytoplasmic mutant binding to ICAM-1

The inability of Abs and phorbol esters to stimulate binding to ICAM-1 on cells bearing β cytoplasmic mutations raised the question of whether the mutations resulted in molecular changes that made LFA-1 incapable of assuming a high avidity conformation. To address this, we tested whether Mn^{2+} was able to enhance binding of LFA-1 independently of the other activation pathways. Stable transfected cell lines expressing either the wild-type β subunit or the mutations outlined in Figure 8 were placed in Mn^{2+} containing media and assessed for binding to ICAM-1. As shown in Figure 8, all the mutants were capable of binding to ICAM-1, indicating that the phenotypic behavior of these mutants is related to disruption of specific interactions or modifications in the cytoplasmic region.

Discussion

The binding of LFA-1-bearing cells to immobilized ICAM-1 is a regulated process. It can be modulated via inside-out signaling (cell surface receptors on T or B cells or protein kinase C) (17–22), or by activating Abs directed against LFA-1 (28–30, 49). The results described in this paper demonstrate that the activation of LFA-1 in JY cells by either Ab binding or inside-out signaling share common requirements at the level of the LFA-1 receptor but that they differ in the manner in which this activation is accomplished.

Activation of LFA-1 by the CBR LFA-1/2 Ab occurs at a comparable rate to that seen with stimulation of inside-out signaling pathways. This rapid induction of ligand binding has been demonstrated with other anti LFA-1- Abs (KIM127, KIM 185, and NKL16) (28, 49).

Activation through mAb CBR LFA-1/2, in contrast to PMA, does not require preservation of protein kinase activity. Inhibition of protein kinase activity by staurosporine does not interfere with cell binding to ICAM-1 brought about by the activating Ab, CBR LFA-1/2. This is similar to what is observed through the α subunit-specific Ab, NKL-16, which was also not affected by treatment of cells with staurosporine(29), but is in contrast to the effects of staurosporine on B cell binding to ICAM-1 previously reported (39).

We extended these studies and demonstrated that inhibition of protein phosphatase activity by okadaic acid blocks the phorbol ester-mediated stimulation of LFA-1 binding to ICAM-1 but did not affect ICAM-1 binding in response to CBR LFA-1/2 mAb. This not only demonstrated that activation through CBR LFA-1/2

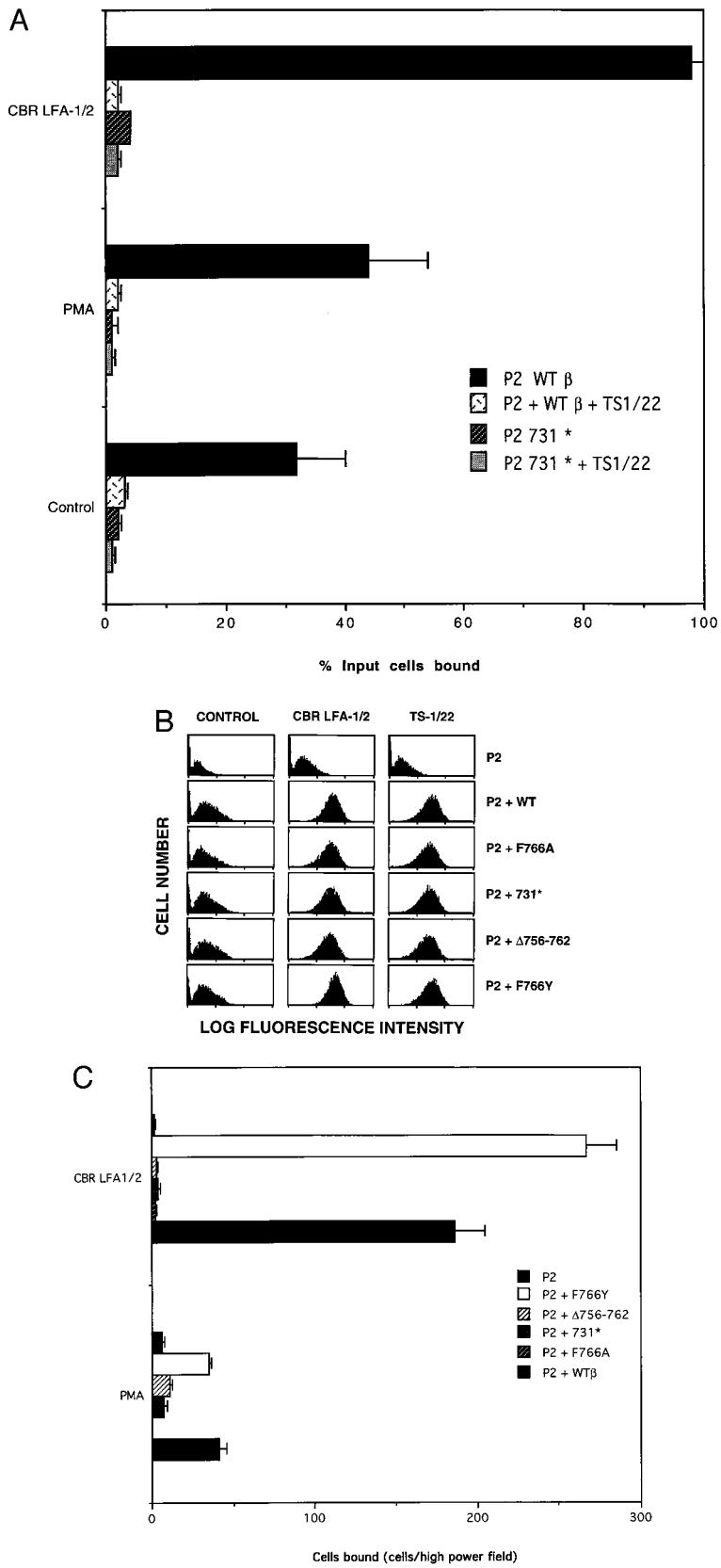

FIGURE 7. Effect of CBR LFA-1/2 on binding to ICAM-1 of EBV cell lines transfected with mutant β subunits. *A*, Patient 2 cells expressing the wild-type β subunit or the cytoplasmic truncation of the β_2 subunit (731*) were labeled with BCECF as described in *Materials and Methods*. Cell binding to ICAM-1 was assessed in the presence of CBR LFA-1/2, phorbol esters, or control Ab. The unbound cells were removed by aspiration after incubation for 10 min at 37°C. *B*, Immunofluorescence flow cytometry of transfected patient cell lines. Transfectants were labeled with α_L-specific (TS 1/22) or β-specific (CBR LFA-1/2) Ab, FITC-conjugated goat anti-mouse IgG, and subjected to flow cytometry. Fluorescence intensity is on a three-decade log scale. *C*, ICAM-1 binding of cells expressing β cytoplasmic mutants. The complete panel of mutants was assayed under less stringent conditions of washing as described in *Materials and Methods*. Background binding mAb was less than five cells/high power field in the presence of TS 1/22. Cells were incubated with the indicated stimulus, and five high-powered fields were assessed and average with SE is reported.

mAb does not require preservation of protein phosphatase PPI and PP2A activity, but also implicates this phosphatase activity in the pathway that leads to activation of LFA-1 by protein kinase C as previously shown in purified B cells (39). In contrast, binding to ICAM-1 substrates stimulated by protein kinase C or the mAb CBR LFA-1/2 was affected by inhibition of protein tyrosine kinase activity with genistein. Experiments with C3 exoenzyme suggest that activation of LFA-1 binding to ICAM-1 by both the Ab and

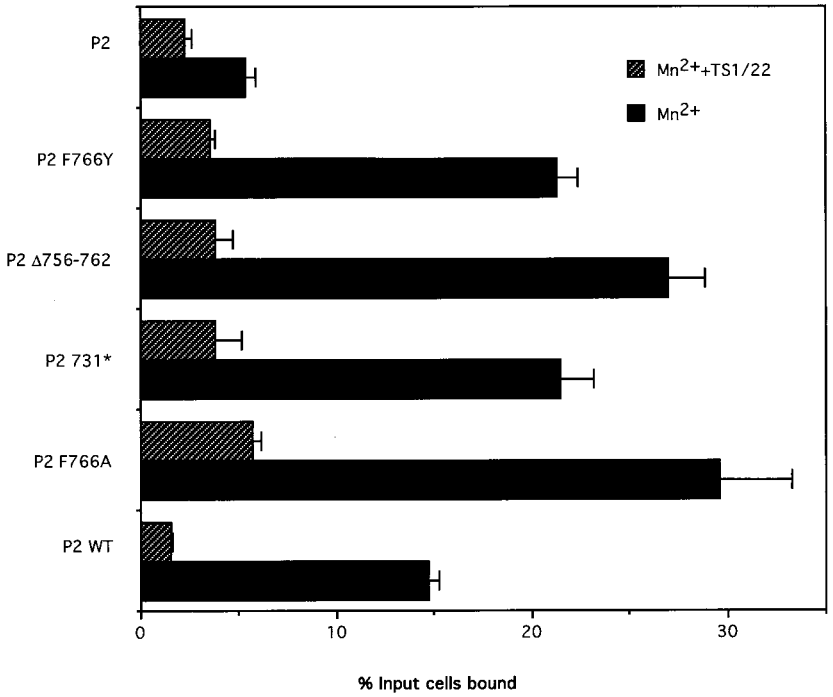

FIGURE 8. Mn^{2+} stimulated binding of transfectants expressing β_2 subunit mutations. Transfectants were labeled with BCECF and resuspended in 20 mM HEPES pH 7.6, 140 mM NaCl, and 2 mg/ml glucose and cell binding was assessed in the presence of 0.5 Mn^{2+} for 10 min at 37°C. Specificity was assessed by the blocking Ab TS1/22. Values are reported in quadruplicate with SE.

protein kinase C does not depend upon activity of the small GTP-binding protein RhoA, which has been implicated in rearrangement of the actin network and disrupts cell-cell aggregation (9, 50). These results suggested that serine/threonine protein kinase activity, but not phosphatase activities, are required for full activation via inside-out signaling, but not by external activation achieved through CBR LFA-1/2 mAb. Neither tyrosine kinase nor RhoA activity is required for ICAM-1 substrate binding induced by inside-out signaling or the activating Ab.

When we analyzed the effect of these inhibitors on LFA-1-mediated cell-cell aggregation, we observed that C3 exoenzyme impaired the ability of both PMA and CBR LFA-1/2 mAb to induce cell aggregation, suggesting that the LFA-1/ICAM interaction initiates a signaling cascade that feeds into the RhoA family of GTP-binding proteins or that multiple pathways acting in parallel are required for LFA-1-dependent homotypic aggregation. Homotypic aggregation was previously reported to be inhibited by C3 exoenzyme (9). The cell aggregation assay also revealed that a staurosporine-sensitive step is required for aggregation stimulated by PMA, but not by CBR LFA-1/2 mAb. The pathway leading to cell aggregation is also sensitive to protein tyrosine kinase and phosphatase inhibition under either condition of stimulation. It is an important observation that intact cellular energy sources are required for both ligand binding and cell aggregation in response to either Ab or PMA activation. This suggests that specific interactions and/or modifications are required for LFA-1 to attain full activation.

Previous studies have demonstrated that specific regions of the β_2 subunit are required for PMA-mediated activation (37, 48); thus, it was critical to test whether the regions of the β subunit that were critical for phorbol ester activation also were involved in inducing activation by this β subunit Ab. The regions between residues 756–762 and the phenylalanine at position 766, were found to be necessary not only for activation by phorbol esters but also for activation by CBR LFA-1/2 mAb. A central question was whether mutations in this region rendered the molecules incapable of achieving the high avidity conformation associated with activation of LFA-1 and enhanced binding to ICAM-1. Therefore, we studied the effect of Mn^{2+} on the ability of these mutants to bind to ICAM-1. Mn^{2+} has been shown to stimulate binding of LFA-1 to ICAM-1 directly in several cell lines (8, 30). All of the LFA-1 mutants exhibited enhanced binding to ICAM-1 in response to Mn^{2+}, demonstrating that the mutants are fully competent to recognize the ligand.

Critical regions in the β subunit are required for ligand binding stimulated by both Ab and inside-out signaling. Our data support the model that the β cytoplasmic domain plays a key role in LFA-1-dependent ligand binding. Since both depletion of intracellular energy stores and critical regions of the β subunit are required for activation by both the Ab and inside-out signaling pathways, one model is that a key energy-dependent interaction must be maintained to mediate ligand binding that is independent of activation of protein kinase C, protein tyrosine kinase, and phosphatase activities. A specific protein-protein interaction, such as the recently described cytohesin-1, is a candidate for modulating the activity of LFA-1 (51). Enhanced ligand binding by PMA stimulation has been proposed to occur through post receptor signaling events that strengthen cytoskeletal interaction (52). By disrupting the β subunit, it is possible that interactions with the cytoskeleton that are critical for enhanced binding are lost. Furthermore, our results showed that the LFA-1/ICAM interaction is not sufficient to induce cell aggregation. Intracellular pathways, as well as specific regions of the LFA-1 molecule, are required for cellular aggregation, consistent with the idea that the LFA-1/ICAM pair is a signaling complex. This is supported by the observation that LFA-1-mediated cell adhesion induces the tyrosine phosphorylation of $p130^{cas}$ and its association with the adapter protein C-crk (53).

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