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Superantigen-Induced T Cell:B Cell Conjugation Is Mediated by LFA-1 and Requires Signaling Through Lck, But Not ZAP-70

Margaret M. Morgan,* Christine M. Labno,* Gijs A. Van Seventer,†‡ Michael F. Denny,† David B. Straus,‡‡ and Janis K. Burkhardt*§§

The formation of a conjugate between a T cell and an APC requires the activation of integrins on the T cell surface and remodeling of cytoskeletal elements at the cell-cell contact site via inside-out signaling. The early events in this signaling pathway are not well understood, and may differ from the events involved in adhesion to immobilized ligands. We find that conjugate formation between Jurkat T cells and EBV-B cells presenting superantigen is mediated by LFA-1 and absolutely requires Lck. Mutations in the Lck kinase, Src homology 2 or 3 domains, or the myristoylation site all inhibit conjugation to background levels, and adhesion cannot be restored by the expression of Fyn. However, ZAP-70-deficient cells conjugate normally, indicating that Lck is required for LFA-1-dependent adhesion via other downstream pathways. Several drugs that inhibit T cell adhesion to ICAM-1 immobilized on plastic, including inhibitors of mitogen-activated protein/extracellular signal-related kinase kinase, phosphatidylinositol-3-kinase, and calpain, do not inhibit conjugation. Inhibitors of phospholipase C and protein kinase C block conjugation of both wild-type and ZAP-70-deficient cells, suggesting that a phospholipase C that does not depend on ZAP-70 for its activation is involved. These results are not restricted to Jurkat T cells; Ag-specific primary T cell blasts behave similarly. Although the way in which Lck signals to enhance LFA-1-dependent adhesion is not clear, we find that cells lacking functional Lck fail to recruit F-actin and LFA-1 to the T cell:APC contact site, whereas ZAP-70-deficient cells show a milder phenotype characterized by disorganized actin and LFA-1 at the contact site. The Journal of Immunology, 2001, 167: 5708–5718.

The formation of a conjugate between a T cell and an APC requires the activation of integrins on the T cell surface via inside-out signaling pathways that are not well understood. The cell surface integrins are not constitutively adhesive, but are able to engage their ligands strongly after stimulation through other cell membrane receptors. Th cell adhesion to B cells, monocytes, and other APCs has been shown to occur via two main pathways: CD2 binds to the ligand LFA-3, and the \( \beta_1 \) integrin LFA-1 binds to the intracellular adhesion molecules ICAM-1, ICAM-2, and ICAM-3 (1–4). In contrast, T cell adhesion to VCAM-1 and extracellular matrix proteins such as fibronectin is mediated by the \( \beta_1 \) integrin subfamily (5).

LFA-1 and CD2 participate in T cell adhesion in distinct ways. The adhesion mediated by CD2 interaction with LFA-3 is present in resting T cells, but becomes more active over a period of hours following T cell activation (4). CD2 ligation can mediate or enhance T cell activation (4), and triggering through CD2 results in the persistent activation of LFA-1 (6). In contrast, LFA-1 binding to ICAMs is transiently up-regulated within minutes of TCR stimulation (2–4). It has been suggested that the CD2 pathway plays a critical role in initiating cell-cell interactions before the engagement of the TCR, and that LFA-1 strengthens the T cell-APC contact following TCR engagement (7).

Activation of both LFA-1 and the \( \beta_1 \) integrins in T cells has been the focus of numerous studies; adhesion mediated by both integrins is quickly up-regulated following TCR cross-linking or activation with phorbol esters (2, 6, 8–10). The stimulation through cell surface receptors is thought to produce intracellular second messengers that alter the adhesive state of integrins, a process termed inside-out signaling. This rapid modulation of adhesion is due to an increase in avidity brought about by qualitative rather than quantitative changes in the cell surface expression of integrins (3, 4, 8, 11–13). Evidence suggests that rather than conformational changes being involved, increased adhesion results from the transient release of the integrins from the actin cytoskeleton, allowing them to move more freely in the membrane to find their ligands and to cluster at the cell-cell contact site, where they reassociate with F-actin in an organized adhesion complex (14–16).

Numerous studies have attempted to identify and characterize the inside-out signaling pathways that regulate integrin avidity in T cells and other cell types (reviewed in Refs. 17 and 18). Although the results of these studies are somewhat variable, they have established that signaling through protein kinase C (PKC) (2) plays an important role in the activation of adhesion (17). Other

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Abbreviations used in this paper: PKC, protein kinase C; CMAC, 7-amino-4-chloronethylcoumarin; ERK, extracellular signal-regulated kinase; GEF, guanine exchange factor; GFP, green fluorescent protein; HE, hydroethidine; LAD, leukocyte adhesion deficiency; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase ERK kinase; PI-3, phosphatidylinositol-3; PLC, phospholipase C; PTK, protein tyrosine kinase; SEE, staphylococcal enterotoxin E; SF-RPMI, serum-free RPMI 1640; SH, Src homology; Tg, transgenic.
late signaling molecules have also been implicated in TCR-induced, LFA-1-mediated adhesion; roles for the Ras/mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol-3 (PI-3) kinase, calpain, and cytohesin-1 have all been reported (19–23). By comparison with these later signaling requirements, relatively little is known about the proximal signaling events that regulate TCR-activated integrin adhesion.

Many studies of integrin adhesion have used as an experimental model the binding of previously activated T cells to purified ligands immobilized on plastic. Others have examined the interaction between T cells and B cells following T cell activation with PMA or CD3 cross-linking. Because it is now recognized that there are significant differences in T cell responses to Abs and immobilized ligands as opposed to bona fide APCs, we tested the role of early signaling molecules in T-B adhesion without prior activation of the T cells. We developed an assay to study superantigen-dependent conjugation of Jurkat cells to EBV-B cells, and used the Lck-deficient mutant JCaM1 and the ZAP-70-deficient mutant P116 to ask about the proximal signaling events involved.

Our results show that adhesion is largely mediated by LFA-1, and requires Lck and downstream signaling molecules, but is independent of ZAP-70 activity. At least part of the role of Lck in adhesion is to organize actin and LFA-1 at the cell-cell contact site in response to TCR engagement.

Materials and Methods

Ab and reagents

The anti-CD5 mAb OKT3 was purchased from OrthoBiotect (Raritan, NJ), and anti-LFA-1 α-chain TS1/22 was obtained from Endogen (Woburn, MA). The anti-LFA-1 β-chain TS1/18, anti-LFA-1 α-chain TS2/4, and anti-class I W6/32 hybridomas were obtained from American Type Culture Collection (Manassas, VA); anti-CD2 Ab 95-5-49 was a gift from R. Gross (National Cancer Institute, Bethesda, MD); and anti-CD29 Ab Lial1/2 was purchased from Biodiagnost (Indianapolis, IN), and other protease inhibitors were purchased from Biodesign International (Saco, ME). The anti-TCR β chain c305 has been described (24), and the rabbit anti-mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) was a gift from M. Rosner (University of Chicago, Chicago, IL). Rabbit polyclonal anti-phospho-MEK and anti-phospho-ERK were obtained from New England Biolabs (Beverley, MA), and anti-phosphotyrosine mAb 4G10 (Cell Signaling Technology, Beverly, MA) was purchased from Cell Signaling Technology (Danvers, MA). The anti-phospho-MEK and anti-phospho-ERK antibodies used for western blotting were purchased from Cell Signaling Technology (Boston, MA). The anti-phospho-ERK and anti-phospho-MEK antibodies used for flow cytometry were obtained from Cell Signaling Technology (Danvers, MA). The anti-phospho-ERK and anti-phospho-MEK antibodies used for western blotting were purchased from Cell Signaling Technology (Danvers, MA). The anti-phospho-ERK and anti-phospho-MEK antibodies used for flow cytometry were obtained from Cell Signaling Technology (Danvers, MA).

Western blotting

Western blots were performed essentially as described previously (25). Jurkat cells were labeled with calcein-AM, and lysates were prepared for western blotting essentially as described previously (25).

Results

Lck have been described (30). The Lck substitution mutants, G2A and K273A, have been described previously (31, 32). Substitutions at these positions block Lck myristoylation or catalytic activity (33, 34). LckG2A and LckK273A were subcloned into pBlu-neo (35) and pBP1 (29), respectively, and transfected into JCaM1 by electroporation. Antibiotic-resistant clones that expressed Lck at levels equivalent to the parental Jurkat E6 subclones were selected for further analysis. Biochemical studies confirmed that LckG2A was mislocalized to the cytosol, and that LckK273A lacked in vitro kinase activity (M. Denny and D. Strauss, unpublished data). Clones expressing either LckG2A or LckK273A failed to initiate signaling in response to TCR stimulation. All Jurkat cell lines were analyzed by fluorescence flow cytometry every 4–6 wk to ensure that they expressed equivalent levels of TCR and LFA-1.

Murine primary T cell blasts specific for OVA peptide residues 323–339 (OVA323–339) presented by I-A d were prepared from DO-11.10 Tg mice (26). Lymph node cells from DO-11.10 mice (2 × 106/well) were expanded by coculture with irradiated splenocytes (2500 rad, 6 × 103/ml) and added to 10 6/well) and 0.15 mg/ml OVA323–339 peptide in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mM L-glutamine, nonessential amino acids, 10 mM HEPES, and 5 × 10−5 M 2-ME), at 10% CO2. Tg cells were harvested and live cells were counted by Ficol-Hypaque gradient separation following PMA stimulation for 7–14 days after activation, by which time they were rested, as judged by small size and requirement for additional stimulation to induce significant proliferation. A20 B cells were cultured in complete DMEM, supplemented as above.

FACS-based conjugation assay

EBV-B cells were used as the APCs were stained with HE or PKH26. For HE staining, EBV-B cells were incubated in RPMI 1640 containing 3 μg/ml HE at 37°C for 30 min, washed twice with RPMI 1640, then reseeded as above with 0.15 mg/ml OVA323–339 peptide in complete medium (DMEM). Following HE staining, EBV-B cells were incubated in RPMI 1640 containing 3 μg/ml HE at 37°C for 1.5 h. B cells were then washed again and resuspended in serum-free RPMI 1640 (SF-RPMI) at 1 × 106 cells/ml. For PKH26 staining, EBV-B cells were stained with SF-RPMI, resuspended in diluent C (Sigma-Aldrich; provided with PKH26) at 1 × 106 cells/ml, then mixed with an equal volume of 0.5 μM PKH26 and incubated at room temperature for 10 min. After an equal volume of FBS was added to quench the staining, diluted in SF-RPMI and added with RPMI 1640 (200 μl/ml) and incubated with or without SEE, as described above. Just before conjugation, EBV-B cells were pelleted and resuspended in SF-RPMI at 1 × 106 cells/ml. Jurkat cells were incubated in RPMI 1640 containing 0.15 μg/ml calcein-AM for 30 min at 37°C, then washed twice with RPMI 1640 and resuspended in SF-RPMI at 1 × 106 cells/ml.

Conjugation

Conjugation was performed by mixing 1 × 106 Jurkat cells with 1 × 106 EBV-B cells in SF-RPMI at 37°C for 15 min. The tubes were vortexed for 5–10 s to resuspend the cells, then fixed by the addition of 0.5 μl of PBS/6% paraformaldehyde. The relative proportion of red, green, and red/green events in each tube was determined by two-color flow cytometry using a BD Biosciences (Franklin Lakes, NJ) FACScan flow cytomter with an excitation wavelength of 492 nm and emission filters for green (FL1, 520 ± 15 nm) and red (FL2, 585 ± 21 nm). For each experiment, gates were set to exclude crenated and unstained cells and to optimize the separation of the two fluorescent signals. The number of gated events counted per sample was at least 15,000. Percentage of conjugation was calculated as the number of dual-labeled events and the unconjugated Jurkat cells (green events), as described previously (36). Conjugation of DO-11.10 T cells and A20 B cells was performed similarly, except that A20 cells were pulsed for 1–2 h with 2 μg/ml OVA323–339 in place of SEE.

Ab-blocking and drug inhibition experiments

For Ab-blocking experiments, the number of APCs and Jurkat cells used in the conjugations was halved, which did not significantly alter the percentage of conjugation. Following labeling with calcein-AM, Jurkat cells were preincubated with 29 μg/ml PKH26 for 10 min at room temperature, before combining with an equal volume of APCs to give a final Ab concentration of 10 μg/ml. Since B cells also express some LFA-1 (37), the APCs were also preincubated with anti-LFA-1 Abs in the experiments using TS1/18 and TS1/22.

For experiments using wortmannin, PD98059, SB202190, U73122, calpeptin C, and calpeptin D, T cells were first labeled with calcein-AM, washed, resuspended in SF-RPMI containing the appropriate concentration of drug, and incubated at 37°C for 30 min. For LY294002 experiments, D0–11.10 T cells were treated as described above, while Jurkat cells were...
incubated in RPMI 1640 containing drug for 6 h at 37°C, then labeled by adding calcein-AM to a final concentration of 0.125 μg/ml and incubating another 30 min at 37°C. Cells were then washed and resuspended in SF-RPMI containing the appropriate concentration of LY294002. Unless otherwise indicated, drug concentrations were adjusted upon addition of APCs to maintain the indicated concentrations throughout the conjugation. Where indicated, T cells or B cells were treated with drugs for 30 min before conjugation, washed, and added to conjugation reactions in the absence of additional drug.

**Immunoblotting of whole cell lysates**

Jurkat cell suspensions (1 × 10^7 cells/ml in PBS or RPMI 1640) were unstimulated, stimulated for 2 min at 37°C with c305, or stimulated for 5 min at 37°C with OKT3. Alternatively, cells were stimulated by conjugation with SEE-coated (or control) EBV-B cells. Cells suspensions of Jurkat and EBV-B cells (2 × 10^7 cells/ml in RPMI 1640) were combined, pelleted at 500 rpm (room temperature), and incubated for 10 min at 37°C. In each case, cells were lysed at 4°C in 1% (v/v) Nonidet P-40, 10 mM TBS (pH 7.8), 150 mM NaCl, 1 mM Pefabloc, 1 mM sodium orthovanadate, 10 mM NaF, 0.5 μg/ml leupeptin, 4 μg/ml aprotinin, 0.5 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone, and 1 μg/ml pepstatin. Debris was removed by centrifugation at 12,500 × g for 10 min at 4°C. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Filters were probed with 4G10 anti-phosphotyrosine, anti-phospho-ERK, anti-phospho-MEK, or anti-MEK1. Primary Abs were detected using HRP-conjugated secondary Abs and chemiluminescence. For quantitation, blots were scanned using a FluorChem imager (Alpha Innotech, San Leandro, CA). The peak area for each band was determined, and only measurements falling within the linear response range were used. Phospho-MEK and ERK values were corrected for protein loading based on total MEK values, and expressed as percentage of the positive control response for each set of conditions.

**Adhesion to ICAM-1-Fc**

Nunc Maxi-Sorp 96-well plates (Naperville, IN) were coated with 0.5 μg/well goat anti-human IgG (Fcγ specific) overnight at 4°C, blocked with 1% BSA in PBS, then incubated with 0.3 μg/well ICAM-1-Fc at room temperature for 90 min. Wells were rinsed with PBS and blocked with PBS/1% BSA for 1 h before use. Jurkat cells were labeled with 5 μM CFSE in SF-RPMI for 20 min at 37°C. The reaction was quenched with 1% HEPES, then the cells were washed twice in RPMI 1640 and suspended in SF-RPMI containing 10 μM CMAC cell tracker blue for 30 min at 37°C, then labeled by incubation in SF-RPMI containing 10 μM CMAC cell tracker blue for 30 min at 37°C, washed, and resuspended in RPMI 1640 with or without 2 μg/ml SEE at 37°C for 1.5 h. The cells were then washed and resuspended in SF-RPMI at 10^7 cells/ml. Before use, live GFP actin-transfected Jurkat cells were separated from dead cells by passage over Ficoll, washed, and resuspended in SF-RPMI at 10^7 cells/ml. Conjugations were performed as above, except that after vortexing, cells were pipetted onto poly-l-lysine-coated slides and incubated at 37°C for 5–10 min. Slides were rinsed briefly in PBS and fixed in 3% paraformaldehyde/PBS for 20 min at room temperature. For the GFP actin transfectants, slides were dipped in water and mounted (see below).

For LFA-1 immunofluorescence, slides were quenched in 50 mM ammonium chloride, then blocked in PBS/0.25% fish skin gelatin (PBS/gel) for 15 min at room temperature. A total of 20 μl of primary Ab diluted in PBS/gel was added to each well, and the slides were incubated for 45 min at room temperature. Slides were washed in PBS/gel and incubated with Alexa-488-conjugated secondary Abs in PBS/gel for 45 min at room temperature. The slides were then washed, dipped in water, and mounted in Mowial 4-88 (Hoechst Celanese) with 10% 1,4 diazobicyclo-[2,2,2]octane (Sigma-Aldrich) as an antifade. Slides were viewed using a Zeiss Axioplan microscope equipped with a Photometrics PXL-cooled CCD camera. Image capture and deconvolution analysis was performed using Openlab version 2.06 (Improvision, Coventry, U.K.).

**Results**

**LFA-1 mediates the conjugation of Jurkat T cells with superantigen-pulsed B cells**

In the course of studying cytoskeletal responses in a microscopy-based assay (38), we observed that the Lck-deficient T cell line JCaM1 was poor at forming conjugates with APCs, but that the ZAP-70-deficient cell line P116 seemed to conjugate normally. To explore this observation, we adapted a FACS-based assay (36) to measure the ability of Jurkat cells to form conjugates with EBV-B cells. B cells were labeled with the red dyes HE or PKH26, and Jurkat cells were labeled with the green dye calcein-AM. Used in this way, these dyes have no effect on conjugation or effector function (36, 39–41). B cells were then incubated with or without SEE and pelleted together with T cells in a 1:1 ratio. After incubation at 37°C, pellets were vortexed, fixed in suspension, and analyzed by flow cytometry. Fig. 1 shows a typical assay. The percentage of T cells in conjugates is increased 3- to 5-fold in the presence of SEE. The conjugates are quite stable; they can be submitted to pipetting and vigorous vortexing without dissociation (data not shown). Moreover, the percentage of conjugation was remarkably consistent from experiment to experiment, provided that the input T:B ratio was maintained at 1:1.

To identify the adhesion molecules involved, function-blocking Abs were added to inhibit conjugate formation. Conjugate formation in the absence of SEE (Fig. 2, III) was unaffected by control Abs reactive to MHC class I and β2 integrins, but was inhibited by anti-LFA-1 and anti-CD2 Abs. This indicates that both LFA-1 and CD2 contribute to the basal superantigen-independent adhesion. In the presence of SEE (Fig. 2, III), anti-LFA-1-α- and β-chain-specific Abs inhibited conjugation to almost background levels, alone or in combination, whereas anti-class I, anti-β1, and anti-CD2 Abs had no effect. When anti-CD2 Abs were combined with anti-LFA-1 Abs, binding was decreased slightly more than with anti-LFA-1 Abs alone, but the difference was not significant (data not shown). Taken together, these results show that the SEE-dependent adhesion is largely mediated by LFA-1.
Adhesion requires the activity of Lck, but not ZAP-70

Wild-type Jurkat cells (E6), or Jurkat lines lacking Lck (JCaM1) or ZAP-70 (P116) were tested for their ability to form stable conjugates with SEE-pulsed B cells. The level of conjugation observed for each of the mutant cell lines in the absence of SEE was not significantly different from that observed for E6 cells (data not shown). In the presence of SEE (Fig. 3A), JCaM1 cells failed to form conjugates above the background levels observed in the absence of SEE. This was specifically due to the absence of Lck, since conjugation was restored in JCaM1 cells stably transfected with wild-type Lck. In contrast, the ZAP-70-deficient cell line P116 formed conjugates as well as wild-type cells. This indicates that the increased adhesion mediated by LFA-1 in response to SEE requires the activity of Lck, but not ZAP-70.

To further study the requirement for Lck, we tested a series of JCaM1 cell lines that stably express functional domain mutants of Lck. These include the Src homology (SH) 2 domain mutant R154K, the kinase-dead mutant K273A, and the myristoylation mutant G2A, all of which fail to initiate TCR signaling (28) (our unpublished data). Also tested was the SH3 domain mutant, W97A, which initiates TCR signaling through ZAP-70, but is defective in the MAPK pathway (29). Finally, since Fyn can restore some aspects of TCR signaling, we tested JCaM1 cells expressing Fyn at levels similar to that of Lck in wild-type Jurkat cells (30) to

FIGURE 2.  SEE-induced conjugation is mediated by LFA-1. Conjugations were conducted after incubation of Jurkat cells (and, in the case of LFA-1 Abs, B cells) with blocking Abs to the indicated proteins, all at 20 μg/ml, before mixing T cells and APCs. In the absence of superantigen (Lady), conjugate formation was inhibited by anti-CD2, and by Abs to the αL- and β2 chains of LFA-1 (alone or in combination). In the presence of SEE (Lady), only the LFA-1-specific Abs inhibited conjugate formation. Data are means of at least three independent experiments ± SD.

FIGURE 3.  SEE-induced LFA-1-mediated adhesion requires Lck, but not ZAP-70. Conjugations were carried in the absence (Lady) or presence (□) of SEE, using a panel of T cell lines. A, In comparison with control Jurkat cells (wild-type, WT) and ZAP-70-deficient cells (P116), Lck-deficient cells (JCaM) fail to show SEE-induced increases in conjugation. This is restored in JCaM cells stably expressing wild-type Lck (JCaM/Lck). B, Lck domain mutants fail to restore conjugation to JCaM1 cells. All proteins are expressed in JCaM1 cells, and unless specified are expressed at levels comparable with Lck in control E6 Jurkat cells. Lck, wild-type Lck; Lck20%, wild-type Lck at 20% normal levels; KD, kinase-dead Lck (K273A); SH3, SH3 domain mutant (WA97); Myrist, myristoylation site mutant (G2A); SH2, SH2 domain mutant (R154K) at 20% normal levels; Fyn, wild-type Fyn. Background levels of conjugation for the mutant cell lines in the absence of SEE were similar to that observed for E6 cells (data not shown). Data are means of at least three independent experiments ± SD.
ask whether Fyn can substitute for Lck in signaling APC-induced adhesion. All cell lines except the SH2 domain mutant R154K express levels of Lck (or Fyn) equivalent to Lck levels in wild-type Jurkat cells (28, 29) (data not shown). Since the SH2 domain mutant expresses Lck at ~20% of normal levels, a transfectant expressing similar levels of wild-type Lck (28) was included as the matched control for this mutant. As shown in Fig. 3B, only wild-type Lck could restore normal conjugation to JCaM1 cells. Even the expression of wild-type Lck at only 20% of normal levels was sufficient to restore a significant level of conjugation. In contrast, none of the altered forms of Lck could rescue the JCaM1 conjugation phenotype. For the Lck myristoylation, SH2 domain, and kinase-dead mutants, the defect in adhesion can be attributed to the failure of these cells to initiate TCR signaling. However, cells expressing the Lck SH3 domain mutant recruit and activate ZAP-70, and flux calcium normally (29), but these cells still failed to form conjugates. Furthermore, although the expression of Fyn in Lck-deficient T cells has been reported in other systems to partially restore TCR signaling (30), we found that Fyn could not compensate for Lck in our conjugation assay. Taken together, these results indicate that adhesion to APCs via LFA-1 depends upon signaling through Lck, but not ZAP-70.

Conjugation does not require MEK activation, but is sensitive to PKC and phospholipase C (PLC) inhibitors

Since expressing the Lck SH3 domain mutant are defective in MEK activation (29), one possibility was that signaling through MEK might be required for conjugation. In keeping with this, it has been reported that inhibition of MEK activity partially inhibits the TCR-mediated adhesion of murine T cells to immobilized ICAM-1 (21). We therefore looked for evidence that the MAPK pathway functions downstream of Lck in the SEE-induced activation of LFA-1-dependent conjugation. When Jurkat E6 cells pretreated with the MEK inhibitor PD98059 were tested, no inhibition of conjugation was observed (Fig. 4). The inhibition of MEK phosphorylation was confirmed by Western blotting with anti-phospho-MEK (data not shown). Thus, if the defect in conjugation requires signaling in the Ras-MAPK pathway, the relevant molecules must lie upstream of MEK.

In a search for alternative signaling pathways, we tested the effect of a panel of inhibitors on conjugation (Table I). An inhibitor of p38 MAPK (42) failed to inhibit conjugation. The PI-3 kinase inhibitors wortmannin and LY294002, which have been reported to inhibit T cell adhesion to immobilized ICAM-1 (19), did not inhibit conjugation, even when the Jurkat cells were treated for extended periods of time. Similarly, the calpain inhibitor calpeptin, which also inhibits binding to immobilized ICAM-1 (23), had no effect.

Based upon studies using immobilized ICAM-1, PKC is thought to play an important role in LFA-1-dependent T cell adhesion. In the conjugation assay, we could not bypass the requirement for TCR signaling by treatment with PMA (see Fig. 7); however, we found that the PKC inhibitor calphostin C profoundly inhibited SEE-dependent conjugation (Table I). Two interpretations of these findings are possible. Activation of PKC may be necessary, but not sufficient, to induce conjugation. Alternatively, conjugation may require the localized activation of PKC at the cell-cell contact site, which cannot be adequately mimicked by treatment with soluble PMA. The other inhibitor that affected conjugation was the general PKC inhibitor U73122. This drug was recently reported to inhibit IL-3-induced integrin activation in BaF3 cells (43). Importantly, calphostin C and U73122 had similar effects on both wild-type Jurkat T cells, and Jurkat cells lacking a functional ZAP-70 (Fig. 5, A and B). This suggests that the PLC isoform required for activation-induced conjugation is not dependent on ZAP-70. To verify that calphostin C and U73122 inhibit conjugation by affecting T cell signaling rather than APC-dependent processes, control experiments were conducted in which T cells or B cells were preincubated with the inhibitors, which were then removed during conjugate formation. Inhibition was observed only when T cells were preincubated with drug (Fig. 5C), showing that the relevant PKC- and PLC-dependent events occur in the T cell.

The strong dependence of conjugate formation on signaling through PKC and PLC family members was not restricted to Jurkat T cells, nor to superantigen-induced conjugate formation. Similar results were obtained with Ag-specific T cells from DO-11.10 TCR Tg mice conjugated to A20 B cells pulsed with OVA25-33 peptide. As shown in Fig. 5D, treatment of the murine T cells with PD98059 had little effect on conjugate formation, and LY294002 inhibited conjugation only partially. By contrast, both U73122 and calphostin C completely blocked conjugation, indicating that the requirement for signaling through PKC and PLC extends to this system as well.

**U73122 inhibits the MAPK signaling pathway**

Since the Lck SH3 domain mutation and the PLC inhibitor U73122 both inhibited conjugation, we looked for evidence that they affect the same signaling pathways. Initially, Jurkat cells were pretreated with U73122 and stimulated with anti-TCR Abs. Western blots of whole cell lysates were then probed with Abs that recognize activated MEK and activated ERK. Under these conditions, inhibition of PLC induced only a partial reduction in MEK

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**Table I. Inhibition of conjugate formation**

<table>
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<tr>
<th>Enzyme Inhibited</th>
<th>Drug</th>
<th>Highest Dose</th>
<th>% Untreated Control</th>
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<tr>
<td>PI-3 kinase</td>
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<td>PLC</td>
<td>U73122</td>
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<td>4</td>
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</tbody>
</table>

*Jurkat T cells were pretreated with inhibitors at concentrations up to the highest dose indicated, and conjugates with EBV-B cells were formed in the continued presence of inhibitor, all as described in Materials and Methods. Percent conjugation was determined and is expressed relative to untreated control conjugates prepared in parallel. Data are representative of results from two or three independent experiments.*
Inhibitors of PLC and PKC inhibit the conjugation of wild-type and ZAP-70-deficient Jurkat T cells, and Ag-specific primary T cell blasts. A, Inhibition of Jurkat: EBV-B conjugation by the PLC inhibitor U73122. Following calcein-AM labeling, wild-type (E6, solid line) and ZAP-70-deficient (P116, dashed line) Jurkat T cells were incubated with the indicated concentrations of U73122 for 30 min, combined with SEE. Calibration of Jurkat: EBV-B conjugation by the PLC inhibitor U73122. Following calcein-AM labeling, wild-type (E6, solid line) and A blasts. By contrast, when T cells were stimulated with SEE-pulsed APCs, MEK phosphorylation was inhibited to near-basal levels (85% inhibition relative to the levels obtained in the absence of drug; Fig. 6D, []). Under these conditions, ERK phosphorylation was inhibited by 52% relative to the levels obtained in the absence of drug (Fig. 6D, []). Although it is not clear whether this partial reduction in ERK phosphorylation would impact gene expression, these results show that activation by superantigen-coated APCs presents a qualitatively different signal to T cells than activation by anti-TCR Ab, and that a PLC activity functions upstream of MEK activation under conjugation conditions.

To exclude the possibility that the defect in MEK activation is due to a failure of the cells to maintain contact long enough to initiate the TCR signaling cascade, Western blots of cells activated in the absence or presence of U73122 were probed with antiphosphotyrosine. Dramatically increased levels of tyrosine phosphorylation were observed in the lysates of all the TCR- or B cell-stimulated samples, irrespective of whether U73122 was present, indicating that the early signaling cascade was initiated (data not shown).

**FIGURE 5.** Inhibitors of PLC and PKC inhibit the conjugation of wild-type and ZAP-70-deficient Jurkat T cells, and Ag-specific primary T cell blasts. A, Inhibition of Jurkat: EBV-B conjugation by the PLC inhibitor U73122. Following calcein-AM labeling, wild-type (E6, solid line) and ZAP-70-deficient (P116, dashed line) Jurkat T cells were incubated with the indicated concentrations of U73122 for 30 min, combined with SEE-

pulsed B cells in the continued presence of inhibitor, and assayed for conjugation efficiency. Both wild-type and ZAP-70-deficient cells showed a dose-dependent inhibition of conjugation to levels found in the absence of SEE. B, Inhibition by the PKC inhibitor calphostin C. Following calcein-AM labeling, wild-type ([]) and ZAP-70-deficient ([]) Jurkat T cells were incubated with 200 nM calphostin C (Calph. C) for 30 min, combined with SEE-presenting or control B cells in the continued presence of inhibitor, and assayed for conjugation efficiency. A and B, drug concentrations were maintained during the conjugation. C, Treatment of T or B cells before conjugate formation. Wild-type Jurkat T cells or EBV-B cells were untreated ([]) or preincubated for 30 min with 1 μM U73122 ([]) or 200 nM calphostin C (Calph. C). Cells were either allowed to form conjugates in the continued presence of inhibitor, as described above (Contin.), or washed, and used in conjugation reactions in the absence of additional inhibitor (B only, treated B cells + untreated T cells; T only, treated T cells with untreated B cells). D, Inhibition of conjugation by Ag-specific primary T cell blasts. Primary T cell blasts from DO-11.10 TCR Tg mice were pre-treated with the indicated inhibitors at the concentrations given in Table I, and allowed to form conjugates with unpulsed ([]) or OVA323-339-pulsed ([]) A20 B cells. Data in A and B represent means of at least three independent experiments ± SD. Data in C and D represent triplicate samples from one of two independent experiments ± SD.

Adhesion involves avidity changes and protein tyrosine kinase (PTK)-dependent cytoskeletal remodeling

In studies of LFA-1-dependent binding of preactivated T cells to immobilized ICAM-1, it has been shown that changes in either avidity or affinity can lead to increased binding. In keeping with this, we find that binding of unactivated wild-type Jurkat cells to immobilized ICAM-1 can be activated by either incubation with Mg-EGTA, under conditions that induce the high-affinity conformation of LFA-1, or by treatment with PMA, which results in enhanced avidity (16). Binding under either of these conditions consistently exceeded that of stimulation with anti-TCR (Fig. 7A).
We then asked whether we could bypass the requirement for TCR signaling in the conjugation assay in each of these ways. As shown in Fig. 7B, PMA treatment failed to activate conjugate formation in the absence of SEE, while incubation with Mg/EGTA induced only a 2-fold increase in conjugation. Varying the conditions under which these experiments were done, for example altering the timing of PMA stimulation or stimulating with PMA + ionomycin, failed to induce additional conjugate formation (data not shown).

Although we saw no enhancement of conjugation by PMA treatment, this could be explained by a requirement for the localized activation of PKCs at the cell-cell contact site that cannot be adequately mimicked by treatment with soluble PMA. However, the finding that induction of the high affinity form of LFA-1 by incubation with Mg-EGTA could not account for the large increase observed with SEE-dependent signaling suggests that adhesion to APCs involves a significant component of increased avidity. These results indicate that the requirements for plate-bound adhesion and conjugate formation differ, and must be assayed in different ways.

It seemed likely that the T cell mutants that fail to conjugate properly might be defective in actin remodeling and recruitment of LFA-1 to the cell-cell contact site. To evaluate the remodeling of actin in the T cell only, without visualizing actin in the bound B cell, wild-type and mutant T cells were transiently transfected with GFP actin and conjugates were analyzed by fluorescence microscopy. Wild-type T cells typically formed a slightly cupped contact which these experiments were done, for example altering the timing of PMA stimulation or stimulating with PMA + ionomycin, failed to induce additional conjugate formation (data not shown).
among conjugates. As expected, JCaM1 T cells formed very few conjugates. In those that did form, the contact site morphology was relatively normal, although on average it was narrower than in wild-type cells, and no actin response was observed (Fig. 8C). JCaM1 T cells expressing either the Lck SH3 domain mutant (Fig. 8D) or Fyn (Fig. 8E) behaved similarly; few conjugates were formed, and little or no actin remodeling was observed. The contact site was less than one T cell diameter, and the cells often formed elongated necklike structures reaching toward the bound B cell (Fig. 8D). The ZAP-70-deficient T cells formed conjugates with high frequency; however, the phenotype of these conjugates was strikingly different from those formed by either the wild-type E6 cells or the Lck mutants. The size and shape of the contact site were quite variable, and the T cells often formed “arms” that reached as much as halfway around the APC (Fig. 8E). The accumulation of actin was generally less dramatic than in E6 cells, and more variable in structure. In some conjugates, normal actin organization was observed; in others, actin accumulated primarily at the tips of the “arms.” Sometimes very little actin response was observed, even in conjugates in which extensive cueing occurred.

To ask whether the defects in actin remodeling in the PTK mutants are accompanied by defects in LFA-1 recruitment to the contact site, conjugates were analyzed for LFA-1 by immunofluorescence microscopy. To minimize the contribution of LFA-1 expression on the B cell to the fluorescence signal at the cell-cell contact site, LAD B cells lacking surface LFA-1 were used as APCs. In conjugates formed with wild-type T cells, a bright, sharp band of LFA-1 was observed at the cell-cell interface (Fig. 9A). The length of the LFA-1 band was typically the same as the length of the contact site. Although Lck-deficient T cells expressed levels of LFA-1 similar to wild-type cells and were equally bright when visualized alone, in many of the conjugates formed using these T cells, no LFA-1 band was observed (Fig. 9B). Similar results were obtained with cells expressing the Lck SH3 domain mutant (Fig. 9, C and D). In both Lck-deficient T cells and cells expressing the SH3 domain mutant, a very dim band could sometimes be detected, but this was also observed in wild-type cells contacting B cells in the absence of SEE (data not shown), and probably represents activation-independent accumulation of LFA-1. Interestingly, some conjugates formed using the Lck mutants showed a relatively bright band of LFA-1, which was quite short in comparison with control conjugates (Fig. 9D). This appears to represent a limited recruitment of LFA-1 to a small contact site, and can
apparently occur in the absence of gross actin remodeling. As observed in the actin studies, conjugates made using the ZAP-70-deficient T cells were more heterogeneous than conjugates made using either wild-type T cells or the Lck mutants. In roughly half of the ZAP-70-deficient T cells, accumulation of LFA-1 at the contact site was apparent (Fig. 9E), although in many of these labeling was dimmer or more disorganized than in wild-type conjugates (Fig. 9F). In the remainder, very little LFA-1 accumulated at the contact site (Fig. 9G), or a short, bright band was formed (Fig. 9H). In general, the actin and LFA-1 phenotype of the Lck mutants was much more severe than the ZAP-70-deficient cells. This finding is consistent with the conjugation data, and suggests that Lck plays a role in LFA-1-dependent adhesion that is somehow distinct from that played by ZAP-70.

Discussion

Although the requirement for inside-out signaling in integrin-dependent adhesion has been demonstrated in numerous studies (17, 45), remarkably little is known about the early signaling events required for stable conjugate formation during T cell:B cell interactions. In this study, we have taken advantage of existing mutant cell lines to address the role of the PTKs Lck and ZAP-70 in T cell:B cell conjugate formation. Conjugate formation in our assay system exhibits the basic properties demonstrated in previous studies of T:B conjugates (7, 46, 47). Both CD2 and LFA-1 contribute to modest Ag-independent T:B adhesion, while LFA-1 is primarily responsible for Ag-dependent conjugate formation. In the presence of Ag, conjugate formation is accompanied by recruitment of LFA-1 and actin to the contact site, consistent with cytoskeleton-dependent avidity increases resulting from inside-out signaling.

The finding that Lck activity is required for conjugate formation is not surprising, since signaling through Lck is required to initiate all aspects of TCR-mediated signaling (27). However, our results show that activation of ZAP-70, the protein normally thought to lie along the stream of Lck that ultimately result in the actin and LFA-1 remodeling in response to B cell binding. In most conjugates formed with Lck-deficient T cells, little or no actin accumulates at the cell-cell contact site, consistent with a failure to activate localized actin polymerization. LFA-1 recruitment also fails to occur. These defects almost certainly affect the avidity of T:B binding, and are likely to account for (or at least contribute to) the adhesion defects in these cells. Importantly, similar defects are also observed in cells expressing the Lck SH3 domain mutant, suggesting that a ZAP-70-independent process is involved. Although cytoskeletal defects were also observed in the ZAP-70-deficient T cells, some remodeling was initiated in most of these cells, and many more achieved a wild-type phenotype. Remarkably, ZAP-70-deficient T cells actually tended to form an abnormally large contact site with the APC, suggesting that the structure of the cortical cytoskeleton was aberrantly organized. Although the nature of the defect in these cells is still unclear, the remodeling that does occur is apparently adequate to allow conjugation at normal levels.

We have begun to characterize the signaling pathways downstream of Lck that ultimately result in the actin and LFA-1 remodeling required for conjugate formation. Clues about what pathways may be involved come from the known defects in T cells expressing the Lck SH3 domain mutant. Exactly how this mutant perturbs TCR signaling is an area of active investigation. Many proteins have been shown to interact with the SH3 domain, and the mutation is expected to disrupt these interactions. In addition, however, Lck activity is regulated by conformational changes, so that the mutation may perturb enzymatic activity through complex effects on tertiary structure. One known defect in cells expressing this mutant is in the activation of MEK (29). Since the MAPK pathway has been shown to be required for murine T cell adhesion to immobilized ICAM-1 (21), this pathway was a good candidate. Using the MEK inhibitor PD98059, we show that MEK and ERK are unlikely to be involved in the activation of LFA-1; however, this does not rule out a role for proteins upstream of MAPK. Indeed, our inhibitor studies suggest that conjugation depends on the activity of members of the PLC and PKC families. Classical models of TCR signaling pathways include ZAP-70-dependent activation of PLCγ1, which leads to downstream activation of PKC, and PKC has been implicated in integrin activation in many cell systems (49–54). We find that conjugation is independent of ZAP-70 activity, and that conjugation of ZAP-70-deficient T cells is as
sensitive as wild-type cells to the PLC inhibitor U73122. Thus, the relevant PLC may not be PLC-1, but rather another isoform such as PLC-ε, which has been shown to have Ras/guanine exchange factor (GEF) activity and to interact with Ras and its antagonist, Rap-1 (55, 56). Alternatively, PLC-1 may be activated in a ZAP-70-independent manner under conjugation conditions. If PLC-1 is the relevant isoform, it could influence adhesion either by activation of PKC, or by activation of the Ras pathway (57–60). In support of the latter possibility, we find that the effects of the PLC inhibitor U73122 on MAPK activation are much more pronounced when APCs are used to stimulate the T cells. In addition to showing defects in MAPK signaling, T cells expressing the Lck SH3 domain mutant are defective in costimulation through CD28 (61).

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