Antigen-Receptor Complex Stimulation Triggers Protein Kinase C-dependent CD11a/CD18-Cytoskeleton Association in T Lymphocytes

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Abstract. Although it is well accepted that intercellular adhesion involving the CD11a/CD18 (LFA-1) complex is critical in a wide array of T cell-dependent processes, recent demonstrations of an LFA-1 high avidity state, induced by triggering the T cell receptor (TCR) complex, has raised questions about the intracellular signals generated and molecular events leading to effective cell coupling, as well as their orderly sequence. In this study, we assessed the effects of T cell activation on the actin-based cytoskeleton, and LFA-1, as well as their interaction. Crosslinking the TCR complex with anti-CD3 mAb resulted in actin polymerization and colocalization with LFA-1, as detected by fluorescence microscopy. This association was confirmed by immunoprecipitating LFA-1 from the detergent insoluble, cytoskeletal-associated membrane fraction after TCR crosslinking. These consequences were inhibited by the protein kinase C (PKC) inhibitor staurosporine or by PKC desensitization, as was a transient CD11a hyperphosphorylation, induced by monoclonal anti-CD3. Furthermore, a small percentage of $\beta 2$-deficient T cells maintained the ability to rearrange the cytoskeleton in response to TCR complex activation, with F-actin-VLA4 colocalization. These results provide evidence that the important consequences of TCR-induced signal transduction include a PKC-dependent cytoskeletal rearrangement, involving an association between leukocyte integrins and F-actin. We discuss the implications of these findings with respect to effective T cell functions.

INTERCELLULAR adhesion between cytotoxic T lymphocytes and their targets or helper T lymphocytes and antigen presenting cells involves the coordinate engagement of a variety of membrane receptors by their respective cell bound ligands. Phenomenologic analysis of lymphocyte-target cell adhesion indicates that when the antigen receptor complex is productively engaged, T cells undergo rapid morphologic changes resulting in increased contact area with adhesion strengthening to the antigen bearing cell. This is followed by reorientation of the lymphocyte microtubule organizing center (MTOC)[1] and Golgi complex towards the contact site (for review see reference 23). Although cell membrane and intracellular organelle redistribution have been demonstrated in virtually all T cell-dependent adhesions involving the engagement of the antigen receptor (39), their molecular and biochemical bases have only partially been elucidated.

Evidence has been obtained demonstrating that a nonspecific adhesion step, mediated by structures such as CD2 or CD11a/CD18 (LFA-1), precedes the T cell receptor/antigen interaction, thereby lowering the ΔG required for such interaction to efficiently trigger T cell activation (30, 40). Recent reports, however, provide new insight for the interpretation of these phenomena, showing that triggering of the T cell receptor (13, 45), CD2 (45), MHC class II (32), and CD44 (22) molecular complexes transiently induces a still incompletely defined "activated" or "high avidity" state in lymphocyte adhesion receptors, such as LFA-1, which results in increased affinity for their ligands. According to these findings, the engagement of the antigen receptor on T lymphocytes would be a prerequisite for the subsequent stabilization of intercellular contact mediated by adhesion molecules.

We have been interested in mechanisms of lymphocyte activation following intercellular adhesion, and have previously reported that alloantigen specific adhesion occurs in the LFA-1/ICAM-1-dependent interaction of lymphocytes with vascular endothelium (4, 5). In addition, we found that LFA-1 is itself capable of signal transduction and second messenger generation upon antibody-mediated crosslinking in T and NK lymphocytes (35). In the present work we attempt to further investigate the molecular basis and intracellular events responsible for the "inside-out" signaling between the antigen receptor and cell adhesion molecules. The results demonstrate that crosslinking of the antigen receptor complex triggers a protein kinase C (PKC)-dependent cyto-

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1. *Abbreviations used in this paper*: LAD, Leukocyte Adhesion Deficiency; MTOC, microtubule organizing center; PBMC, peripheral blood mononuclear cells; PKC, protein kinase C; PMA, phorbol myristate acetate; T, temperature.

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skeletal rearrangement in T cells, with rapid co-localization, co-clustering, and physical association of the F-actin-based cytoskeleton with leukocyte integrins, but not other membrane molecules, at the cell surface. We postulate that these events may be involved in the T cell receptor-induced signal transduction process ultimately leading to adhesion strengthening and polarization of the lymphocyte protein secretory apparatus toward the antigen-bearing cell.

Materials and Methods

Cell Isolation

Leukocyte enriched residuals from healthy adult blood donors were obtained from the Red Cross Blood Center (West Haven, CT) and the University of Milan Blood Center (Milan, Italy). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation and were monocyte- and B cell-depleted by plastic adherence and serial passages over nylon wool columns. Monocyte-depleted PBMC were further depleted of CD16+ NK cells by negative selection using a panning technique (47). Cells obtained by this method were >98% CD3+CD16− as detected by direct immunofluorescence cytophluorographic analysis using a FACscan (a registered trademark of Becton Dickinson & Co., Mountain View, CA) flow cytometer. T cells were obtained from heparinized venous blood drawn from a 2-y-old patient affected by Leukocyte Adhesion Deficiency (LAD) syndrome using the same procedure.

Antibodies and Reagents

Anti-CD3 murine mAbs OKT3 (IgG_{2a}), IVD3 (IgG_1), and 289 (IgG_1) were affinity purified from ascites generated in our laboratory. Murine mAbs S3.5 (anti-CD4; IgG_1), W6/32 (anti-MHC class I framework; IgG_{2b}), IA.1 (anti-CD8; IgG_1) were gifts from E. G. Engleman (Stanford University, Stanford, CA); anti VLA 4α chain antibody HP2/1 (IgG_1) was purchased from Immunotech (Marseille, France), TS2.9 (anti-CD58; IgG_1), TS1.18 (anti-CD18; IgG_1), and TS1.22 (anti-CD11a; IgG_1) were kindly provided by A. Krensky (Stanford University); 2F12 and CLB LFA/1/2, both anti-CD11a monoclonal IgG_1s were generous gifts from R. E. Schmidt and R. Van Lier, respectively; mouse monoclonal anti-vinculin (IgGl) and rabbit polyclonal anti-α-actinin were purchased from Sigma Chemical Co. (St. Louis, MO); (CD2.1 and 9.6) (anti-CD2; both IgG_1) were gifts from D. Olive; 3A1 (anti-CD7; IgG_1) was a gift from L. Moretta (University of Genoa, Genoa, Italy). Staurosporine was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN); AMG, Cytochalasin-B, fluoresceine- and rhodamine-conjugated phalloidin, poly-L-lysine and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co.

Cytoskeletal Rearrangement Assay

Glass slides pre-cleaned by serial rinses in acetone and ethanol were precoated with PBS 1% FBS for 60 min at room temperature (T°) and subsequently treated with 10 μg/ml of the indicated antibodies or 100 μg/ml poly-L-lysine in PBS for 30 min at room T°. Slides were rinsed with PBS 1% FBS and soaked in the same buffer at 4°C for storage. Cells (1×10^6/ml) were resuspended in PBS 5% FBS and layered over the coated slides followed by incubation at 37°C for 15–25 min. Non-adherent cells were removed by gentle washing with PBS and adherent cells were fixed with 3% paraformaldehyde, 2% sucrose in PBS for 30 min at room T°. Cells were then permeabilized with permeabilization buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100) for 1 min at 0°C, rinsed with PBS, and treated with 2 μg/ml fluorochrome-conjugated phalloidin for 20 min at room T°. For colocalization studies, FITC-conjugated antibodies specific for various surface antigens were added to the adherent cells after fixation to avoid antibody-induced capping of membrane molecules and incubated for 20 min at 4°C, after which the slides were processed for TRITC-PHD treatment as described above. Slides were mounted in glycerol-based mounting medium and examined with a Dialux microscope (E. Leitz, Rockleigh, NJ) equipped for epifluorescence analysis.

^{32}P-Labeling

Cells were washed three times in phosphate-free, serum-free incubation medium (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 6 mM Glucose, 2 mM $CaCl_2$, 25 mM Hepes, pH 7.4, 0.005% soybean trypsin inhibitor, 5% dialyzed FBS), and resuspended (10^7/ml) in the same medium containing 5% dialyzed FBS and 2.5 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). ^{32}P (10–20 μCi/ml as carrier-free orthophosphate; New England Nuclear, Boston, MA) was added and cells were incubated for 180 min at 37°C to allow complete equilibration of the intracellular ATP pool. At the end of the incubation period cells were pelleted and resuspended in phosphate-free medium with 10 μg/ml of the indicated antibodies followed by incubation at 4°C for 15 min. After washing, cells were resuspended in warm, ^{32}P-containing medium with 20 μg/ml polyclonal goat anti-mouse Ig antiserum and incubated at 37°C for various time intervals. In PKC inhibition experiments, staurosporine (20 nM) or AMG (100 nM) was added before the primary antibody treatment and kept in the cell suspension throughout the assay. In PMA treatment experiments, PMA (50 nM) was added to the cell suspension after the labeling procedure. At the end of each treatment, cells were washed three times in stop buffer (25 mM Hepes, pH 7.9, 100 mM NaCl, 10 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA) and lysed in the same buffer containing 1% NP-40 and a protease inhibitor mix (see below). Lysates were processed for immunoprecipitation as described below.

^{125}I Labeling, Immunoprecipitation, SDS-PAGE, and Immunoblotting

Cells were surface labeled by lactoperoxidase-catalyzed iodination as described (35). Lysis was carried out in buffer containing 1% NP-40 as described except for experiments investigating membrane protein-cytoskeleton associations. In these experiments, cells were lysed in stabilizing buffer (300 mM sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 1% CHAPS, and a protease inhibitor mix containing 1 μg/ml of leupeptin, chymostatin pepstatin, 1 mM benzamidine, 1 mM iodoacetamide, 10 KIU/ml aprotinin, and 5 mM PMSF) and the detergent soluble fraction was collected after 15-min incubation at 4°C and centrifugation at 10,000 g for 2 min. The insoluble pellet was resuspended in solubilization buffer (340 mM sucrose, 0.5 mM ATP, 0.5 mM DTT, 1 mM EDTA, 0.5% NP-40, and protease inhibitor mix) and incubated for 18 h at 4°C. Immunoprecipitation was carried out using a combination of anti-CD11a and anti-CD18 antibodies and samples were subjected to SDS-PAGE analysis as described (35). For co-precipitation assays, cells were lysed in buffer containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris HCl, pH 7.4, 0.02% NaN_3, 0.1% BSA, 1% CHAPS, and the above-described protease inhibitor mixture. For Western blot analysis, samples were electrophoresed, transferred onto nitrocellulose paper, and immunoblotted using HRP-conjugated swine anti-rabbit or goat anti-mouse antisera.

Results

Antigen Receptor Crosslinking Induces Cytoskeletal Rearrangement in T Lymphocytes: Involvement of PKC

Crosslinking of the antigen receptor complex on freshly isolated T lymphocytes by various solid-phase bound anti-CD3 mAbs induces dramatic morphologic changes on the adherent lymphocytes. Immediately after contacting the anti-CD3-coated support, the vast majority (>90%) of T cells undergo a spreading process, with increased area of contact to the solid surface and frequent pseudopode formation (Fig. 1 *b*). This process is selectively induced by anti-CD3 and by stimulating pairs of anti-CD2 antibodies, as T lymphocytes do not adhere to the uncoated solid support (not shown) and maintain their round-shaped, refractile appearance when immuno-adhered to supports coated with non-activating, isotype-matched antibodies, such as anti-CD4 (Fig. 1 *a*), anti-CD8, or anti-CD58 (Table I). Staining of cytoplasmic F-actin with fluorochrome-labeled phalloidin in anti-CD3 stimulated cells reveals rearrangement of the actin-based cytoskeleton, in form of polarized, peripheral patches or central clustering with radiating filaments connected to the cell membrane (Fig. 1 *d*). The reasons for the occurrence

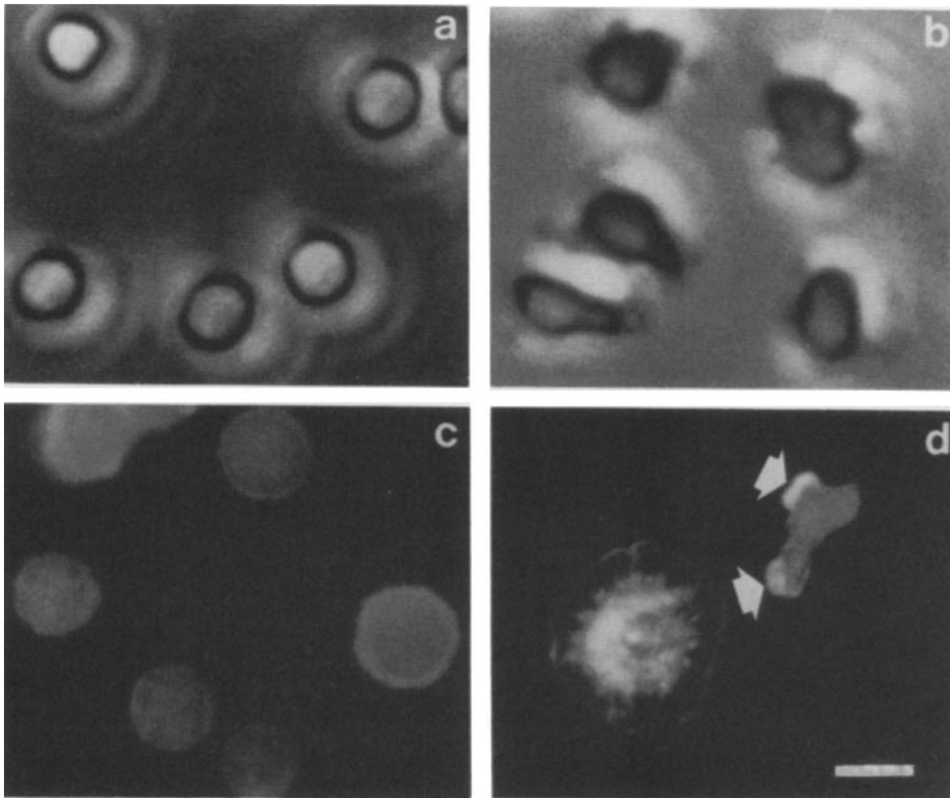


Figure 1. Morphologic and cytoskeletal rearrangement in antigen receptor complex–stimulated T lymphocytes. Cells were layered onto anti-CD4 (*a* and *c*) or anti-CD3 (*b* and *d*) antibody-coated solid supports for 15 min at 37°C, after which unbound cells were removed and bound cells were fixed, permeabilized and stained for cytoplasmic F-actin as described in the Materials and Methods section. Cells were examined under an inverted phase contrast (*a* and *b*) or fluorescence (*c* and *d*) microscope. Panels represent different fields (arrows indicate patches of rearranged actin-based cytoskeleton). Bar, 10 μm.

of multiple, discrete patterns of rearrangement are unclear, but may lie in the uneven spatial distribution of the ligand (anti-CD3 mAb) on the solid support. A diffuse, homogeneous staining is visible in lymphocytes bound to nonstimulating antibody coated dishes (Fig. 1 *c*). Anti-CD3–induced cytoskeletal rearrangement appears to be temperature dependent, although it occurs, at a much slower rate, at T° below 37°C (not shown). As expected, cytochalasin-B pretreatment completely abrogates the process, confirming the involvement of a transition between monomeric and polymerized actin in the observed rearrangement (Table I).

To explore the intracellular events and mediators responsible for this phenomenon, T cells were pre-treated with activating agents capable of bypassing the requirements for membrane receptor engagement and second messenger generation. Of the reagents tested, PMA, but not the Ca^{2+} ionophore ionomycin, appeared to reproduce the anti-CD3–induced rearrangement, suggesting a role for PKC activation in coupling the membrane triggering event to actin polymerization and cytoskeletal rearrangement (Table I). To confirm this, cells were pre-treated with PKC inhibitors staurosporine or AMG before CD2 or CD3 crosslinking and analysis of cytoskeletal rearrangement. Staurosporine inhibits the catalytic domain of PKC at a site different from the phorbol ester binding site (33, 43). AMG selectively inhibits PKC at wide concentration range (22). Table I shows that the inhibitor pre-treatment can indeed abrogate the effects of antigen receptor or CD2 triggering on T cell cytoskeletal rearrangement. Similar inhibition was observed after desensitization of PKC by prolonged incubation with micromolar concentrations of PMA (Table I). Although neither staurosporine nor AMG are absolutely PKC specific, the noted effects at the relatively low concentrations used, in conjunction with the PKC desensitization results, strongly support a role for PKC in this phenomenon.

Selective Association of the CD11a/CD18 Heterodimer with Cytoskeletal Elements in Antigen Receptor-stimulated T Cells

Integrins have been shown to co-localize with cytoskeletal and sub-cortical proteins at focal plaques and adherent junc-

Table I. Induction of Cytoskeletal Rearrangement in T Lymphocytes

	Crosslinked antigen					
Pre-treatment	None	CD2	CD3	CD4	CD8	CD58
None	–	+	+	–	–	–
Cytochalasin-B 20 μM 30′	–	–	–	–	–	–
PMA 50 nM 15′	+	ND	ND	ND	ND	ND
Ionomycin 1 μM 15′	–	ND	ND	ND	ND	ND
Staurosporine 50 nM 15′	–	–	–	–	–	–
AMG 100 nM 15′	–	–	–	–	–	–
PMA 1 μM 24 h	–	–	–	–	–	–

Freshly isolated T cells were bound to antibody-coated or (for treatments with PMA and the Ca^{2+} ionophore ionomycin) poly-L-lysine–coated solid supports after treatment at 37°C with the indicated reagents. (+) Score was assigned when >90% of the cells displayed actin polymerization as shown in Fig. 1 *b*; (–) Score indicates that >90% of the cells displayed diffuse, homogeneous staining with fluorochrome-conjugated PHD as shown in Fig. 1 *c*.

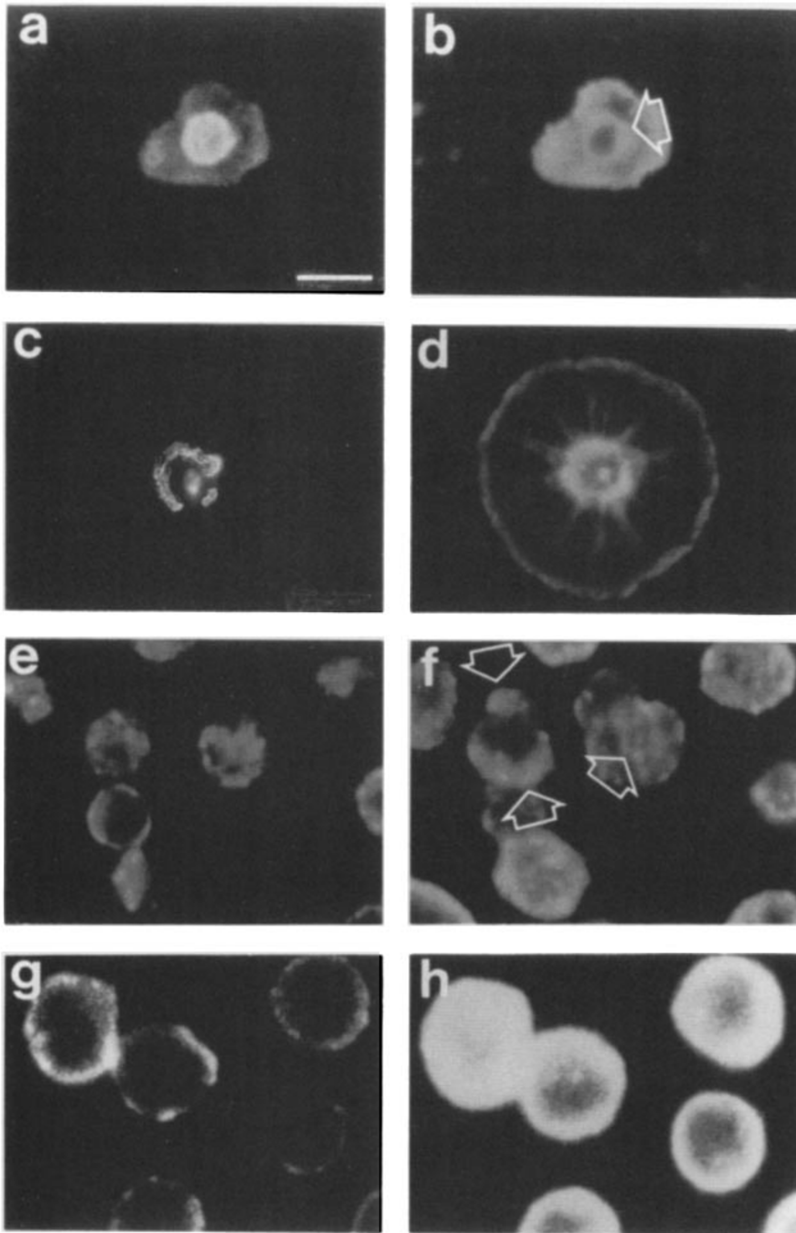


Figure 2. Selective CD11a/CD18-F-actin colocalization in antigen receptor complex–stimulated T cells. Cells were allowed to bind to two anti-CD3 (*a* and *b* OKT3, IgG_{2a}; *c–f* 289, IgG_1) or to anti-CD4 (*g* and *h*) antibody-coated supports and processed as described in Fig. 1. Bound cells were fixed and stained for surface CD11a/CD18 (*a*, *c*, and *g*) or class I MHC (*e*) with FITC-conjugated TS1.22 and W6/32 antibodies, respectively, and counterstained for cytoplasmic F-actin with TRITC-conjugated phalloidin (*b*, *d*, *f*, and *h*) after permeabilization. Paired panels represent the same microscopic field. Arrows indicate patches of rearranged actin-based cytoskeleton. Bar, 10 μm.

tions, and have been proposed to serve as connecting elements between the plasma membrane and the cytoskeleton in matrix-adherent cells such as fibroblasts (7, 10, 18). To investigate whether antigen receptor–triggered lymphocytes display a similar association, anti-CD3–stimulated, solid-phase bound T cells were stained with TRITC-labeled phalloidin to detect filamentous actin and counterstained with FITC-conjugated anti–LFA-1 antibodies to detect redistribution of the heterodimer at the cell surface. Fig. 2 (*a–d*) shows that in anti-CD3–stimulated cells LFA-1 is detected as a central or peripheral cluster, superimposed on and frequently surrounded by F-actin patches. This process is not related to nonspecific capping or patching of the molecule, as lymphocytes immuno-adhered to nonstimulating antibody–coated supports maintain an essentially diffuse distribution of the molecule at the cell surface (Fig. 2, *g* and *h*). Moreover, the co-localization of CD11a/CD18 with F-actin in antigen receptor–stimulated cells appears to be selective, as demonstrated by the diffuse distribution of MHC class I molecules (Fig. 2, *e* and *f*) or other membrane structures such as CD7 and CD58 (not shown) at the cell surface under the same experimental conditions.

To ascertain whether the observed co-clustering of LFA-1 and cytoskeletal elements was due to independent, spatial co-localization or to direct physical association of the two structures, the heterodimer was immunoprecipitated from the detergent-soluble membrane fraction or the detergent-insoluble, cytoskeletal matrix–associated membrane fraction of antigen receptor-stimulated, surface iodinated T cells (17, 28). Fig. 3 shows that the CD11a/CD18 molecular complex can be entirely recovered from the detergent soluble membrane fraction in unstimulated T lymphocytes, suggesting the lack of association to cytoskeletal elements under these conditions. In PMA-stimulated lymphocytes and, even more dramatically, in anti-CD3–triggered cells, however, a significant proportion of LFA-1 molecules could be detected

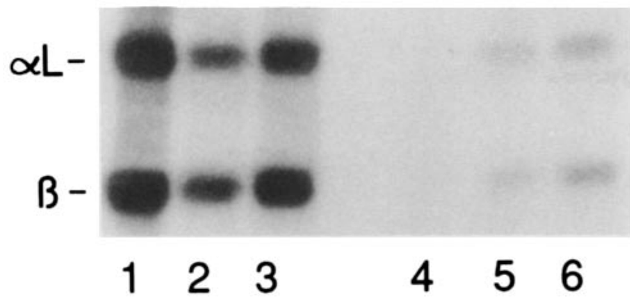


Figure 3. CD11a/CD18-cytoskeletal association induced by anti-CD3 or PMA stimulation in T lymphocytes. Cells (5 × 10^{6}/sample) were treated with medium (lanes *1* and *4*), 50 nM PMA (lanes *2* and *5*) or anti-CD3 plus goat anti-mouse IgG antibodies (lanes *3* and *6*) as described in the Materials and Methods section, followed by lactoperoxidase-catalyzed surface iodination and lysis in cytoskeleton-stabilizing buffer. CD11a/CD18 was immunoprecipitated from the detergent soluble (membrane-associated) fraction (lanes *1–3*) or the detergent insoluble (cytoskeletal-associated) fraction (lanes *3–6*) after overnight incubation in ATP-containing solubilization buffer.

in the detergent-insoluble membrane fraction after overnight incubation in ATP-containing solubilization buffer. Densitometric analysis of the precipitated molecules after SDS-PAGE and autoradiography suggests that the stoichiometry of the two subunits of the CD11a/CD18 complex in the cytoskeleton associated heterodimer is unchanged compared to the membrane-bound form (not shown). Although the relative amounts of membrane-bound versus cytoskeleton-associated LFA-1 molecules in PMA and anti-CD3-stimulated cells would indicate that only a minor proportion of the molecular complex undergoes cytoskeletal association, this interpretation could be misleading because of the intrinsically poor efficiency of the solubilization procedure in preserving low affinity associations between cytoskeletal and membrane proteins. In fact, cytoskeleton-associated proteins could only be detected in our system using lysis buffer containing low salt concentration and the mild zwitterionic detergent CHAPS, instead of conventional nonionic detergents (such as NP-40), suggesting a weak, noncovalent association of membrane LFA-1 with subcortical and/or cytoplasmic cytoskeletal elements (not shown).

To further confirm this induced association, co-precipitation assays were performed using antibodies specific for α-actinin and vinculin. These are subcortical proteins which, among others (such as talin and fimbrim) have been shown to be directly or indirectly involved in connecting the actin-based cytoskeleton to the plasma membrane in several cell types (10). Fig. 4 shows that both α-actinin (*A*, lanes *1–3*) and vinculin (*B*, lanes *1–3*), co-precipitate molecules co-migrating with the LFA-1 heavy and light chains in CD3-stimulated T cells. The identity of the co-precipitated bands with LFA-1 is confirmed by their dramatic and selective reduction in samples pre-cleared with anti-LFA-1 antibodies before immunoprecipitation with anti-α-actinin or anti-vinculin reagents (Fig. 4, *A* and *B*, lane *2*). Of the several anti-LFA-1 antibodies tested, all of which efficiently recognize the heterodimer in resting lymphocytes, some (including 2F12, LFA-1/2, and TS1.18), but not others (i.e., TS1.22) were effective in pre-clearing the cytoskeletal-associated bands, suggesting

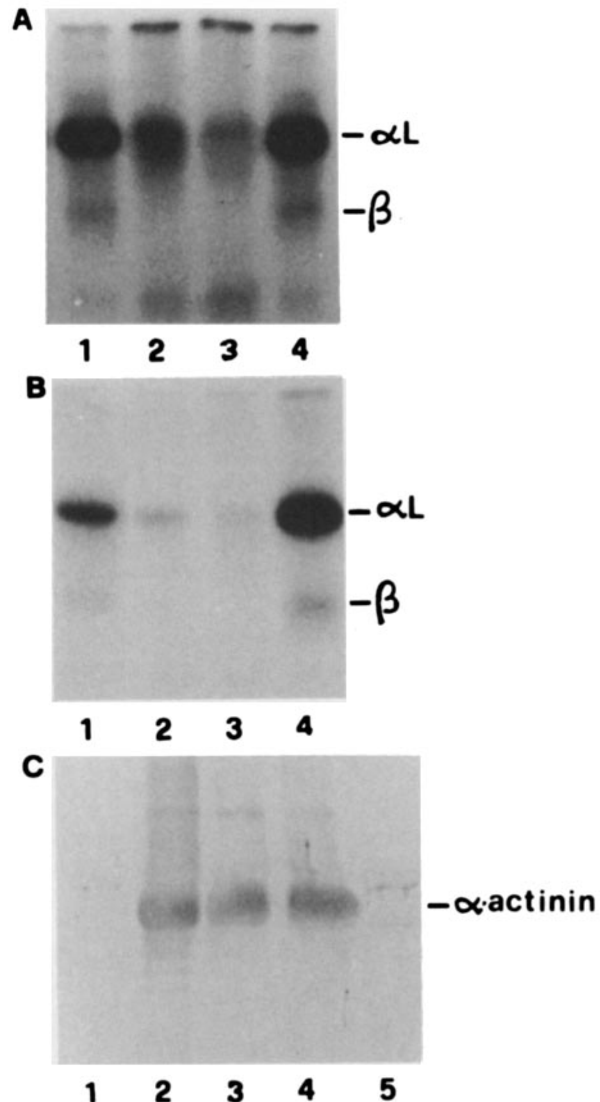
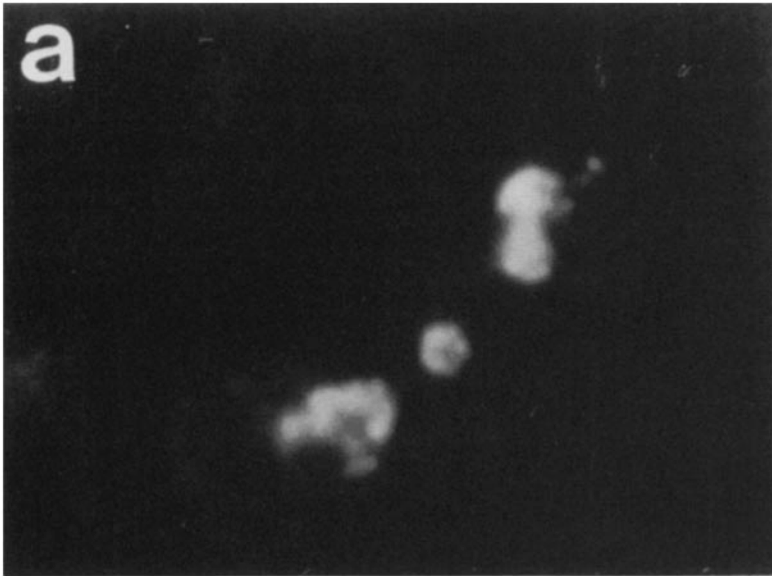


Figure 4. CD11a/CD18 association with the cytoskeletal proteins α-actinin and vinculin upon antigen receptor complex stimulation in T lymphocytes. (*A* and *B*) cells (30 × 10^{6}) were treated with anti-CD3 (lanes *1*, *2*, and *4*) or anti-CD4 (lane *3*) plus goat anti-mouse IgG antisera, followed by lactoperoxidase-catalyzed iodination and lysis in buffer containing 1% CHAPS and divalent cations. Immunoprecipitation was carried out with anti-α-actinin (*A*, lanes *1–3*), anti-vinculin (*B*, lanes *1–3*) or a combination of anti-LFA-1 antibodies (2F12 and TS1.18, lane *4*), In lanes *1* and *2*, the lysate was precleared twice with a negative control antibody or the anti-LFA-1 combination, respectively, before immunoprecipitation with the anti-cytoskeletal protein antibodies. Samples were subjected to SDS-PAGE followed by autoradiography. (*C*) anti-CD3 treated cells were lysed as described above and immunoprecipitation was carried out with anti-vinculin (lane *1*), anti-α-actinin (lane *2*), the anti-LFA-1 antibodies TS1.22 (lane *3*), and 2F12 plus TS1.18 (lane *4*) or a negative control antibody (lane *5*). Samples were reduced, applied to SDS-PAGE, transferred to nitrocellulose and analyzed by immunoblotting with anti-α-actinin polyclonal antiserum.

that the fraction of LFA-1 which becomes physically associated to cytoskeletal proteins in CD3-stimulated cells is conformationally altered (not shown). Reciprocal evidence of the association of LFA-1 with α-actinin was provided by Western blots showing the presence of the 95-kD α-actinin



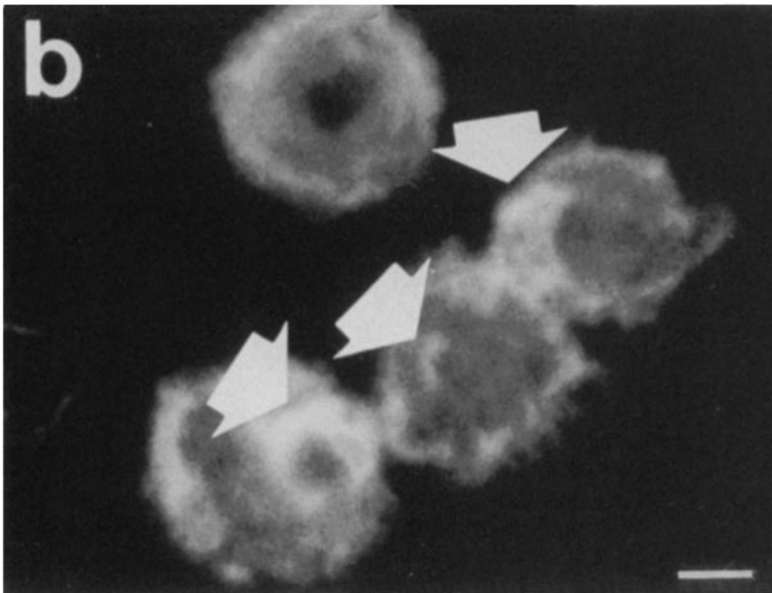


Figure 5. VLA-4-F-actin colocalization in antigen receptor complex–stimulated T cells from a LAD patient. Cells were processed as described in Fig. 1 (*b* and *d*), after which they were fixed and stained for surface VLA4α with FITC-conjugated VLA4α antibody (*a*) and counterstained for cytoplasmic F-actin with TRITC-conjugated phalloidin (*b*) after permeabilization. Bar, 10 μm.

molecule in LFA-1 immunoprecipitates upon CD3 stimulation (Fig. 4 *C*). Note that no α-actinin could be detected in the anti-vinculin precipitates (Fig. 4 *C*, lane *1*) under the conditions used for the co-precipitation assay. A similar experiment could not be performed to confirm the presence of vinculin in LFA-1 immunoprecipitates, as the available anti-vinculin mAb does not recognize the denatured protein in immunoblots (not shown). The association of LFA-1 with α-actinin and vinculin is clearly inducible by the anti-CD3 treatment, although faint bands co-migrating with the leukocyte integrin are visible in unstimulated cells (Fig. 4, *A* and *B*, lane *3*). This may be a consequence of a pre-existing partial association of the two proteins in resting conditions, or reflect the activation of a fraction of T cells during the separation procedure.

Integrin–Cytoskeleton Association in Leukocyte Adhesion Deficiency T Lymphocytes

To assess whether surface expression of LFA-1 is required for anchoring cytoskeletal elements to the cell membrane upon CD3 stimulation, T cells from a LAD patient were used in the solid phase-bound anti-CD3 triggering assay. T lymphocytes from this patient expressed less than 5% membrane LFA-1 molecules compared to normal control cells, but had normal density of CD3 and other coreceptor structures, such as CD4 and CD8 (not shown). Upon binding to anti-CD3–coated dishes, a small (15–20%) but significant proportion of LAD T cells underwent cytoskeletal rearrangement with a pattern undistinguishable from that of normal T cells. When counterstained with anti-LFA-1 antibodies, the rearranged cells did not display any detectable surface LFA-1 molecules (not shown). To test the hypothesis that other leukocyte integrins, normally expressed on LAD T cells (37, 41), could replace LFA-1 in connecting cytoskeletal proteins to the cell membrane, LAD lymphocytes were counterstained with antibodies to the VLA-4 alpha chain after cross-linking of the antigen receptor complex. Fig. 5 demonstrates that membrane VLA-4 selectively co-localized with F-actin with

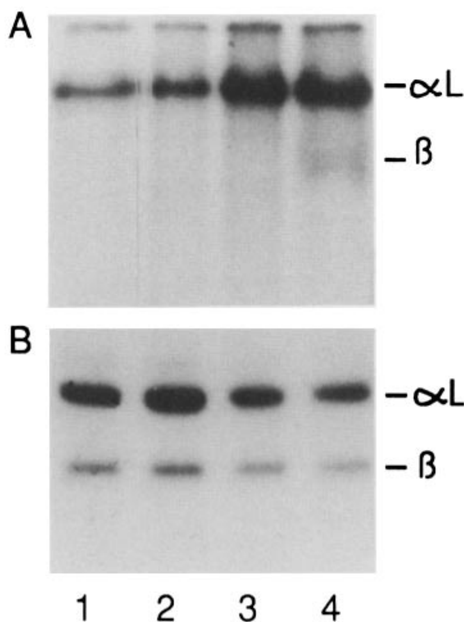


Figure 6. CD11a/CD18 phosphorylation in PMA and antigen receptor complex-stimulated T cells. (*A*) ^{32}P-labeled T cells were treated with polyclonal goat anti-mouse mAb alone (lane *1*), anti-CD3 plus GAM in the presence (lane *2*), or in the absence (lane *3*) of 50 nM staurosporine or 50 nM PMA (lane *4*) after which CD11a/CD18 was immunoprecipitated and detected using PAS-conjugated TS1.22 and SDS-PAGE. *B* shows control CD11a/CD18 immunoprecipitates from surface iodinated cell aliquots identical to *A*.

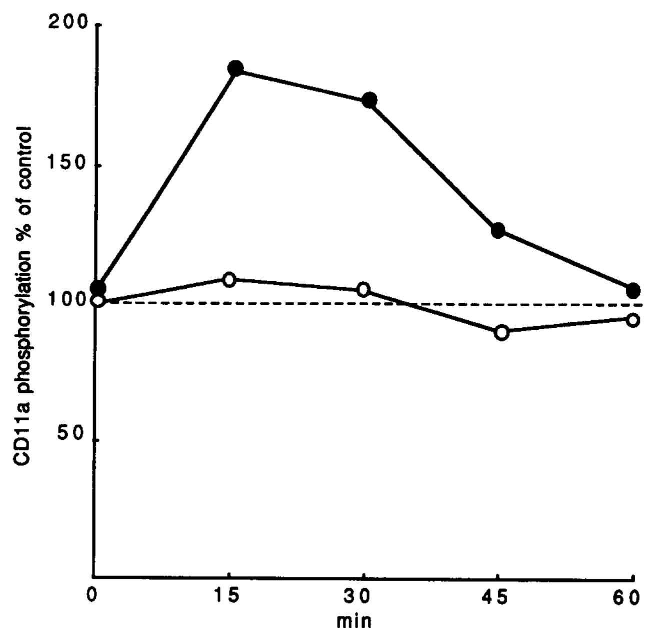


Figure 7. Time course of anti-CD3-induced CD11a hyperphosphorylation in T cells. ^{32}P-labeled T cells were untreated (*O*) or treated with anti-CD3 plus polyclonal goat anti-mouse IgG antibodies (*O*) as described in Materials and Methods after which they were washed and incubated for the indicated times followed by immunoprecipitation with PAS-conjugated TS1.22 mAb, SDS-PAGE, and densitometric analysis of autoradiograms.

a pattern similar to the one displayed by LFA-1 in normal T cells. Nonrearranged LAD T lymphocytes, regardless of the antibody used for the immuno-adherence assay, showed an irregular but essentially diffuse distribution of VLA-4 on the cell membrane (not shown).

Antigen Receptor Stimulation Induces a Transient, PKC-dependent LFA-1 Alpha Subunit Hyperphosphorylation in T Cells

The CD3-induced cytoskeletal rearrangement described above appears to be dependent on PKC activation, as it is induced by phorbol ester treatment and virtually abrogated by PKC desensitization or inhibition (Table I). As both subunits of the LFA-1 heterodimer have been shown to be a substrate for PKC in phorbol ester-stimulated lymphocytes and neutrophils (11, 12, 31), we sought to determine if this enzyme is involved in the heterologous phosphorylation of LFA-1 molecules in antigen receptor-stimulated T cells. Fig. 6 demonstrates that CD3-stimulated T cells indeed display a marked hyperphosphorylation of the CD11a subunit which, as previously reported, appears to be constitutively phosphorylated in unstimulated lymphocytes. Interestingly, no phosphorylation of the CD18 subunit could be detected in anti-CD3-stimulated T cells, although a weakly phosphorylated β subunit was detectable after a short pulse of lymphocytes with nanomolar concentrations of PMA. A role for PKC in the observed CD11a hyperphosphorylation was suggested by its virtual abrogation in staurosporine treated cells, which nevertheless displayed basal phosphorylation levels similar to untreated control cells. Analogous results were obtained by desensitizing PKC with chronic PMA treatment (not shown). This observation would suggest that a kinase different from PKC is responsible for the constitutive phosphorylation of the LFA-1 alpha subunit observed in unstimulated lymphocytes. Analysis of the kinetics of the phosphorylation process (Fig. 7) demonstrated that the hyperphosphorylation of the CD11a subunit induced by antigen receptor triggering is transient, reaching a maximum between 15 and 30 min after the initial stimulus and returning to basal levels within ~45 min.

Discussion

This work was undertaken to investigate some of the intracellular events occurring in T lymphocytes when they adhere to autologous or allogeneic cells bearing specific membrane antigen-MHC complexes capable of triggering the T cell antigen receptor. These events include increased contact area, adhesion strengthening, as measured by biophysical means (19), and polarization of the MTOC and cell secretory apparatus toward the bound cell, all of which are required to spatially and vectorially focus the release of functionally relevant mediators (i.e., lymphokines or cytolytic proteins) from the effector lymphocyte to the target cell (36). These processes are apparently dependent on intracellular signaling driven by the antigen receptor, as they do not occur in cell conjugates lacking antigen specificity (23), but their biochemical and molecular bases are as yet largely undefined. Recent findings have provided a link between the engagement of the antigen receptor and the adhesion strengthening process in T cells, showing that direct cross-linking of CD3 (13) or CD2 (45) structures by activating antibodies induces a transient increase in the avidity of LFA-1 for its ligand, ICAM-1, in purified (13) or cell-bound form (45). The im-

portance of these findings lies in the demonstration of a cross-communication between membrane molecular complexes that are not physically associated with each other but rather interact functionally in the cell adhesion process.

In this study, we show here that direct stimulation of T cells through the antigen receptor complex triggers two independent, but functionally related processes, namely the rearrangement of the actin cytoskeleton, with transition from monomeric (phalloidin nonreactive) to polymerized (phalloidin reactive) actin, and physical association of cytoskeletal elements to membrane leukocyte integrins. Two such elements were directly identified by coprecipitation assays as α-actinin and vinculin (Fig. 4), although it is likely that other proteins (such as talin) may be part of the intermolecular network connecting membrane LFA-1 to the cytoskeleton in antigen–receptor complex stimulated T cells. The apparent absence of α-actinin in the anti-vinculin precipitates, as detected by Western blot analysis (Fig. 4 *C*, lane *I*), raises the possibility that independent LFA-1/α-actinin and LFA-1/vinculin complexes may form upon CD3 stimulation in T cells. This would be at variance with the commonly accepted view that α-actinin and vinculin are part of the same intermolecular chain connecting the plasma membrane to F-actin (10, 15) and with the observation that the two proteins are actually associated with each other with low affinity in fibroblasts (46). An alternative possibility is that the extraction procedure used by us disrupts the weak interactions taking place amongst sub-cortical proteins, although being relatively efficient in preserving their association with LFA-1. Finally, lymphocyte-specific isoforms of either subcortical protein, lacking the appropriate binding site for the other protein, may account for their independent behavior in lymphocytes. This interpretation is supported by the existence of a talin-binding site in cytoskeletal proteins such as vinculin, which may or may not be present in the mature protein depending on the cell type, probably as a consequence of alternative splicing of the relevant mRNA (10). Kupfer and coworkers (26) have reported the clustering of talin, but not α-actinin or vinculin, into the specific cell/cell contact region in cytotoxic lymphocyte:target cell couples. Our contrasting data may reflect actual differences in the triggering process. Alternatively, the different fixation–permeabilization procedures used in the two studies may have contributed to the apparently distinct subcortical elements defined in these associations. Likewise, Kupfer and Singer (24) have shown the co-clustering of LFA-1 and talin in the region of the plasma membrane of helper T cells juxtaposed to antigen-bearing APCs. Our data confirm the general significance of these observations and provide more direct evidence that LFA-1 is not simply co-localized, but it is physically associated to cytoskeletal elements under these conditions. In addition, we show that a similar process can be induced in T cells (as well as NK cells, unpublished data) by stimulating antibodies directed toward the CD2 molecule, which has long been considered a T cell activation structure involved in intercellular adhesion (1) and which has recently been shown to be physically associated with the antigen receptor complex in mature T cells (6).

The common biochemical pathway underlying these events appears to be the activation of PKC, as they can be abrogated by selective inhibition or desensitization of the enzyme. Although antigen receptor triggering, by promoting phosphoinositide hydrolysis, also effects a transient intracellular (Ca^{2+}) increase (for review see reference 1), the ability of PMA alone to induce cytoskeletal rearrangement and LFA-1/F- actin co-clustering, in addition to the inability of the Ca^{2+} ionophore ionomycin to induce the same phenomenon, suggest that extracellular Ca^{2+} influx and/or Ca^{2+} redistribution from internal stores are not strictly required for these processes to occur. In fact, extracellular Ca^{2+} has been reported to induce a polarized signal for reorientation of the cell MTOC and Golgi apparatus in CTL/target cell couples (23), but the co-localization of LFA-1 and talin in such couples seems to be Ca^{2+} independent (25). In agreement with our observations, a role for PKC in the adhesion strengthening process has been proposed by Dustin et al. (13), who demonstrated that phorbol esters can induce a long-lasting high avidity state of LFA-1 for its purified ligand ICAM-1, and that staurosporine abrogates the transient, but quantitatively similar phenomenon induced by antigen receptor crosslinking in T cells. In an earlier report (29), the same group had shown that integrity of the cytoskeleton is required for LFA-1$^+$ cells to adhere to plastic-bound liposomes containing purified ICAM-1. Several, non-mutually exclusive PKC-mediated intracellular events could account for these findings. By phosphorylating intracellular substrates belonging to the cytoskeletal network, PKC could increase their affinity for one or both integrin subunits, thereby facilitating their association at the cell surface, and creating a polarized leading edge which would ensure the apposition of new membrane (and, consequently, new adhesion receptors) at the site of cell contact. The subcortical protein talin has indeed been shown to be a substrate for PKC in phorbol ester–stimulated cells (9, 44). Alternatively (see below), discrete regions in both integrin subunit cytoplasmic domains could serve as substrates for PKC and, as a result of a phosphorylation-induced allosteric transition, increase the affinity of the heterodimeric complex for extracellular ligands or cytoskeletal elements.

Our results demonstrate that the CD11a subunit can be a substrate for PKC-mediated phosphorylation following antigen receptor triggering or PMA treatment in T lymphocytes. Both subunits of the LFA-1 heterodimer have consensus sequences for phosphorylation by serine/threonine kinases in their cytoplasmic domains (27). The alpha subunit, in particular, has two serine residues (positions 1,138 and 1,140) surrounded by charged or polar residues, in the unique carboxy-terminal region of the cytoplasmic domain, in addition to a serine residue (position 1,119) conserved with the p150 (or αX) subunit expressed in polymorphonuclear cells. Although our data do not demonstrate a causal relationship between PKC-induced cytoskeletal rearrangement, the association of LFA-1 with cytoskeletal elements and the adhesion strengthening process, the time course of the observed CD11a hyperphosphorylation (Fig. 6) parallels those of the above mentioned events, suggesting their functional interdependence. Phosphorylation experiments also show that, unlike treatment with phorbol esters, crosslinking of the antigen receptor complex does not induce a detectable phosphorylation of the CD18 subunit in T cells. This could either be a consequence of the different relative potency of the two stimuli, or possibly reflect the involvement of a distinct, staurosporine-sensitive PKC isoform in the process (20, 34). Finally, the possibility that LFA-1 can serve as a substrate for

kinases different from PKC is raised by the persistence of basal, constitutive phosphorylation levels of the CD11a subunit in cells subjected to PKC-blocking or -desensitizing treatments, although we recently observed that CD11a does not appear to be phosphorylated by a cAMP dependent protein kinase in lymphocytes (manuscript in preparation). Direct demonstration of a role for these LFA-1 phosphorylation events in the overall function of the molecular complex awaits the completion of site-directed mutagenesis studies.

Experiments with LAD T cells were conducted to test the hypothesis that membrane LFA-1 is required as a connecting element between the cell membrane and rearranging cytoskeletal elements in antigen receptor–stimulated cells. Results demonstrate that, at least in a subset of T lymphocytes, this function can be carried out by other integrins, such as VLA-4, that are expressed in T lymphocytes as well as in other cells of lympho-hematopoietic origin (41, 42). Although we did not analyze in detail the biochemical events responsible for the VLA-4-cytoskeleton association process, it is possible that they reflect the existence of a common mechanism coupling antigen receptor stimulation to integrin-mediated cell/cell adhesion. These results are of interest, as they could provide an explanation for the relative efficiency of T cell–mediated immunity, as opposed to polymorphonuclear cell mediated immunity, in LAD patients (for review see reference 2). Lymphocytes from these patients show normal expression of members of the $\beta 1$ integrin subfamily and retain the ability to migrate across the endothelium at inflammatory sites (41). In fact, integrity of VLA-4–mediated adhesion has been recently demonstrated to be responsible for the presence of relatively efficient in vitro T cell adhesion to vascular endothelial cells expressing the proposed ligand, VCAM-1 (42), in LFA-1 deficient lymphocytes (37). Little is known, however, about the involvement of this T cell adhesion pathway in more selective lymphocyte effector functions involving the antigen receptor, such as target cell lysis and antigen presentation, although recent reports would suggest that it plays a role in regulatory homotypic and heterotypic interactions in T cells (3, 16, 38).

In conclusion, based on the data presented here, we propose a model in which the triggering of the antigen receptor complex in T lymphocytes initiates a signal transduction process, at least partially dependent on PKC activation, leading to a transient association of LFA-1 or other integrins with the cytoskeletal network. This could result in adhesion strengthening and cytoskeletal-mediated reorientation of the protein secretory apparatus toward the bound cell, with possible active participation of the cytoskeleton in the polarized secretion process (8, 21). Mutagenesis studies will be needed to define in detail the molecular basis for these essential activities of T lymphocytes, and the regulatory events involved in their termination, leading to detachment of the bound lymphocyte and recycling of its effector function.

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References

1. Altman, A., K. M. Coggeshall, and T. Mustelin. 1990. Molecular events mediating T cell activation. *Adv. Immunol.* 48:227–244.
2. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1 and p 150,95 glycoproteins. *Ann. Rev. Med.* 38:175–194.
3. Bednarczyk, J. L., and B. W. McIntrye. 1990. A monoclonal antibody to VLA4α chain (CD49d) induces homotypic lymphocyte aggregation. *J. Immunol.* 144:777–783.
4. Bender, J. R., R. Pardi, J. Kosek, and E. G. Engleman. 1989. Evidence that cytotoxic lymphocytes alter and traverse allogeneic endothelial cell monolayers. *Transplantation (Baltimore).* 47:1047–1053.
5. Bender, J. R., R. Pardi, and E. G. Engleman. 1990. T cell receptor negative natural killer cells display antigen-specific cytotoxicity for microvascular endothelial cells. *Proc. Natl. Acad. Sci. USA.* 87:6949–6953.
6. Brown, M. H., D. A. Cantrell, G. Brattsand, M. J. Crumpton, and M. Gullberg. 1989. The CD2 antigen associates with the T cell antigen receptor CD3 antigen complex on the surface of human T lymphocytes. *Nature (Lond.).* 339:551–553.
7. Buck, C., and A. F. Horwitz. 1987. Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. *J. Cell Sci. Suppl.* 8:231–242.
8. Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion of proteins. *Ann. Rev. Cell Biol.* 3:243–293.
9. Burn, R., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:497–501.
10. Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann. Rev. Cell Biol.* 4:487–525.
11. Buyon, J. P., S. G. Slade, J. Reibman, S. B. Abramson, M. R. Philips, G. Weissmann, and R. Winchester. 1990. Constitutive and induced phosphorylation of the α- and β- chains of the CD11/CD18 leukocyte integrin family. *J. Immunol.* 144:191–197.
12. Chatila, T. A., R. S. Geha, and A. Arnaut. 1989. Constitutive and stimulus induced phosphorylation of CD11/CD18 leukocyte adhesion molecules. *J. Cell Biol.* 109:3435–3444.
13. Dustin, M. L., and T. A. Springer. 1989. T cell receptor cross-linking transiently stimulates adhesiveness through LFA1. *Nature (Lond.).* 341: 619–624.
14. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyi, M. C. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 60:577–584.
15. Geiger, B. 1989. Cytoskeleton-associated cell contacts. *Curr. Op. Cell Biol.* 1:103–109.
16. Groux, S., H. Huet, H. Valentin, D. Pham, and A. Bernard. 1989. Suppressor effects and cyclic AMP accumulation by the CD29 molecule of $CD4^{+}$ lymphocytes. *Nature (Lond.).* 339:152–154.
17. Gupta, S. K., and B. A. Woda. 1988. Ligand-induced association of surface immunoglobulin with the detergent insoluble cytoskeleton may involve actinin. *J. Immunol.* 140:176–182.
18. Horwitz, A. F., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectine receptor with talin. A transmembrane linkage. *Nature (Lond.).* 320:531–533.
19. Hubbard, B. B., M. W. Glacken, J. R. Rodgers, and R. R. Rich. 1990. The role of physical forces on cytotoxic T cell: target cell conjugate stability. *J. Immunol.* 144:4129–4138.
20. Isakov, N., P. McMahon, and A. Altman. 1990. Selective post-transcriptional down regulation of protein kinase C isoenzymes in leukemic T cells chronically treated with phorbol esters. *J. Biol. Chem.* 265:2091–2097.
21. Koffer, A., P. E. R. Tatham, and B. D. Gomperts. 1990. Changes in the state of actin during the exocytic reaction of permeabilized rat mast cells. *J. Cell Biol.* 111:919–927.
22. Koopman, G., Y. Van Kooyk, M. DeGraaff, C. J. L. M. Myer, C. G. Figdor, and S. T. Palls. 1990. Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through LFA-1 pathway. *J. Immunol.* 145:3589–3593.
23. Kupfer, A., and S. J. Singer. 1989*a*. Cell biology of cytotoxic and helper T cell functions. *Ann. Rev. Immunol.* 7:309–337.
24. Kupfer, A., and S. J. Singer. 1989*b*. The specific interaction of helper T cells and antigen-presenting B cells. IV. Membrane and cytoskeletal reorganizations in the bound T cell as a function of antigen dose. *J. Exp. Med.* 170:1697–1713.
25. Kupfer, A., G. Dennert, and S. J. Singer. 1985. The reorientation of the Golgi apparatus and the MTOC in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells. *J. Mol. Cell Immunol.* 2:37–46.
26. Kupfer, A., S. J. Singer, and G. Dennert. 1986. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. *J. Exp. Med.* 163:489–498.

27. Larson, R. S., A. L. Corbi, L. Berman, and T. A. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1α subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* 108:703–712.
28. Marano, N., D. Holowka, and B. Baird. 1990. Bivalent binding of an anti-CD3 antibody to Jurkat cells induces association of the T cell receptor complex with the cytoskeleton. *J. Immunol.* 143:931–938.
29. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function associated antigen-1 (LFA-1). *Cell.* 51:813–819.
30. Mentzer, S. J., B. R. Smith, J. A. Barbora, M. A. V. Crimmins, S. H. Hermann, and S. J. Borakoff. 1987. CTL adhesion and antigen recognition are discrete steps in the human CTL-target cell interaction. *J. Immunol.* 138:1325–1330.
31. Merril, J. T., S. G. Slade, G. Weissmann, R. Winchester, and J. P. Buyon. 1990. Two pathways of CD11b/CD18-mediated neutrophil aggregation with different involvement of protein kinase C dependent phosphorylation. *J. Immunol.* 145:2608–2615.
32. Mourad, W., R. S. Geha, and T. Chatila. 1990. Engagement of major histocompatibility complex class II molecules induces sustained lymphocyte function associated molecule-1 dependent cell adhesion. *J. Exp. Med.* 172:1513–1516.
33. Nakadate, T. A., Y. Jeng, and P. M. Blumberg. 1988. Comparison of protein kinase C functional assay to clarify mechanisms of inhibitor action. *Biochem. Pharmacol.* 37:1541–1549.
34. Nishizuka, T. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.).* 334:661–665.
35. Pardi, R., J. R. Bender, C. Dettori, E. Giannazza, and E. G. Engleman. 1989. Heterogeneous distribution and transmembrane signaling properties of LFA-1 in human lymphocyte subsets. *J. Immunol.* 143:3157–3166.
36. Poo, W., L. Conrad, and C. A. Janeway, Jr. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature (Lond.).* 32:378–380.
37. Schwartz, B. R., E. A. Wayner, T. M. Carlos, H. D. Ochs, and J. M. Harlan. 1990. Identification of surface proteins mediating adherence of CD11/CD18 deficient lymphoblastoid cells to cultured human endothelium. *J. Clin. Invest.* 85:2019–2022.
38. Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Costimulation of proliferative responses of resting $CD4^+$ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. *J. Immunol.* 145:59–67.
39. Singer, S. J., and A. Kupfer. 1988. On membrane dynamics in cell:cell interactions, with application to immunology. *In* The T cell receptor. M. Davis and J. Kappler, editors. A. R. Liss, Inc., New York. 361–376.
40. Spits, H., W. Van Shooten, H. Ketzer, G. Van Seventer, M. Van de Rijn, C. Terhorst, and J. E. DeVries. 1986. Alloantigen recognition is preceded by non specific adhesion of cytotoxic T cells and target cells. *Science (Wash. DC).* 232:403–405.
41. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:426–434.
42. Takada, Y., M. J. Elices, C. Crouse, and M. C. Hemler. 1989. The primary structure of the α-subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1361–1368.
43. Tamadki, T., H. Nomoto, T. Isami, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397–402.
44. Turner, R. S., and J. F. Kuo. 1985. Phospholipid-sensitive, Ca^{2+}-dependent protein kinase (protein kinase C): the enzyme, substrates and regulation. *In* Phospholipids and cellular regulation. J. F. Kuo, editor. Vol. 2. CRC Press, Boca Raton, FL. 75–99.
45. Van Kooyk, Y., P. Van De Wiel, Van Kemenade, P. Weder, T. W. Kuijpers, and C. G. Gifoor. 1989. Enhancement of LFA1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature (Lond.).* 342:811–813.
46. Wachsstock, D. H., J. A. Wilkins, and S. Lin. 1987. Specific interaction of vinculin with a-actinin. *Biochem. Biophys. Res. Commun.* 146:554–560.
47. Wysocki, L. J., and V. L. Sato. 1978. Panning for lymphocytes. A method for cell separation. *Proc. Natl. Acad. Sci. USA.* 75:2844–2848.