Integrin Leukocyte Function-associated Antigen-1-mediated Cell Binding Can Be Activated by Clustering of Membrane Rafts*

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The leukocyte function-associated antigen-1 (LFA-1) integrin (CD11a/CD18) is an important adhesion molecule for lymphocyte migration and the initiation of an immune response. At the cell surface, LFA-1 activity can be regulated by divalent cations that enhance receptor affinity but also by membrane clustering induced by treatment of cells with substances such as phorbol esters. Membrane clustering leads to increased LFA-1 avidity. We report here that LFA-1-mediated binding of mouse thymocytes or activated T lymphocytes to intercellular adhesion molecule 1 can be rapidly induced by clustering of membrane rafts using antibodies to the glycosphatidylinositol-anchored molecule CD24 or cholera toxin (CTx). CD24 and CD18 were found to colocalize in rafts and cross-linking with CTx lead to enhanced LFA-1 clustering. We observed that disruption of raft integrity by lowering the membrane cholesterol content abolished the CTx and the phorbol 12-myristate 13-acetate (PMA) effect to activate LFA-1 with Mg2+/EGTA unpaired. In contrast to activation with Mg2+/EGTA, activation via raft clustering was dependent on PI3-kinase, required cytoskeletal mobility, and was accompanied by Tyr phosphorylation of a 18-kDa protein. Our results support the notion that rafts as preformed adhesion platforms could be important for the rapid regulation of lymphocyte adhesion.

An essential feature of immune cells is their continuous recirculation through the body. Lymphocytes usually travel in a nonadhesive form through the blood and lymph but become adherent when they have to interact with other cells. The LFA-11 (CD11a/CD18) integrin and its cellular ligands ICAM-1–3 play a crucial role in this process because they are involved in lymphocyte adhesion and transmigration through endothelial cells of the blood vessels and the stabilization of cell-cell contacts with antigen-presenting cells (for review see refs. 1–5). Thus, knowledge about the regulation of LFA-1 activity is important for the understanding of lymphocyte function.

Lymphocytes can modulate LFA-1 binding without changing cell surface expression levels. Instead, the LFA-1 molecule can be rapidly converted from an inactive into an active state. In principle, the strength of LFA-1-mediated binding can be up-regulated in two ways: altering the affinity of the individual integrin molecule or increasing the avidity because of clustering of many LFA-1 molecules in the plane of the membrane (4). Experimentally, increased LFA-1 binding can be induced from the inside or the outside of the cell. For example, activation from the inside occurs following triggering of certain membrane receptors like the antigen-specific T cell receptor (6), CD40 in B lymphocytes (7), or CD14 for Mac-1 activation in monocytes (8). Activation of LFA-1 from the outside can be achieved with certain divalent cations like Mn2+ or Mg2+/EGTA, which are thought to bypass the physiological activation pathway and directly affect the conformation of the integrin receptor (4, 9). LFA-1 cluster formation accompanied with increased LFA-1-mediated cell binding can artificially be induced by treatment of lymphocytes with phorbol esters and other substances that increase cellular Ca2+ concentration (9). This type of regulation depends on lateral diffusion of LFA-1 in the membrane and interaction with the cytoskeleton (4, 5, 9).

Much attention has recently been drawn to the role of membrane microdomains termed as rafts (for review see Refs. 10–12). These specialized membrane regions have a distinct composition and are enriched in sphingolipids, cholesterol, certain transmembrane proteins, and GPI-anchored proteins (13–15). Most of the evidence that rafts exist as a physical entity at the cell surface is functional, and convincing physical evidence is still necessary (12). Initially, microdomains containing glycosphingolipids, GPI-anchored proteins and cholesterol were shown to mediate the vesicle transport and sorting of membrane proteins in polarized cells (10, 16, 17). At the cell surface, rafts could serve as platforms for signaling molecules like Src family members, G-proteins, PI3-kinase, and adaptor proteins (10–12, 18, 19). Indeed, recent studies have demonstrated that rafts are required for efficient T cell activation (19–23). However, there is no direct evidence that rafts are involved in the regulation of T lymphocyte adhesion.

Here we have investigated the role of rafts and raft clustering in LFA-1 activation. Clustering was induced using two markers, i.e. antibodies against the GPI-anchored molecule CD24 that is confined to rafts or cholera toxin that binds to the raft marker GM1 lipid (11, 19, 24). We find that cross-linking leads to a rapid increase in LFA-1-mediated cell binding to purified ICAM-1 in thymocytes and activated T cells. We present evidence that CD24 and CD18 co-localize in rafts and that raft integrity is essential for cross-linking-induced but also basal LFA-1 binding. Our results suggest that membrane rafts are important platforms for the regulation of LFA-1 avidity.

MATERIALS AND METHODS

Chemicals and Antibodies—The following mAbs were used and have been described before: mAb 12–15 to CD2, mAb 500A12 to CD3, mAb 79...
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or M1.69 to mouse CD24, mAb 30G12 to CD45, mAb TIB 218 to CD18, mAbs FD441 (TIB 213) and FD 18.5 to CD11a, and mAb YN.1.1/7 against ICAM-1 (25). The Thy-1-specific mAb G7 and FITC-anti-CD3 mAb were obtained from Pharmingen (Hamburg, Germany). The anti-phosphotyrosine mAb 4G10 was obtained from Biomol (Biomol, Hamburg, Germany). Antibodies to the Thy-1 binding site were titrated against Thy-1 from Sigma. Antibodies were used in purified form for functional studies. CTX, CTX B subunit, and MCD were obtained from Sigma. Wortmannin, genistein, and bistindolylmaleimide I were obtained from Calbiochem (Bad Soden, Germany). Jasplakinolide was purchased from Molecular Probes (Leiden, The Netherlands).

Cell Culture and Animals—Thymus and spleen were collected from 6–8-week-old female C57Bl mice. The CD24+/− mice were originally obtained from Dr. Peter Nielsen (Max-Planck Institut für Immunobiologie, Freiburg, Germany) and bred in the DKFZ mouse colony. Erythrocytes were lysed by brief incubation in 155 mM NH₄Cl, 0.1 mM EDTA, 10 mM KHCO₃ solution followed by washing of the cells. T lymphocytes were activated with concanavalin A or immobilized mAb to CD3 as described previously (26). Blasts were isolated by density centrifugation using histopaque 1077 (Sigma). The purity of the T cells was assessed by staining with FITC-conjugated anti-mouse CD3 and was approximately 95%. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 10 mM Hepes, and 50 mM 2-mercaptoethanol. All cells were cultivated at 37 °C, 5% CO₂, and 100% humidity.

Fluorescent Staining—The staining of cells with mAbs and FITC-conjugated goat antibodies to rat Ig has been described (25, 26). Stained cells were analyzed with a FACScan (Becton & Dickinson, Heidelberg, Germany). For confocal microscopy, cells were immobilized on poly-L-lysine-coated glass slides for 20 min and fixed with 1% freshly prepared paraformaldehyde in phosphate-buffered saline for 10 min. Fluorescent staining was then performed using the mAb to CD18 or CD11a followed by FITC-conjugated goat anti-rat Ig. For CTX-cross-linked binding, cells were preincubated with CTX (15 μg/ml) for 10 min before immobilization to the glass slide. After staining the cells were imbedded in levamisol and analyzed with a confocal microscope (Zeiss, Oberkochem, Germany).

Affinity Purification—Mouse ICAM-1 was purified by affinity chromatography on a mAb YN.1.1/7 Sepharose column. Briefly, ES-CH MP cells (25) were lysed in 20 mM Tris/HCl, pH 8.0, containing 2% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation and passed over a normal rat IgG-Sepharose column followed by the specific antibody column. The column was washed and then eluted with 100 mM diethylamine/HCl, pH 11.5, 150 mM NaCl. ICAM-1 preparation was developed with CD24- and CD18-specific mAbs followed by peroxidase-conjugated goat anti-rat Ig and ECL detection (Amersham Pharmacia Biotech). Tyr phosphorylation of cellular proteins was tested by incubating thymocytes for the indicated length of time with mAb to CD24, CTX, Mg²⁺/EGTA, or PMA at 37 °C. Cells were then lysed in the presence of Na₃VO₄ for blocking of phosphatases, and lysates were separated by SDS-PAGE, blotted to Immobilon membrane, and analyzed for Tyr phosphorylation using a specific mAb and ECL as described previously (25).

RESULTS

CD24 Cross-Linking Induces LFA-1-mediated Binding of Thymocytes—Previous studies have shown that antibodies to the GPI-anchored molecule CD24 can modulate integrin activity; however, the underlying mechanism remained unclear (28, 29). In the thymus, CD24 is expressed by nearly all cells, but expression ends before the cells leave the thymus and enter the periphery. We investigated the effects of anti-CD24 antibodies on the binding of thymocytes to purified mouse ICAM-1. As shown in Fig. 1A, the basal level of binding was significantly enhanced in the presence of mAb M1.69 or other mAbs to CD24 (not shown). Adhesion was blocked by mAbs to LFA-1 or ICAM-1 in a dose-dependent manner, indicating that it was mediated by the LFA-1/ICAM-1 binding pathway (not shown).

Engagement of CD24 was required for the LFA-1 activation as thymocytes derived from CD24−/− mice did not show this effect (Fig. 1A). However, both CD24−/− and CD24+/- thymocytes showed equal levels of LFA-1-mediated binding in response to Mg²⁺/EGTA, which is known to increase LFA-1 affinity (2, 9).

CD24 Abs to other cell surface antigens expressed by thymocytes were tested in a dose range of 1–50 μg/ml. In addition to CD24, another GPI-anchored molecule, Thy-1, was able to stimulate LFA-1-mediated cell binding. Antibodies to the transmembrane proteins CD45, CD3, or CD2 were without effect in the short term binding assay (not shown).

CD24 and LFA-1 Co-localize in Membrane Rafts—GPI-anchored proteins are enriched in rafts (10–12, 24). It is generally accepted that these microdomains can be isolated in the form of DIGs, cell membrane rafts, and that cellular proteins from the GPI-anchored molecules are separated from the cytoplasmic domain of another GPI-anchored molecule, Thy-1, was able to stimulate LFA-1-mediated cell binding. Antibodies to the transmembrane proteins CD45, CD3, or CD2 were without effect in the short term binding assay (not shown).
Cross-linking of Rafts Using Cholera Toxin Induces LFA-1

Binding—We assumed that the CD24 mAb cross-linked membrane rafts and because of the co-localization led to LFA-1 cluster formation. To test this hypothesis, another raft marker, the GM1 lipid, was used. CTx consists of an A subunit surrounded by five B subunits that can bind to GM1 and that has been used to cluster rafts (21, 24). We treated thymocytes with CTx or, for control, the monovalent B subunit and analyzed the effect on ICAM-1 binding. Fig. 1B shows that CTx cross-linking triggered ICAM-1 binding in both CD24+/− and CD24+/+ thymocytes in a dose-dependent manner. The monomeric B subunit was unable to mediate the activating effect. However, when the B subunit was cross-linked with specific antibodies, LFA-1 activation was observed with similar magnitude as the intact CTx (not shown). These observations supported the notion that raft clustering was a prerequisite for the induction of LFA-1-mediated cell binding and that the A subunit was not involved in the process.

Fluorescence-activated cell sorter analysis revealed that the level of CD11a or CD18 expression on thymocytes did not change following preincubation with CTx (not shown). As shown in Fig. 3, using confocal microscopy, untreated cells revealed a patchy membrane staining of CD18, and these patches were significantly enlarged and appeared more condensed in CTx-pretreated cells. These observations are in support of the notion that CTx induces raft cluster formation.

Activation of LFA-1 by Raft Cross-linking or Phorbol Ester Has Distinct Features—Having established that raft cross-linking can induce LFA-1-mediated cell binding, we set out to further characterize this activation pathway. Previous studies have indicated that rafts are enriched in Src kinases (15, 24). A possible role of these kinases in CD24- or CTx-induced cell binding was therefore investigated. As summarized in Fig. 4, pretreatment of thymocytes with the Tyr kinase inhibitor genistein could prevent cell binding induced by CTx and the mAb to CD24 but did not affect the Mg2+/EGTA- or PMA-induced binding to ICAM-1. Similar results were obtained by pretreatment of cells with wortmannin, an inhibitor of PI3-kinase. In contrast, the protein kinase C inhibitor bisindolylmaleimide I blocked only the PMA-induced LFA-1 activation but did not affect any other activation pathway.

Integrin binding induced by receptor clustering involves re-arrangement of the cytoskeleton (9, 30). To analyze the role of the cytoskeleton in raft cross-linking, cells were pretreated with jasplakinolide, which stabilizes pre-existing actin filaments, promotes actin polymerization, and prevents actin depolymerization (9, 31). As shown in Fig. 4, Jasplakinolide did not cause inhibition of thymocyte binding in response to Mg2+/EGTA treatment of the cells. In contrast, the binding seen following activation by the mAb to CD24, CTx, or PMA was drastically reduced.

Raft Clustering Causes Tyr Phosphorylation—Given the observation that inhibitors of Tyr kinases could block raft clustering-induced LFA-1 binding, we analyzed the effect of raft clustering on the phosphorylation of cellular proteins using phosphotyrosine blots of whole cell lysates. As shown in Fig. 5, binding of CTx or the mAb to CD24 specifically induced Tyr phosphorylation of a 16–18-kDa protein. This protein band was not detected in thymocytes treated with Mg2+/EGTA or PMA. The nature of the pp16/18 protein remains to be determined. Collectively, these data and the results presented in Fig. 4 suggest that the different pathways of LFA-1 activation are distinct but partially overlapping.

Effect of Cholesterol Depletion on LFA-1-mediated Binding—Membrane rafts are enriched in cholesterol, and several reports have shown that depletion of cellular cholesterol can disperse rafts and destroy function (13, 14, 32, 33). To further
analyze the role of rafts for LFA-1-mediated cell binding, we established conditions to extract membrane cholesterol from thymocytes using MCD. As shown in Table I, pretreatment of thymocytes with 1 mM MCD reduced the level of binding seen after cross-linking with CTx or the mAb to CD24 by approximately 80 or 70%, respectively. The PMA-induced LFA-1 binding was reduced by approximately 42%. The ability of cells to bind ICAM-1 in response to Mg\(^{2+}\)/EGTA was not impaired.

We also investigated the effect of MCD treatment on the binding of activated T lymphocytes to ICAM-1. In contrast to thymocytes, activated mouse T lymphocytes showed a higher level of basal binding (see below) that was further enhanced by cross-linking with CTx, Mg\(^{2+}\)/EGTA, or PMA treatment (between 20–45%). Cross-linking with mAbs to CD24, which is transiently expressed by activated T lymphocytes (26), lead to increased cell binding of similar magnitude as CTx. When activated T cells were exposed to 10 mM MCD before the binding assay, the level of CTx- and mAb CD24-induced binding was reduced by approximately 60% and the level of PMA binding was reduced by approximately 50%. The treatment, however, did not affect the Mg\(^{2+}\)/EGTA-induced ICAM-1 binding. Thus, the results obtained with activated T cell blasts were very similar to those obtained with thymocytes.

**Constitutive LFA-1 Activity Requires Raft Integrity**—These results suggested that rafts were important for the LFA-1 activity induced by cross-linking of rafts or by treatment of cells with PMA, respectively, yet did not play an essential role in the regulation by divalent cations. As mentioned above, activated T cells had a much higher level of constitutive binding to ICAM-1 than thymocytes. This allowed us to investigate whether rafts were also essential for the basal activity of LFA-1 in activated T cells. Indeed, in the absence of exogenous cross-linking, MCD treatment significantly reduced the constitutive binding activity of LFA-1 (Fig. 6A). Again, the regulation of LFA-1 affinity by Mg\(^{2+}\)/EGTA was not affected at the concentrations of MCD tested.

The biochemical composition of membrane rafts can be affected by inhibitors of the cholesterol biosynthesis pathway (32, 33). We used lovastatin, an effective blocker of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Treatment of activated T lymphocytes for 20 h with lovastatin at 1 \(\mu\)M did not affect cell viability as assessed by trypan blue staining, nor did it affect LFA-1 expression levels (not shown). When tested in functional assays, the ability of CTx to induce ICAM-1 binding...
To test this hypothesis we used several ways to perturb membrane and raft integrity. We found that cholesterol depletion, which causes disruption of raft integrity, destroyed the cross-linking-induced LFA-1 activation in thymocytes and in T lymphoblasts. In principle, the failure to activate could be due to the loss of CD24 or GM1 from raft association. However, the finding that not only the cross-linking-dependent activation but also the basal, constitutive level of LFA-1 binding was decreased argues against this interpretation. The manipulation of the cellular cholesterol content also reduced the LFA-1 binding in response to PMA treatment of the cells. In contrast, there was no effect on the Mg$^{2+}$/EGTA activation of LFA-1, which binds ICAM-1 apparently independent of intact rafts. Thus, the raft cross-linking- and PMA-induced binding as well as the constitutive LFA-1-mediated adhesion of T lymphoblasts was critically dependent on intact rafts.

To further dissect the characteristics of the different LFA-1 activation protocols, specific inhibitors for kinases were used. The induction of thymocyte binding with mAb to CD24 or CTx was blocked by the PI3-kinase inhibitor wortmannin and the Tyr kinase inhibitor genistein. PI3-kinase has been implicated in the activation of the $\alpha_{\beta_3}$ integrin of platelets and megakaryocytes (35) and appears to be involved in phosphorylation events during cytoskeletal reorganization, growth factor-dependent mitogenesis, prevention of apoptosis, cytokine production, and endocytic trafficking (36–38). Recent studies have implicated PI3-kinase in the regulation of $\beta_2$-integrin-mediated adhesion of Jurkat T cells to ICAM-1 (39, 40). Expression of a constitutively active PI3-kinase was sufficient to activate LFA-1 adhesion and enhance membrane recruitment of cytohesin-1 (40). The effects could be blocked in the presence of wortmannin (40). Cytohesin-1 is a 47-kDa intracellular protein that interacts specifically in several systems with the cytoplasmic domain of LFA-1 (39). Overexpression of cytohesin-1 in Jurkat cells was shown to have profound effects on the binding of LFA-1 to ICAM-1 (40). In our experiments wortmannin was effective only in raft cross-linking-induced but not in PMA- or divalent cation-induced LFA-1 activation, suggesting a close association of the cytohesin-1 system with raft cluster formation.

Interestingly, in a recent study Hmama et al. (41) have investigated the LFA-1-mediated monocyte adherence induced by LPS. LPS binds to the cell surface receptor CD14. The signaling pathway involved PI3-kinase, cytohesin, and the Rho family of small GTP-binding proteins (41). Although CD14 is a GPI-anchored molecule and expected to localize in membrane rafts, Hmama et al. did not consider a functional role of rafts in this process. Our findings that LFA-1 is functionally associated with rafts and can be regulated by raft cross-linking is in agreement with this previous study.

Recently, Harder and Simons (24) investigated cellular responses to raft patching in Jurkat cells and in particular changes in the actin cytoskeleton. It was shown that clustering of GM1 or GPI-anchored molecules lead to activation of Src kinases and the accumulation of filamentous actin, which was dependent on Tyr phosphorylation in the rafts (24). The study also suggested that phosphotyrosine accumulation is an upstream event occurring before the recruitment of actin filaments in these cells (24). Our finding that genistein could block the mAb to CD24- or CTx-induced LFA-1 activation indicates that the cytoskeleton plays a critical role in the process. The inhibition of LFA-1 activation in cells pretreated with the actin stabilizer jasplakinolide further supports this view.

Our results also showed that jasplakinolide was effective in blocking the PMA-induced LFA-1 activation, consistent with previous studies on human T lymphoblasts (9). In this publication, the mechanism of LFA-1 activation following treatment of T cells with Ca$^{2+}$ mobilizing agents like ionomycin, thapsigargin, cross-linking of the TCR/CD3 complex, or phorbol esters, which also activates protein kinase C, were investigated (9). It was observed that a general feature of these adhesion-activating protocols, in contrast to Mg$^{2+}$/EGTA treatment, was the induction of local LFA-1 clustering in the membrane (9).
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Thymocytes were treated with MCD for 15 min at room temperature, and activated T lymphoblasts were incubated for 25 min. Pretreated cells were then activated with the indicated substances and tested for adhesion to ICAM-1. Data are given as the percentage of binding ± S.E. of non-MCD-treated control.

|                | Thymocytes | T cell blasts |
|----------------|------------|--------------|
|                | Untreated  | MCD-treated (1 μM) | Untreated | MCD-treated (10 μM) |
| Mg/EGTA (5 mM/1 mM) | 100 ± 1.7 | 97.8 ± 1.4 | 100 ± 2.7 | 93.7 ± 3.6 |
| CTx (15 μg/ml)    | 100 ± 3.8 | 17.6 ± 2.7 | 100 ± 3.1 | 37 ± 3.2 |
| mAb CD24 (15 μg/ml) | 100 ± 10 | 31 ± 1.9 | 100 ± 2.9 | 36.1 ± 2.7 |
| PMA (50 ng/ml)    | 100 ± 6.1 | 58.7 ± 1.2 | 100 ± 2.8 | 51.5 ± 1.0 |

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