LFA-1-dependent lipid raft recruitment of DNAM-1 (CD226) in CD4⁺ T cell

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Abstract

Upon antigen recognition by the TCR, both the leukocyte adhesion molecules DNAM-1 and leukocyte function-associated antigen-1 (LFA-1) associate with lipid rafts and form peripheral supra-molecular activation clusters that surround central-supra-molecular activation clusters at the immunological synapse. The serine residue in the cytoplasmic tail of DNAM-1 is responsible for this association of DNAM-1 with lipid rafts. The TCR-mediated signal also induces physical association of DNAM-1 with LFA-1, for which the serine phosphorylation of DNAM-1 is also responsible. However, how the serine residue is involved in lipid raft recruitment of DNAM-1 has remained unclear. Here, we show that, although the TCR-mediated signal induced the serine phosphorylation of DNAM-1, DNAM-1 did not associate with lipid rafts in CD4⁺ T cells derived from mice deficient in LFA-1 expression, indicating that lipid raft recruitment of DNAM-1 depends on LFA-1 expression. These results suggest that the serine phosphorylation of DNAM-1 primarily induces physical association of DNAM-1 with LFA-1, which then takes DNAM-1 into lipid raft compartment.

Introduction

Lipid rafts are specialized plasma membrane microdomains, containing sphingolipids with saturated acyl chains and cholesterol in the outer leaflet (1, 2). These are primarily involved in the initiation and propagation of signal transduction cascades associated with lymphocyte activation (3–5).

The αLβ2 integrin adhesion molecule leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) expressed on a variety of immune cells not only mediates intercellular binding but also transduces signals for their activation (6). Activation of LFA-1 on CD4⁺ T cells mediates co-stimulatory signals and induces various cellular responses, including apoptosis, proliferation, cytokine production, cytoskeletal re-organization and T₁,₂ differentiation (7). Recent reports demonstrated that, upon antigen recognition by the TCR, LFA-1 is recruited into lipid rafts in primary T cells as well as T cell lines (8, 9) and involved in the formation of peripheral supra-molecular activation clusters (p-SMACs) that surround central supra-molecular activation clusters at the immunological synapse (IS) (10, 11) [for review, see (12, 13)].

The leukocyte adhesion molecule DNAM-1 is constitutively expressed on the majority of T lymphocytes, NK cells, monocytes and platelets (14). Stimulation of peripheral T cells with anti-CD3 induces the physical association of DNAM-1 with LFA-1, for which the serine phosphorylation of DNAM-1 at residue 329 is responsible (15, 16). Once LFA-1 and DNAM-1 associate with each other, cross-linking LFA-1 induces tyrosine phosphorylation of DNAM-1 at residue 322, for which the Fyn protein tyrosine kinase is responsible (16), and mediates co-stimulatory signals for triggering naïve T cell differentiation into T₁,₂ phenotype and proliferation (17). The TCR-mediated signal also induces lipid raft recruitments of DNAM-1 (17) as well as LFA-1 and Fyn (18). Recently, we have reported that the serine at residue 329 of DNAM-1 is essentially required for lipid raft recruitment of DNAM-1 (19). However, how the serine residue is involved in lipid raft recruitment of DNAM-1 has remained unclear.

In the present study, we investigated a role of LFA-1 in lipid raft recruitment of DNAM-1 in CD4⁺ T cells, by using mice deficient in LFA-1 expression. We demonstrate here that the serine phosphorylation of DNAM-1 is primarily required for the association of DNAM-1 with LFA-1 that is recruited into lipid rafts, suggesting that DNAM-1 is taken into the lipid raft compartment by LFA-1.
CD4+ T cells were lysed with 1% digitonin lysis buffer containing Co-immunoprecipitation mouse IgG at 37°C transferred onto poly-L-lysine pre-coated cover slips to allow as analyzed by flow cytometry. For CD4+ T cell stimulation, cells were incubated with 3 mg ml−1 of anti-CD3, CD4, CD11a, and phosphoserine mAbs were purchased from BD Biosciences (San Jose, CA, USA). Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-Rap1 ligand (anti-RAPL) polyclonal antibody was kindly provided by Dr T. Kinashi (Kansai Medical University, Moriguchi, Japan). Alexa555-conjugated anti-rat IgG and streptavidin–Alexa647 conjugate were purchased from Molecular Probes (Eugene, OR, USA). FITC-conjugated cholera toxin (CTx) subunit B was purchased from Sigma (St Louis, MO, USA). Mouse DNAM-1–Fc fusion protein was described previously (20).

Cells

CD4+ T cells were separated from splenocytes by positive selection using MACS. The purity of CD4+ T cells was >95%, as analyzed by flow cytometry. For CD4+ T cell stimulation, cells were incubated with 3 mg ml−1 of anti-CD3 and anti-CD28 mAbs on ice for 20 min, followed with 5 mg ml−1 of anti-mouse IgG at 37°C for 20 min.

Co-immunoprecipitation

CD4+ T cells were lysed with 1% digitonin lysis buffer containing 0.12% Triton X-100, 150 mM NaCl, 20 mM triethanolamine, protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF) and 15.3 IU aprotinin] and phosphatase inhibitors (1 mM EGTA, 10 mM NaF, 1 mM Na4PO7, 0.1 mM b-glycerophosphate and 1 mM Na3V04). Lysates were immunoprecipitated with Protein L agarose beads (Pierce, Rockford, IL, USA) conjugated with anti-CD11a or anti-DNAM-1 mAb. Immunoblotting analyses were performed, as described (17, 19).

Immunofluorescence

Receptor clustering was induced as previously described (19). In brief, CD4+ T cells purified from spleen were incubated with 3 mg ml−1 anti-CD3 on ice for 20 min, followed with 6 mg ml−1 of goat anti-rat IgG at 37°C for 20 min. T cells were then transferred onto poly-L-lysine pre-coated cover slips to allow attachment at 37°C for 5 min, fixed with 2% formaldehyde and stained with FITC-conjugated CTx subunit B (Sigma), biotin-conjugated anti-DNAM-1 (TX42) and anti-mCD11a, followed with streptavidin–Alexa647 and anti-mouse IgG–Alexa555 (Molecular Probes). Cover slips were mounted with Slow Fade (Molecular Probes) and analyzed by Carl Zeiss LSM 510 META confocal laser scanning microscopy with a C-APOCHROMAT ×63/1.2 water objective lens. For co-localization analyses, weighted co-localization coefficients (WCCs) were calculated with Carl Zeiss LSM 510 META software, and >0.7 or <0.2 in WCC on each channel was defined as co-localization or separation.

Lipid raft isolation and biochemistry

Lipid rafts were isolated by discontinuous sucrose density gradient ultracentrifugation, by a modified protocol as described (21). In brief, 5 × 107 splenic CD4+ T cells were lysed in 1 ml of ice-cold 1% Brij-35 in TN buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.5 mM EDTA) with protease inhibitors (1 mM PMSF and 15.3 IU aprotinin) for 30 min, gently mixed with an equal volume of 85% in TN buffer and placed in the bottom of a SW41i centrifuge tube (Becton Dickinson, Mountain View, CA, USA). The sample was then overlaid with 5 ml 35% sucrose and 4 ml 5% sucrose in TN buffer and spun for 17–19 h at 200 000 × g at 4°C. Each 1-ml gradient fraction was sequentially collected from the top of the gradient. Sucrose density fractions of cell lysates were immunoprecipitated with anti-mDNAM-1 (TX42) and proteins were separated by SDS-PAGE. Immunoprecipitation was performed with Protein L agarose beads conjugated with anti-DNAM-1 mAb (TX42). Immunoblotting analyses were performed, as described (17, 19).

Result

Physical association of DNAM-1 with LFA-1 in lipid rafts in mouse CD4+ T cell

We have recently identified the mouse homologue of DNAM-1 (20) and found that the cytoplasmic tail of DNAM-1, containing the serine and the tyrosine residues that are involved in DNAM-1-mediated signaling, was well conserved between humans and mice (Fig. 1). We observed that cross-linking CD3 and CD28 on CD4+ T cells with antibodies against these molecules induced the serine phosphorylation of DNAM-1 in mice (data not shown) as well as humans (16), suggesting that the serine residues might be involved in physical association of DNAM-1 with LFA-1 and lipid raft recruitment of DNAM-1 not only in humans but also in mice. In fact, we observed that DNAM-1 was co-immunoprecipitated with LFA-1 in mouse CD4+ T cells after stimulation with anti-CD3 and anti-CD28 mAbs (Fig. 2A). Moreover, DNAM-1 was co-localized with LFA-1 in ganglioside type 1 (GM1)-rich lipid rafts in CD4+ T cells upon stimulation with anti-CD3 and anti-CD28 mAbs (Fig. 2B). These results suggest that the TCR-mediated signal induces the serine phosphorylation of DNAM-1, resulting in both physical association of DNAM-1 with LFA-1 and lipid raft recruitment of DNAM-1 in mouse as well as human CD4+ T cells.

Human: **-**

Mouse: **-**

Fig. 1. Amino acid sequences of the cytoplasmic tail of human and mouse DNAM-1. The last 19 amino acids in the cytoplasmic tail are well conserved in human and mouse DNAM-1. The tyrosine and the serine residues that are phosphorylated are boxed. The amino acids identical to each other are indicated with asterisks.

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**References:**

(17) Mak, T.W. and Rockford, IL, USA (1997). Anti-DNAM-1 (TX42) was generated in our laboratory by standard method, as described (20).


Lipid raft recruitment of DNAM-1 is dependent on LFA-1

Although the serine residue is required for lipid raft recruitment of DNAM-1 (19), how it is involved in this event has been unclear. To address this issue, we examined whether LFA-1 is required for lipid raft recruitment of DNAM-1, using CD4+ T cells lacking LFA-1 expression on cell surface from mice deficient in CD11a (the α chain of LFA-1) gene, because the serine phosphorylation of DNAM-1 results in physical association of DNAM-1 with LFA-1 (16). While LFA-1+/− CD4+ T cells completely lack LFA-1 expression on cell surface, CD4+ T cells from heterozygous littersmates (LFA-1+/−/−) expressed LFA-1 comparable to those of wild-type (WT) littermates (LFA-1+/++) (Fig. 3A). In contrast, CD4+ T cells from WT and both LFA-1+/−/− and LFA-1−/− mutant mice expressed comparable amounts of DNAM-1 (Fig. 3A). Moreover, DNAM-1 expressed on CD4+ T cells from these mutant as well as WT mice were functional, as determined by ligand-binding ability using CD155–Fc fusion protein (Fig. 3A), indicating that expression and ligand-binding function of DNAM-1 were independent of LFA-1 expression on CD4+ T cells. Therefore, we compared CD4+ T cells from LFA-1+/−/− and LFA-1−/− mice to determine the role of LFA-1 for lipid raft recruitment of DNAM-1. CD4+ T cells purified from spleen of these mutant mice were stimulated with anti-CD3 and anti-CD28 mAbs and then stained with FITC-conjugated CTx subunit B and biotin-conjugated anti-DNAM-1 and anti-CD11a, followed by streptavidin–Alexa647 (blue) and anti-rat IgG–Alexa555 (red), and analyzed by confocal laser scanning microscopy. Data are representative of three independent experiments.

Fig. 2. Physical association of DNAM-1 with LFA-1 in lipid rafts. Splenic CD4+ T cells were stimulated or not stimulated with anti-CD3 plus anti-CD28 mAbs, followed by cross-linking with the secondary antibody. (A) The stimulated CD4+ T cells were lysed in 1% digitonin lysis buffer and immunoprecipitated with anti-CD11a (the α chain of LFA-1) or anti-CD18 mAb (the β chain of LFA-1) and were analyzed by immunoblotting with anti-DNAM-1 mAb. (B) Otherwise, the stimulated CD4+ T cells were fixed and stained with FITC-conjugated CTx subunit B and streptavidin–Alexa647 (blue) and anti-rat IgG–Alexa555 (red), and analyzed by confocal laser scanning microscopy. Data are representative of three independent experiments.
after stimulation in LFA-1+/− CD4+ T cells (Fig. 4A). Taken together, these results indicated that lipid raft recruitment of DNAM-1 depends on LFA-1 expression.

The tyrosine residue in the cytoplasmic tail is conserved also in mouse DNAM-1 (Fig. 1), which was phosphorylated by stimulation with pervanadate in mouse as well as human DNAM-1 (data not shown). Of note, mouse DNAM-1 was tyrosine phosphorylated after stimulation with anti-CD3 and anti-CD28 only in Brij-35-insoluble lipid raft fractions (Fig. 4A), as observed also in human CD4+ T cells (19), suggesting that association of DNAM-1 with lipid rafts was necessary for tyrosine phosphorylation of DNAM-1.

Recent evidences demonstrated that T cell stimulation induces the association of Rap1 with RAPL, followed by binding of these associating molecules with LFA-1 (22). We examined whether Rap1 or RAPL is involved in the physical association of DNAM-1 with LFA-1 upon stimulation of CD3 and CD28, as shown in Figs 2(A) and 4(B). While both Rap1 and RAPL physically associates with DNAM-1 after stimulation of CD3 and CD28 in LFA-1+/− CD4+ T cells, the associations

Fig. 3. Co-localization of DNAM-1 with lipid rafts is dependent on LFA-1. (A) LFA-1+/+ (dotted open histograms), LFA-1+/− (solid open histograms) and LFA-1−/− (gray filled histograms) CD4+ T cell were stained with anti-CD11a, anti-DNAM-1 or CD155-Fc fusion protein, followed by PE-conjugated secondary antibody, and analyzed by flow cytometry. (B and C) LFA-1−/− and LFA-1+/− CD4+ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs, followed by cross-linking with anti-hamster IgG. The stimulated CD4+ T cells were fixed and stained with FITC-conjugated CTx subunit B and anti-DNAM-1, followed by anti-rat IgG–Alexa555 (red), and analyzed by confocal laser scanning microscopy. Data are representative of three independent experiments (B). The percentage of co-localization of DNAM-1 with GM1-rich lipid rafts in 400 LFA-1+/− and LFA-1−/− CD4+ T cells were calculated after stimulation with anti-CD3 and anti-CD28 mAbs.
were not observed in LFA-1−/− CD4+ T cells (Fig. 4C). These results suggest that DNAM-1 does not directly bind to Rap1 and RAPL but indirectly associates with these molecules via LFA-1 upon T cell activation. Thus, the association of LFA-1 with DNAM-1 was not mediated by Rap1 and RAPL.

The serine phosphorylation of DNAM-1 is independent of LFA-1

To examine the role of LFA-1 in the serine phosphorylation of DNAM-1, CD4+ T cells from WT and LFA-1 mutant mice were stimulated with anti-CD3 and anti-CD28 mAbs and examined for the serine phosphorylation. As demonstrated in Fig. 5, stimulation with anti-CD3 and anti-CD28 mAbs induced the serine phosphorylation of DNAM-1 in both LFA-1+/+ and LFA-1−/− CD4+ T cells. These results indicate that the serine phosphorylation of DNAM-1 induced by the TCR-mediated signal was independent of LFA-1 expression.

Discussion

Our prior studies demonstrated that the TCR-mediated signal induced physical association of DNAM-1 with not only LFA-1 (16) but also lipid rafts (19), for both of which the serine residue in the cytoplasmic tail of DNAM-1 was responsible. In this study, we have demonstrated that the TCR-mediated signal could not induce association of DNAM-1 with lipid rafts in CD4+ T cells derived from mice deficient in LFA-1 expression (Figs 3 and 4), indicating that LFA-1 is essentially required for recruitment of DNAM-1 into lipid rafts. Because the serine phosphorylation of DNAM-1 induced by the TCR-mediated signal was independent of LFA-1 expression (Fig. 5), these results suggest that the serine phosphorylation of DNAM-1 primarily induces physical association of DNAM-1 with LFA-1, which takes DNAM-1 into lipid raft compartment.

Others and we previously reported that both LFA-1 and DNAM-1 form the p-SMAC (12, 13, 19). Fukui and colleagues (23) also reported that the translocation of LFA-1 to the IS was

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**Fig. 4.** LFA-1 is required for lipid raft recruitment of DNAM-1. (A) LFA-1+/+ and LFA-1−/− CD4+ T cells were stimulated or not stimulated with anti-CD3 plus anti-CD28 mAbs and were lysed with 1% Brij-35 and fractionated on a sucrose density gradient. Each fraction was immunoblotted with HRP-conjugated CTx B subunit. Otherwise, each fraction was immunoprecipitated with anti-DNAM-1 and immunoblotted with anti-DNAM-1 or anti-phosphotyrosine (p-Y) mAbs. As a positive control for tyrosine phosphorylation, CD4+ T cells were stimulated with sodium pervanadate, lysed with 1% Brij-35, immunoprecipitated with anti-DNAM-1 and immunoblotted with p-Y antibodies, denoted by asterisks. (B and C) LFA-1+/+ and LFA-1−/− CD4+ T cells were stimulated or not stimulated with anti-CD3 plus anti-CD28 mAbs and were lysed with 1% digitonin buffer. The proteins were immunoprecipitated with the antibodies indicated and were immunoblotted with antibodies against DNAM-1, Rap1 or RAPL.
independent of GM1-rich lipid rafts, which could be detected by binding of CTx, in mouse primary T cells. We have recently described a similar result that the translocation of DNAM-1 to the IS did not require GM1-rich lipid rafts (19). The relationship of LFA-1 with lipid rafts was controversial in primary T cells because LFA-1 could not be detected in Triton X-100-insoluble fractions from lysates of primary T cells (8, 24), although it could be detected in those of both human and mouse T cell lines, such as Jurkat and EL-4, respectively. However, Takei and colleagues (8) reported that LFA-1 could be detected in 1% Brij-35-insoluble fractions from lysates of mouse primary T cells, suggesting that lipid rafts may be heterogeneous (i.e. GM1-rich and non-rich rafts or Triton X-100- and Brij-35-insoluble rafts) in primary T cells. Interestingly, DNAM-1 was also detected in 1% Brij-35-insoluble, but not Triton X-100-insoluble, fractions in T cells (19). Together, these reports suggest a hypothesis that LFA-1 and DNAM-1 associate with the same subset of lipid rafts, which may be involved in the formation of p-SMAC.

Based on the data obtained from our previous and present studies and others, we propose a model for lipid raft recruitment of DNAM-1, as illustrated in Fig. 6. T cell activation by TCR-mediated signaling quickly induces the formation of compartmentalized clusters with lipid rafts (3, 21) (Fig. 6A). The TCR-mediated signal also induces the association of the small GTPase Rap with its activator Rap1L, which then binds to and activates LFA-1 (22), resulting in the complex formation with Rap–RapL–LFA-1 (Fig. 6A and B). Our studies indicated that the TCR-mediated signal also induces the serine phosphorylation of DNAM-1, resulting in the physical association of DNAM-1 with LFA-1 (Fig. 6A and B). The complex with Rap–RapL–LFA-1 then accumulates in the lipid raft compartment, into which DNAM-1 associating with LFA-1 may also be taken by LFA-1, and relocates to the outer side of the IS, resulting in the formation of p-SMAC (Fig. 6C). Once activated LFA-1 binds its ligand ICAM expressed on antigen-presenting cells, the LFA-1-mediated signal induces tyrosine phosphorylation of Fyn present in lipid raft compartment, by which DNAM-1 is tyrosine phosphorylated, and mediates co-stimulatory signal for CD4+ T cell proliferation and differentiation (16, 17) (Fig. 6D).

Recent studies have demonstrated that, in resting T cells, DNAM-1 binds the carboxyl-terminal domain of isoforms of the
actin-binding protein 4.1G, which is known to associate with the membrane-associated guanylate kinase homologue human discs large (hDlg) (25). Upon T cell stimulation with phorbol ester, a ternary complex between DNAM-1, hDlg, 4.1G and the cytoskeleton is formed. On the other hand, T cell activation by the TCR-mediated signal also induces the complex formation with RAP–RAPL–LFA-1 (22), which is united with another complex with DNAM-1–hDlg–4.1G–cytoskeleton through the direct interaction of RAPL with 4.1G (25), resulting in the larger complex formation upon T cell activation. Therefore, the serine phosphorylation of DNAM-1 may play a role in the formation of these complexes to physically associate with LFA-1. However, we have demonstrated that the physical association of LFA-1 and DNAM-1 was not mediated by Rap1 and RAPL (Fig. 4C). It was described that the TCR-mediated signal also induces the complex formation with Lck–hDlg–Zap70–Wiskott Aldrich syndrome protein and promotes antigen-induced actin polymerization, lipid raft clustering at the IS, nuclear factor-activated T cell activity and cytokine production (26). These dynamic associations with many molecules provide the structural basis for physical and functional relationship among LFA-1, DNAM-1, lipid rafts and other associated molecules in T cells. Further studies are required to clarify the signaling events mediated by LFA-1 and DNAM-1.

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Abbreviations

CTx
ganglioside type 1
hDlg
human discs large
IS
immunological synapse
LFA
leukocyte function-associated antigen
PMSF
phenylmethylsulfonylfluoride
RAPL
Rap1 ligand
WCCs
weighted co-localization coefficient
WT
wild-type

References