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CUTTING EDGE

Cutting Edge: Localization of Linker for Activation of T Cells to Lipid Rafts Is Not Essential in T Cell Activation and Development[1]

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It has been proposed that upon T cell activation, linker for activation of T cells (LAT), a transmembrane adaptor protein localized to lipid rafts, orchestrates formation of multiprotein complexes and activates signaling cascades in lipid rafts. However, whether lipid rafts really exist or function remains controversial. To address the importance of lipid rafts in LAT function, we generated a fusion protein to target LAT to nonraft fractions using the transmembrane domain from a nonraft protein, linker for activation of X cells (LAX). Surprisingly, this fusion protein functioned well in TCR signaling. It restored MAPK activation, calcium flux, and NFAT activation in LAT-deficient cells. To further study the function of this fusion protein in vivo, we generated transgenic mice that express this protein. Analysis of these mice indicated that it was fully capable of replacing LAT in thymocyte development and T cell function. Our results demonstrate that LAT localization to lipid rafts is not essential during normal T cell activation and development. The Journal of Immunology,* 2005, 174: 31–35.

Lipid rafts, subdomains of the plasma membrane, were initially identified by their relative resistance to Triton X-100 extraction (1, 2). In contrast to the rest of the plasma membrane, lipid rafts are enriched in sphingomyelins, glycosphingolipids, and cholesterol. The long saturated acyl chains in sphingolipids allow tight molecule packing, forming a liquid-ordered phase; the enriched cholesterol in lipid rafts further facilitates this process (3). Many studies have suggested that lipid rafts play a key role in vital cellular processes including signaling, apoptosis, and cell adhesion (1, 2).

Lipid rafts have also been implicated to play a critical role in immune receptor signaling (4, 5). In TCR-mediated signaling pathways, many signaling proteins have been found in lipid rafts; one such protein is the adaptor protein, linker for activation of T cells (LAT)[3] (6, 7). LAT is palmitoylated and constitutively localized in lipid rafts (8). Palmitoylation of LAT is essential for its localization to lipid rafts, tyrosine phosphorylation, and function in TCR signaling (8).

In addition to proteins that are constitutively localized in lipid rafts, other signaling proteins, such as the TCR/CD3 complexes, are recruited to lipid rafts after T cell activation (9). Because lipid rafts contain many signaling proteins, they are postulated to function as essential platforms to initiate signaling cascades. However, despite widespread acceptance of the fundamental role of rafts in immunoreceptor signaling, the very existence of rafts remains a topic of debate (10). Detergent extraction of membranes could induce formation of nonphysiological structures. Proteins might be extracted from the membrane based on their physical properties, such as insolubility in Triton, rather than their real distribution on the plasma membrane (10). Depletion of cholesterol by methyl-β-cyclodextrin, one method to used to demonstrate the importance of lipid rafts, could alter the physical properties of cellular membranes such as permeability and fluidity (11, 12); thus, the effects on TCR signaling by cholesterol depletion do not prove that rafts or protein localization to rafts is essential in T cell activation.

Because LAT plays an essential role in T cell activation, likely by nucleating signaling complexes in lipid rafts to transmit signals from the TCR to the Ras-MAPK and Ca^{2+} pathways, we chose to study the role of lipid rafts in T cell activation by targeting LAT to nonraft fractions and then examine whether T cell activation still proceeds normally. Our results indicate that LAT localization to lipid rafts is not essential in LAT-mediated signaling and function during T cell activation.

Materials and Methods

Constructs

The pMSCV and pCEFL constructs, used to reconstitute LAT-deficient cells, expressed a linker for activation of X cells (LAX)-LAT fusion protein comprising the extracellular and transmembrane domains of human LAX (residues 1–68) and the cytoplasmic domain of human LAT (residues 35–233). The LAX-LAT fusion protein in the transgenic construct contained human LAX (residues 1–68) and the cytoplasmic domain of murine LAT (residues 36–242).

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[3] Abbreviations used in this paper: LAT, linker for activation of T cells; LAX, linker for activation of X cells; NP, nitrophenylacetyl; NP-CGG, nitrophenylacetyl chicken γ-globulin; WT, wild type.

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Antibodies

The following Abs were used in the experiments: anti-human CD3ϵ (OKT3), human TCR (C305), myc (9E10), anti-LAT (6), phosphotyrosine (PY-20), and Grb2 (BD Pharmingen); phosphotyrosine (4G10), LATpY191, and PLC-γ1 (Upstate Biotechnology); pErk (Cell Signaling, Beverly, MA); and CD3, CD28, FITC-CD4, allophycocyanin-CD8, and PE-TCRβ (eBioscience, San Diego, CA).

T cell activation, immunoprecipitation, and Western blot

MAPK activation, Ca^{2+} flux, and luciferase assay in Jurkat cells were performed as previously described (13). Lipid raft and nonraft fractions were separated on sucrose gradients (8). Jurkat cells (1×10^8 cells/ml) were stimulated with anti-TCR Abs (C305) for 1.5 min before lysis. Thymocytes and splenocytes were incubated with biotinylated anti-CD3 (5 μg/ml) and CD4 (1 μg/ml) Abs, followed by cross-linking with streptavidin (25 μg/ml). For Ca^{2+} flux in primary T cells, thymocytes, and splenocytes were loaded with indo-1 for 30 min and then stained with PE-CD4. After addition of biotinylated anti-CD3 (5 μg/ml) and CD4 (1 μg/ml) Abs followed by streptavidin cross-linking (25 μg/ml), Ca^{2+} flux was analyzed by flow cytometry.

For anti-CD3 and -CD28-mediated IL-2 production and T cell proliferation, purified $CD4^+$ T cells were stimulated with plate-bound anti-CD3 alone at 1 and 10 μg/ml or anti-CD3 (1 μg/ml) and anti-CD28 (1, 5, and 10 μg/ml). Twenty hours after stimulation, the supernatants were used in ELISA to quantitate IL-2 concentration, and [^{3}H]thymidine was added for 6–8 h. Incorporation of thymidine was measured by a liquid scintillation counter.

For Western blotting, samples were separated by SDS-PAGE and transferred onto nitrocellulose. After incubation with primary Abs, nitrocellulose membranes were washed three times and probed with either anti-mouse or -rabbit Ig-conjugated to Alexa Fluor 680 (Molecular Probes) or IRDye800 (Rockland). Membranes were then analyzed and relative levels of LAX-LAT and LAT proteins were quantitated by Odyssey infrared imaging system (LI-COR, Lincoln, NE).

NP-specific Ab response

For T-dependent Ab response, 5- to 8-wk-old mice were immunized i.p. with 50 μg of alum-precipitated nitrophenylacetyl chicken γ-globulin (NP-CGG) and challenged with 50 μg of NP-CGG with alum at day 21. Sera were collected at different time points after immunization. Nitrophenylacetyl (NP)-specific IgG1 production was determined by ELISA. High-affinity NP-specific Abs were measured on plates coated with NP5-BSA. Total NP-specific Abs were measured on plates coated with NP25-BSA.

Results

To target LAT to nonraft fractions on the plasma membrane, we made a fusion protein (LAX-LAT) with the extracellular and transmembrane domains of LAX and the cytoplasmic domain of LAT (Fig. 1*A*). In contrast with LAT, LAX is not localized to lipid rafts either before or after Ag receptor stimulation (13). Thus, the LAX-LAT fusion protein should not localize to lipid rafts but has the potential to bind signaling proteins that normally interact with LAT. This fusion protein was expressed from a recombinant retroviral vector that allows simultaneous expression of GFP under the control of an internal ribosomal entry site element. The function of this fusion protein was analyzed in LAT-deficient cells (J.CaM2.5 and ANJ3) that are defective in TCR-mediated Ras-MAPK activation, Ca^{2+} flux, and NFAT activation (7, 14). Introduction of the wild-type (WT) LAT into these cells corrects these signaling defects. J.CaM2.5 cells were transduced by recombinant retroviruses expressing LAT and LAX-LAT proteins. GFP^+ cells were sorted by FACS and expanded for subsequent studies.

We first analyzed localization of these proteins in lipid rafts using sucrose gradients after solubilization of transduced cells with 0.5% Triton X-100. As shown in Fig. 1*B*, most of the WT LAT protein was localized in lipid rafts (fractions 2–4), whereas LAX was found in nonraft fractions (fractions 8–12) as described (8, 13). As predicted, LAX-LAT was also localized exclusively in nonraft fractions like LAX (Fig. 1*B*). Localization of LAX-LAT was not changed after TCR engagement (data not shown). As controls for raft fractionation, we analyzed the distribution of Lck and Grb2 by Western blotting. Lck was primarily found in raft fractions, whereas Grb2 was only present in nonraft fractions as expected.

To determine whether the LAX-LAT protein could be phosphorylated upon T cell activation, cells expressing LAT or LAX-LAT were activated with anti-TCR Abs, and cell lysates were subjected to immunoprecipitation. As shown in Fig. 1*C*, both the LAT and LAX-LAT proteins were highly phosphorylated upon T cell activation, even though LAX-LAT was not localized to lipid rafts. This result indicated that LAT localization to lipid rafts is not essential for its phosphorylation.

We next examined whether the LAX-LAT protein could interact with Grb2 and PLC-γ1, two proteins that the WT LAT binds after TCR engagement. Grb2 and PLC-γ1 were immunoprecipitated and analyzed by Western blotting with anti-pTyr Abs. As shown in Fig. 1*C*, anti-Grb2 and -PLC-γ1 Abs

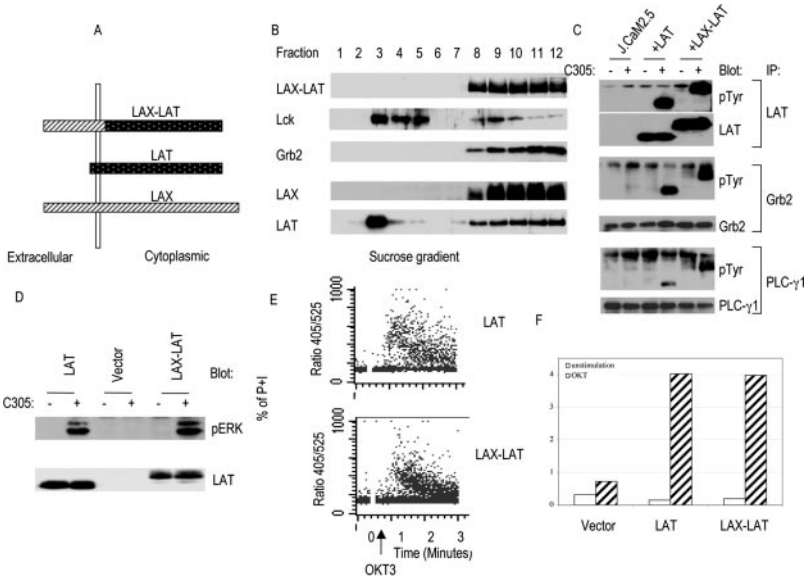

FIGURE 1. Biochemical analysis of the LAT and LAX-LAT proteins. *A*, A schematic diagram of LAT, LAX, and LAX-LAT proteins. *B*, Raft localization of the LAX-LAT fusion protein. *C*, Association of LAT and LAX-LAT with Grb2 and PLC-γ1. *D*, Erk activation detected by blotting with anti-pErk. *E*, Ca^{2+} flux in J.CaM2.5 cells reconstituted with LAT or LAX-LAT. *F*, NFAT activation. NFAT-luciferase activity was normalized by *Renilla*-luciferase activity and represented as the percentage of maximal activity.

coprecipitated phosphorylated LAT and LAX-LAT. Thus, the LAT-LAX protein can associate with Grb2 and PLC-γ1 as efficiently as the WT LAT.

We then determined whether the LAX-LAT protein could restore TCR signaling in J.CaM2.5 cells. Twenty-four hours after retroviral transduction, GFP^+ cells were sorted by flow cytometry and were expanded. After stimulation with anti-TCR Abs or left untreated, total lysates were analyzed by Western blotting with anti-active Erk (pErk). As shown in Fig. 1*D*, Erk activation in LAT-deficient cells was restored by either LAT or LAX-LAT, indicating that this fusion protein is able to function in TCR-mediated Erk activation. We also investigated whether LAX-LAT could reconstitute TCR-mediated Ca^{2+} flux in LAT-deficient cells. Twenty-four hours after retroviral transduction, LAT-deficient cells were loaded with the calcium indicator indo-1 and analyzed by flow cytometry. The anti-human CD3ε mAb (OKT3) was used to induce Ca^{2+} flux in these cells. As shown in Fig. 1*E*, Ca^{2+} flux in J.CaM2.5 cells was restored by both LAT and LAX-LAT. We further examined whether this fusion protein could function in TCR-mediated NFAT activation by transfecting LAT-deficient cells with plasmids expressing either LAT or LAX-LAT together with NFAT/AP-1 luciferase. As reported (6, 7), NFAT activation in LAT-deficient cells (ANJ3) was restored by introduction of WT LAT. LAX-LAT was equally competent in the restoration of TCR-mediated NFAT activation (Fig. 1*F*). Collectively, these results indicated that the LAX-LAT protein, although not localized to lipid rafts, is phosphorylated upon TCR engagement, interacts with Grb2 and PLC-γ1, and functions in Ras-MAPK activation, Ca^{2+} flux, and NFAT activation.

To this point, we have demonstrated that the LAX-LAT fusion protein was indistinguishable from the WT LAT when it was tested in LAT-deficient Jurkat cells. Can LAX-LAT also function in vivo to support thymocyte development and normal T cell activation? Transgenic mice were generated that express the LAX-LAT protein under the control of the CD2 promoter; this promoter successfully drives expression of transgenes in T cells (15). Transgenic mice were crossed with $LAT^{-/-}$ mice to generate a $LAT^{-/-}Tg^+$ strain. As shown in Fig. 2*A*, the LAX-LAT transgene fully rescued thymocyte development in $LAT^{-/-}$ mice. $CD4^+CD8^+$ (double-positive) and $CD4^+$ and $CD8^+$ (single-positive) thymocytes appeared in thymuses from $LAT^{-/-}Tg^+$ mice. Mature single-positive T cells were also found in spleens from these mice. The percentage of each population in $LAT^{-/-}Tg^+$ mice was similar to that in $LAT^{+/-}$ mice. Transgenic T cells expressed normal levels of TCRβ chain on their cell surface (data not shown).

We compared expression levels of the LAX-LAT protein with endogenous LAT. As shown in Fig. 2*B*, expression of the LAX-LAT fusion protein was similar to endogenous LAT in thymus and spleen. Like in LAT-deficient Jurkat cells, this fusion protein was also localized in nonraft fractions in both thymocytes and splenocytes (Fig. 2*C*). Upon activation of thymocytes and splenocytes with anti-CD3 and -CD4 Abs, LAX-LAT localization did not change (data not shown).

TCR-mediated phosphorylation of cellular proteins proceeded normally in T cells from $LAT^{-/-}Tg^+$ mice (Fig. 2*D*). Upon activation of thymocytes and splenocytes by cross-linking of CD3 and CD4, both LAT and LAX-LAT proteins were phosphorylated as detected by Western blotting with Abs against pTyr and phosphorylated LAT at Y191 (LATpY191). Although most of the phosphorylated LAT was localized to rafts as previously reported (8), phosphorylated LAX-LAT was exclusively found in nonraft fractions (data not shown). We also examined TCR-mediated Erk activation and Ca^{2+} mobilization in thymocytes and splenocytes. We did not observe any significant differences between cells from WT and $LAT^{-/-}Tg^+$ mice (data not shown and Fig. 3*A*).

Cross-linking via CD3 and CD28 induces production of IL-2 and T cell proliferation, two physiological consequences in normal T cells. T cells from WT and $LAT^{-/-}Tg^+$ mice were activated via plate-coated anti-CD3 alone or anti-CD3 and anti-CD28 Abs. IL-2 secreted by T cells was assayed using ELISA. As shown in Fig. 3*B*, T cells from WT and $LAT^{-/-}Tg^+$ mice produced similar amounts of IL-2 in response to anti-CD3 stimulation at 10 μg/ml. At a suboptimal concentration of anti-CD3 (1 μg/ml), there was a minimal production of IL-2; however, co-cross-linking of CD28 at 5 and 10 μg/ml induced a dramatic increase of IL-2 production compared with anti-CD3 alone, suggesting that CD28-mediated costimulation is also normal in T cells expressing the LAX-LAT fusion protein. TCR-mediated proliferation under these stimulation conditions was examined by

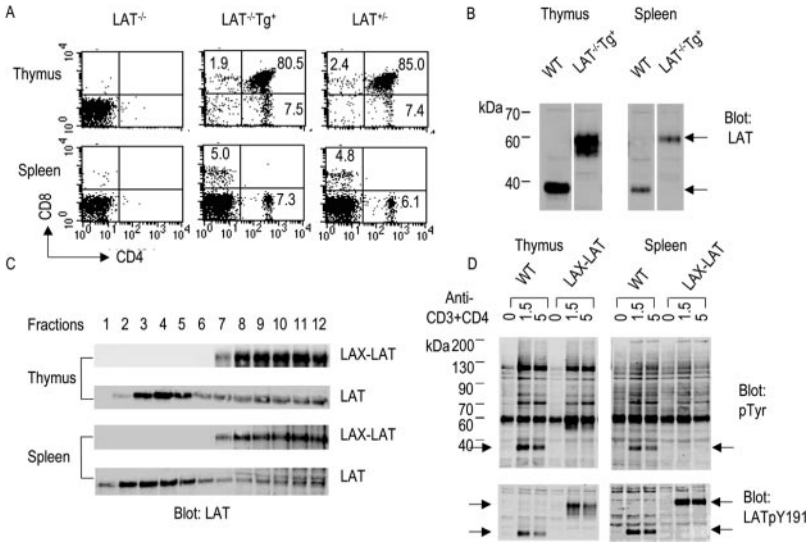

FIGURE 2. Rescue of thymocyte development by the LAX-LAT protein. *A*, Thymocytes and splenocytes were stained with FITC-CD4, allophycocyanin-CD8, and PE-TCRβ Abs and analyzed by flow cytometry. *B*, Expression of the LAX-LAT protein in thymus and spleen. *C*, Distribution of the LAT and LAX-LAT proteins in raft and nonraft fractions. *D*, TCR-induced protein phosphorylation. Arrows indicate where LAT or LAX-LAT migrated on SDS-PAGE. The LAX-LAT protein was not indicated on the pTyr blot, because it comigrated with other proteins.

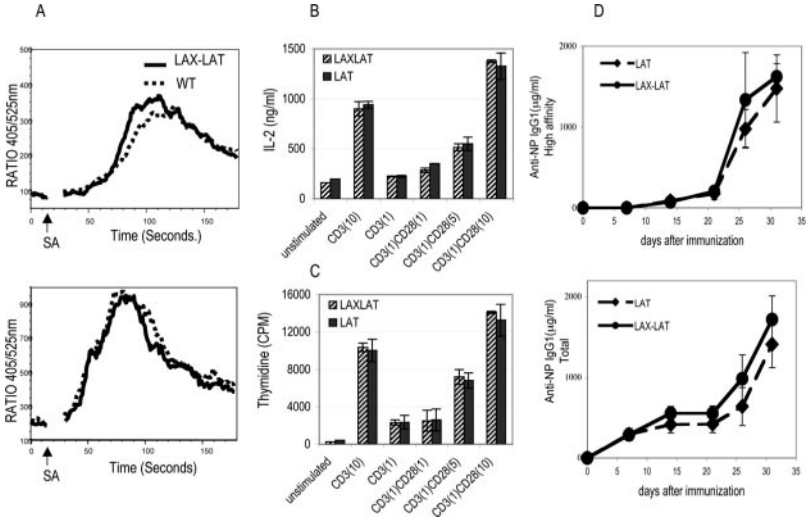

FIGURE 3. T cell activation, cell proliferation, and T-dependent Ab response in $LAT^{-/-}Tg^{+}$ mice. *A*, Normal calcium flux in thymocytes (*top*) and splenocytes (*bottom*) expressing the LAX-LAT protein. *B*, IL-2 production. Purified T cells from $LAT^{-/-}Tg^{+}$ (LAX-LAT) and the WT (LAT) mice were stimulated with plate-coated CD3 alone or anti-CD3 and -CD28. Concentrations of anti-CD3 and -CD28 in micrograms per milliliter were indicated. *C*, T cell proliferation. T cells were stimulated in the same way as in *B*. *D*, T-dependent Ab response.

thymidine incorporation. T cells from both control and $LAT^{-/-}Tg^{+}$ mice proliferated similarly in response to anti-CD3 alone or anti-CD3 and anti-CD28 (Fig. 3*C*).

Finally, we investigated whether T cells from the $LAT^{-/-}Tg^{+}$ mice function normally in vivo. $LAT^{-/-}Tg^{+}$ and WT mice were immunized with a T-dependent Ag, NP-CGG, and then challenged 21 days later with a second dose of NP-CGG. NP-specific Ab titers were assayed by ELISA. Production of NP-specific IgG1, including high-affinity anti-NP IgG1, was virtually the same in these mice (Fig. 3*D*). T cells from $LAT^{-/-}Tg^{+}$ mice, like those from control mice are fully capable of helping B cells undergo isotype switching and affinity maturation to produce high-affinity IgG1 Abs, indicating that the LAX-LAT protein functions indistinguishably from the WT LAT in T-dependent Ab response in vivo.

Discussion

We showed previously that two cysteine residues (C26 and C29) near the transmembrane domain of LAT are critical in LAT palmitoylation. Nonpalmitoylated LAT (C26/29A) fails to localize to lipid rafts and is not tyrosine phosphorylated upon T cell activation (8). In this study, we showed that the LAX extracellular and transmembrane domain could be used to bypass the requirement of palmitoylation and raft localization in LAT function. We have also compared the LAX-LAT and the LAT C26/29A mutant by expressing them in the $LAT^{-/-}$ bone marrow cells using retroviruses; only cells expressing the LAX-LAT could develop into T cells (data not shown). Although it may appear that our present findings do not support our previous work, in fact they do not contradict each other. It is possible that, without palmitoylation, the LAT transmembrane domain alone is not sufficient to tether LAT stably to the plasma membrane, whereas the LAX transmembrane domain can do so. Palmitoylation of LAT might be necessary for membrane targeting of LAT. Due to the physical properties of palmitate attached to LAT via cysteine residues, LAT becomes Triton-insoluble, a crucial criteria for lipid rafts, and localizes to lipid rafts; however, its localization to rafts might not be necessary in LAT function.

The physiological role and even the existence of lipid rafts remain controversial. Visualization of lipid rafts in vivo yielded conflicting results (10). Detergent extraction, which is normally used to identify lipid rafts biochemically, could possibly induce aggregation of structures that do not exist before extraction. Depletion of cholesterol could certainly cause changes in plasma membrane such as membrane permeability and fluidity (10). Interestingly, depletion of cholesterol with methyl-β-cyclodextrin at a low concentration that is sufficient to disrupt rafts has no effect on TCR-mediated tyrosine phosphorylation (16). Our results are in line with those findings and further demonstrated whether lipid rafts, specifically LAT localization to lipid rafts, is important in TCR-mediated MAPK activation, calcium flux, NFAT activation, and IL-2 production. Although we cannot exclude the possibility that the LAX-LAT transiently or loosely associates with rafts, our data show that, even though the LAX-LAT protein is not detected in lipid rafts, it is fully functional in T cell activation and development. Thus, LAT-mediated signaling cascades, including Ras-MAPK activation and Ca^{2+} flux, do not have to be initiated from lipid rafts. Although our results here do not address the importance or existence of lipid rafts directly, they raised the possibility that lipid rafts may not be fundamental in T cell activation.

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