JCB Report

Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells

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Formation of the immunological synapse (IS) in T cells involves large scale molecular movements that are mediated, at least in part, by reorganization of the actin cytoskeleton. Various signaling proteins accumulate at the IS and are localized in specialized membrane microdomains, known as lipid rafts. We have shown previously that lipid rafts cluster and localize at the IS in antigen-stimulated T cells. Here, we provide evidence that lipid raft polarization to the IS depends on an intracellular pathway that involves Vav1, Rac, and actin cytoskeleton reorganization. Thus, lipid rafts did not translocate to the IS in Vav1-deficient (*Vav1*$^{-/-}$) T cells upon antigen stimulation. Similarly, T cell receptor transgenic Jurkat T cells also failed to translocate lipid rafts to the IS when transfected with dominant negative Vav1 mutants. Raft polarization induced by membrane-bound cholera toxin cross-linking was also abolished in Jurkat T cells expressing dominant negative Vav1 or Rac mutants and in cells treated with inhibitors of actin polymerization. However, Vav overexpression that induced F-actin polymerization failed to induce lipid rafts clustering. Therefore, Vav is necessary, but not sufficient, to regulate lipid rafts clustering and polarization at the IS, suggesting that additional signals are required.

Introduction

Stimulation of T cells by peptide-presenting antigen-presenting cells (APCs)* induces formation of a highly organized complex of receptors, intracellular signaling molecules, and F-actin at the contact site between T cells and APCs, the so-called immunological synapse (IS) or supramolecular activation cluster (SMAC) (Monks et al., 1998; Grakoui et al., 1999). Formation of the IS is a multistep process that is initiated by conjugate formation between T cells and APCs. After this event, micrometer-scale molecular movements occur in the T cell plasma membrane and the actin cytoskeleton undergoes reorganization (Dustin and Cooper, 2000). T cells depend on actin cytoskeleton reorganization to induce lipid raft clustering (Gomez-Mouton et al., 2001; Rodgers and Zavzavadjian, 2001), but B cells probably have different requirements (Cheng et al., 2001).

PKCθ is selectively localized in the core of the SMAC (cSMAC) in antigen-stimulated T cells (Monks et al., 1997, 1998). This property and recent studies documenting the specific and important role of PKCθ in activating the transcription factors AP-1 and NF-κB in T cells (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Lin et al., 2000; Sun et al., 2000) have clearly demonstrated that PKCθ plays an obligatory role in mature T cell activation leading to IL-2 production (Altman et al., 2000).

PKCθ undergoes Vav1/Rac-dependent translocation to the membrane and cytoskeleton in activated T cells (Villalba et al., 2000a; Bi et al., 2001). More recently, we found that PKCθ colocalizes with clustered membrane lipid rafts after T cell receptor (TCR)/CD28 engagement in T cells and, furthermore, that these rafts also aggregate at the IS (Bi et al., 2001). Lipid rafts are specialized microdomains enriched in sphingolipids and cholesterol, which are thought to serve as platforms for assembly of signaling complexes (Simons and Ikonen, 1997; Moran and Miceli, 1998). Recent studies have provided substantial evidence suggesting an important role for membrane rafts in T cell signaling (Montixi et al.,

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*Abbreviations used in this paper: APC, antigen-presenting cell; CTx, cholera toxin; GFP, green fluorescent protein; HA, hemagglutinin; IS, immunological synapse; LCMV, lymphocytic choriomeningitis virus; SMAC, supramolecular activation cluster; TCR, T cell receptor.

Key words: T cell; immunological synapse; lipid raft; Vav1; cytoskeleton

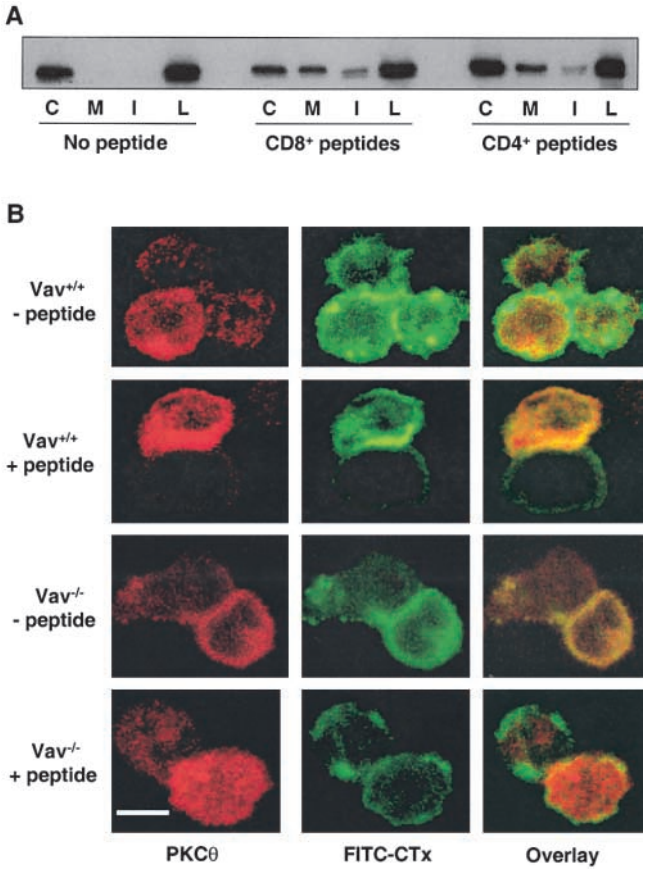

Figure 1. **Antigen-induced translocation of PKCθ and lipid rafts to the IS requires Vav1**. (A) Splenocytes from LCMV-infected normal mice were incubated for 6 h with $CD8^+$ or $CD4^+$ T cell–activating peptides. PKCθ present in cytosol (C), membrane (M), or cytoskeleton (I) compartments was analyzed by immunoblotting. L, whole lysates. (B) Splenocytes prepared as in (A) from wild-type or Vav1-deficient mice were pulsed with specific LCMV peptides to activate $CD8^+$ T cells. After 2 h, cells were fixed, stained with FITC-CTx (green) or anti-PKCθ (red) and analyzed by confocal microscopy. The overlay images are shown in the right panels. Bar, 5 μm.

1998; Xavier et al., 1998; Janes et al., 1999). A variety of cytoplasmic and membrane-associated proteins involved in T cell signaling are present in the detergent-insoluble raft fractions either constitutively or after T cell activation, and disruption of these rafts attenuates T cell activation (Xavier and Seed, 1999; Janes et al., 2000; Bi and Altman, 2001). In this study, we investigated the mechanism that regulates the clustering and IS translocation of lipid rafts and PKCθ in T cells.

Results and discussion

Defective lipid raft clustering in Vav1-deficient T cells

To determine the effect of antigen stimulation on the intracellular localization of PKCθ, we restimulated in vitro lymphocytic choriomeningitis virus (LCMV)-primed spleen cells prepared at the peak of the antiviral T cell response with the relevant LCMV-derived $CD4^+$ or $CD8^+$ T cell–activating peptides. In nonrestimulated cells (Fig. 1 A) or in cells cultured with an irrelevant control peptide (unpublished data), PKCθ was exclusively found in the cytosol fraction; after stimulation with the relevant CD4 or CD8 T cell–specific peptides, 15% and nearly 50% of the total expressed PKCθ, respectively, translocated to membrane and cytoskeleton fractions (Fig. 1 A). These percentages match the fraction of interferon-γ (IFNγ)-producing activated $CD4^+$ or $CD8^+$ T cells measured by intracellular cytokine staining of similarly stimulated cells (unpublished data; Varga and Welsh, 1998; Slifka et al., 1999, 2000), indicating that the antigen-induced translocation of PKCθ occurs at a very high stoichiometry. Furthermore, the finding that this translocation was observed 6 h after peptide stimulation indicates that it is very stable.

TCR triggering induces rapid reorganization of actin cytoskeleton (Penninger and Crabtree, 1999; Dustin and Cooper, 2000), which is intimately tied to T cell activation and proliferation (Valitutti et al., 1995). The finding that *Vav1*$^{-/-}$ T cells display a defect in antibody-induced TCR capping (Fischer et al., 1998; Holsinger et al., 1998) implicates an important role for Vav1 in regulating this actin cytoskeleton reorganization. To determine whether Vav1 is also necessary for lipid raft clustering and PKCθ localization at the IS, we compared PKCθ and lipid raft clustering in LCMV-primed, wild-type, or Vav1-deficient spleen cells. Since a larger fraction (~50%) of $CD8^+$ T cells is activated under these conditions (Fig. 1 A), we restimulated the cells with the relevant $CD8^+$ T cell–activating peptides. The cells were fixed and the intracellular localization of lipid rafts and PKCθ was determined by confocal analysis (Fig. 1 B).

In unstimulated cells, PKCθ was expressed in the cytoplasm and the lipid rafts were present mostly in the membrane in a patchy manner (Fig. 1 B). Peptide stimulation of wild-type T cells induced clustering and translocation of both PKCθ and lipid rafts, and their colocalization, at the T cell–APC contact area. In contrast, no such clustering was observed in the peptide-stimulated Vav1-deficient T cells. These results are consistent with a recent study documenting a defect in antibody-induced lipid raft aggregation in *Vav1*$^{-/-}$ T cells (Krawczyk et al., 2000).

Dominant negative Vav1 mutants interfere with lipid raft clustering

Vav1$^{-/-}$ mice display a reduced primary cytotoxic T lymphocyte (CTL) response, but a near normal secondary CTL response, to LCMV infection (Penninger et al., 1999). Thus, the absence of PKCθ and lipid rafts clustering in Vav1-deficient T cells may reflect the failure of these cells to become adequately primed in vivo. Therefore, we used an alternative approach to assess the role of Vav1, taking advantage of dominant negative Vav1 mutants and a TCR transgenic Jurkat T cell variant, CH7C17, expressing a hemagglutinin (HA)-specific TCR. The two Vav1 mutants, ΔPH or L213A, block TCR-induced activation of NFAT or AP-1, as well as PKCθ membrane translocation, in CH7C17 or in wild-type Jurkat cells (Villalba et al., 2000a; Kaminuma et al., 2001). We determined whether dominant negative Vav1 mutants inhibit antigen-induced PKCθ and lipid rafts localization to the IS. Cotransfection of the same cells with a green fluorescent protein (GFP) plasmid served to visualize and identify the Vav1-transfected cells.

As shown before (Bi et al., 2001), in unstimulated T cells, PKCθ remained localized in the cytoplasm (Fig. 2 A).

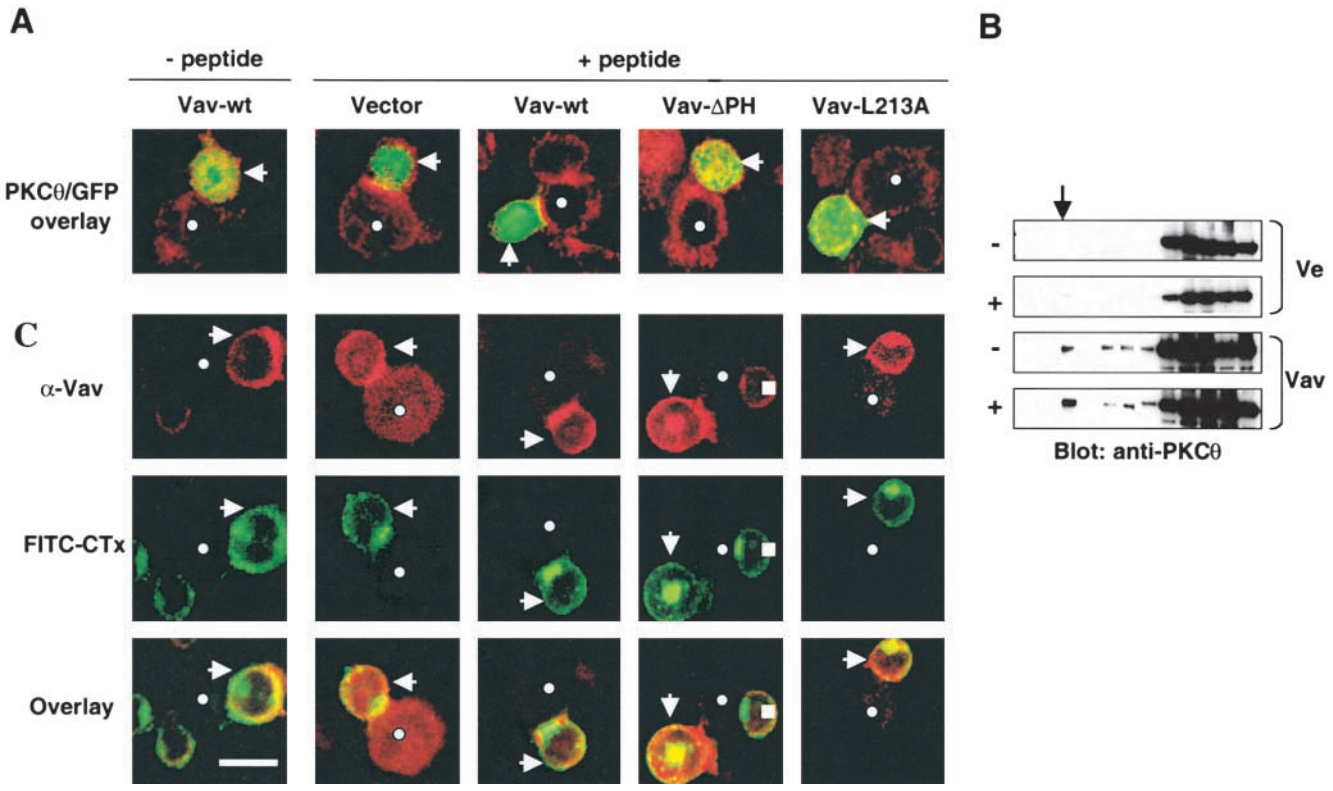

Figure 2. **Vav1 is required for peptide/APC-induced PKCθ and lipid raft polarization at the IS**. (A) CH7C17 T cells were cotransfected with empty vector or the indicated Vav1 plasmids plus a GFP expression vector, and incubated with LG-2 APCs that have (+ peptide) or have not (− peptide) been pulsed with an HA peptide. After 30 min, cells were fixed, permeabilized, and stained with anti-PKCθ mAb; overlay of the PKCθ (red) and GFP (green) images shown. The arrows indicate T cells, the dots show the position of LG-2 APCs. (B) Jurkat E6-1 cells were cotransfected with empty vector (Ve) or with pEF-Vav1 plus an Xpress-tagged PKCθ plasmid. 2 d later, the cells were left unstimulated (−) or stimulated with anti-CD3 plus anti-CD28 antibodies (1 μg/ml each; +). Detergent-insoluble and soluble fractions were prepared, and PKCθ expression analyzed by immunoblotting using an anti-PKCθ antibody. The arrow indicates fraction no. 4 from the gradient, which expresses the detergent-insoluble PKCθ fraction. (C) Cells were transfected as in A and stained with anti-Vav1 mAb (red) or FITC-CTx (green). The bottom row shows the overlay image of the anti-Vav1 and FITC-CTx staining. The arrows indicate T cells, the dots show the position of LG-2 APCs, and the squares mark the position of an untransfected T cell. Bar, 10 μm.

In contrast, peptide stimulation induced translocation of PKCθ rafts to the IS, consistent with our recent findings (Bi et al., 2001), and additional overexpression of wild-type Vav1 induced a tighter clustering of rafts in this contact area. In accordance with this result, anti-CD3/CD28 stimulation caused minor translocation of PKCθ to the biochemically isolated detergent-insoluble cell fraction (which corresponds to the lipid rafts), and this translocation was greatly enhanced by transfected wild-type Vav1 (Fig. 2 B). Endogenous and transfected Vav1 colocalized with lipid rafts at the IS (Fig. 2 C), consistent with findings that T cell activation induces Vav1 translocation to detergent-insoluble cell fractions (Xavier et al., 1998; Zhang et al., 1998). In contrast, expression of the two dominant negative Vav1 mutants blocked the antigen-induced translocation of lipid rafts (Fig. 2 C). The localization of Vav in cells expressing the two Vav1 mutants was similar to that observed in unstimulated cells, i.e., mostly cytoplasmic. Although the Vav1-ΔPH–transfected T cell appears to extend filopodia toward the APC in an attempt to establish contact, lipid rafts do not localize to this area and, instead, they (as well as a significant fraction of Vav1) undergo internalization. However, when Vav1-L213A did induce lipid raft aggregation at the membrane this aggregation did not occur at the IS but, rather, at the opposite side of the T cell (Fig. 2 C). The specificity of the inhibitory effect is evident from the finding that an untransfected cell in the same population displayed intact peptide-induced lipid raft aggregation and localization at the IS.

The inhibitory effect of dominant negative Vav1 mutants could reflect inhibition of conjugate formation between T cells and APCs (Huang et al., 2000). Therefore, we further addressed the role of Vav1 by inducing APC-independent raft polarization using cholera toxin (CTx)-mediated cross-linking of glycosphingolipid GM1, which is enriched in the rafts (Harder and Simons, 1999; Janes et al., 1999). These membrane patches have similar properties to membrane rafts isolated biochemically, and membrane patching can induce some signaling events similar to those induced by TCR stimulation (Harder and Simons, 1999; Janes et al., 1999).

In noncross-linked cells, GM1 was evenly distributed on the plasma membrane in all transfection groups (Fig. 3 A, left). After anti-CTx cross-linking for 15 min, ~70% of the empty vector–transfected cells displayed increased raft polarization, and >90% of Vav1-transfected cells showed a very tight raft clustering. However, the two dominant negative Vav1 mutants failed to induce any clustering. In marked contrast, and as an internal control for the inhibitory effect

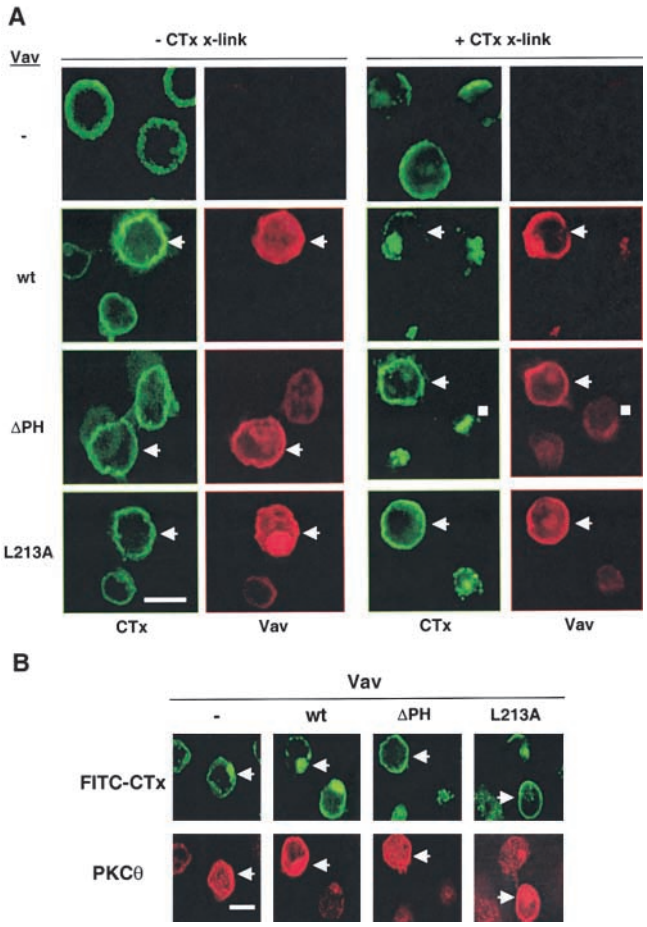

Figure 3. **Vav1 is essential for CTx-induced membrane patch formation**. (A) Jurkat-TAg cells were transfected with empty vector (−) or the indicated Vav1 plasmids. After 2 d, cells were labeled with FITC-CTx and cross-linked with anti-CTx mAb for 0 or 30 min at 37°C. Cells were then fixed, permeabilized, and stained with an anti-Vav1 antibody. Vav1 (red) and FITC-CTx (green) localization were analyzed by confocal microscopy. The arrows and squares indicate Vav-transfected or untransfected T cells, respectively. Note that the untransfected T cell displays intact Vav and lipid raft "capping." (B) Cells cotransfected with Xpress-PKCθ plus the indicated Vav1 plasmids were processed as in A, and stained with an anti-Xpress antibody. The localization of lipid rafts (green) or PKCθ (red) was analyzed as before. Arrows indicate transfected T cells. Bars, 10 μm.

of Vav1-ΔPH, an adjacent, non-Vav1–transfected cell displayed intact raft clustering (Fig. 3 A).

As shown before (Bi et al., 2001), anti-CTx cross-linking induced PKCθ translocation to the site of lipid raft clustering in cells transfected with a control vector or with wild-type Vav1 (Fig. 3 B, four left panels). This clustering appeared "tighter" and more pronounced in the presence of Vav1. However, the dominant negative Vav1 mutants disrupted the effect of anti-CTx cross-linking, as indicated by the even distribution of GM1 at the cell membrane and the mostly cytoplasmic expression of PKCθ (four right panels). These findings are consistent with our previous results that Vav1 is required for PKCθ membrane translocation and activation (Villalba et al., 2000a). Together, these results (Figs. 1–3) suggest that Vav1 is required for lipid raft polarization induced by T cell activation.

Lipid raft clustering depends on Rac and actin cytoskeleton

Since Vav1 regulates actin polymerization and TCR capping in T cells (Fischer et al., 1998; Holsinger et al., 1998), we wished to determine whether actin polymerization and lipid raft clustering are functionally linked. To address this question, we studied the effect of cytochalasin B, an inhibitor of actin polymerization, on anti-CTx–induced lipid raft clustering (Fig. 4 A). GM1 cross-linking for 5 min induced redistribution of membrane rafts from a relatively uniform pattern to distinct patches. At 15 min, most of the cells showed a highly polarized pattern of GM1 expression in cap-like structures, and F-actin accumulated in similar caps. As expected, cytochalasin B pretreatment prevented this actin capping process. However, in addition, this drug also prevented the capping of GM1-enriched lipid rafts observed at 15 min, even though some patching was still apparent.

Vav1 is coupled to both the Rac/Cdc42 and Ras signaling pathways in T cells (Wu et al., 1995; Collins et al., 1997; Costello et al., 1999; Bustelo, 2000; Villalba et al., 2000b), and p21-activated kinases (Paks) are Rac/Cdc42 effectors (Bagrodia and Cerione, 1999). To determine whether the regulation of lipid clustering by Vav1 requires Rac1, Pak, and/or Ras, we transiently transfected T cells with the corresponding dominant negative mutants, all of which were previously found to block the function of the relevant endogenous proteins (del Pozo et al., 2000; Villalba et al., 2000a,b; Kaminuma et al., 2001). Parallel staining of the cells with antibodies specific for the transfected gene products served to identify transfected cells within the total cell population since these cells appeared much brighter than the untransfected cells.

None of the dominant negative mutants affected the pattern of GM1 expression in the absence of anti-CTx crosslinking (Fig. 4 B, left). After crosslinking, however, ~75% of the empty vector–transfected cells displayed lipid raft clustering (Fig. 4, B and C). Dominant negative Pak or Ras mutants had negligible effects on this clustering, but dominant negative Rac1 caused a marked reduction in the percentage of transfected cells displaying lipid raft clustering (Fig. 4, B and C). These results strongly suggest that raft polarization mediated by Vav1 proceeds through the enzymatic activation of Rac, which in turn leads to cytoskeleton changes, but is independent of Pak1 or Ras. The apparent lack of requirement for Pak1 suggests that another Rac1 effector links Vav1/Rac1 to lipid raft clustering. In fact, we have consistently found an increase in lipid raft clustering in cells transfected with DN-Pak (Fig. 4 C). We are currently studying the nature of these findings.

Under resting conditions, Vav overexpression consistently failed to induce lipid raft clustering (Figs. 2 and 3). On the other hand, Vav overexpression induced F-actin polymerization (Fig. 4 D; Villalba et al., 2000a), leading to PKCθ translocation to the membrane and lipid rafts clustering (Fig. 2; Villalba et al., 2000a). To induce maximal Vav activation, we cotransfected Vav with constitutively active mutants of Lck and the catalytic subunit of the PI-3 kinase p110. These two proteins are known positive regulators of Vav activity (Bustelo, 2000). Both plasmids induce tyrosine phosphorylation on Vav (Fig. 4 E), but they failed to induce

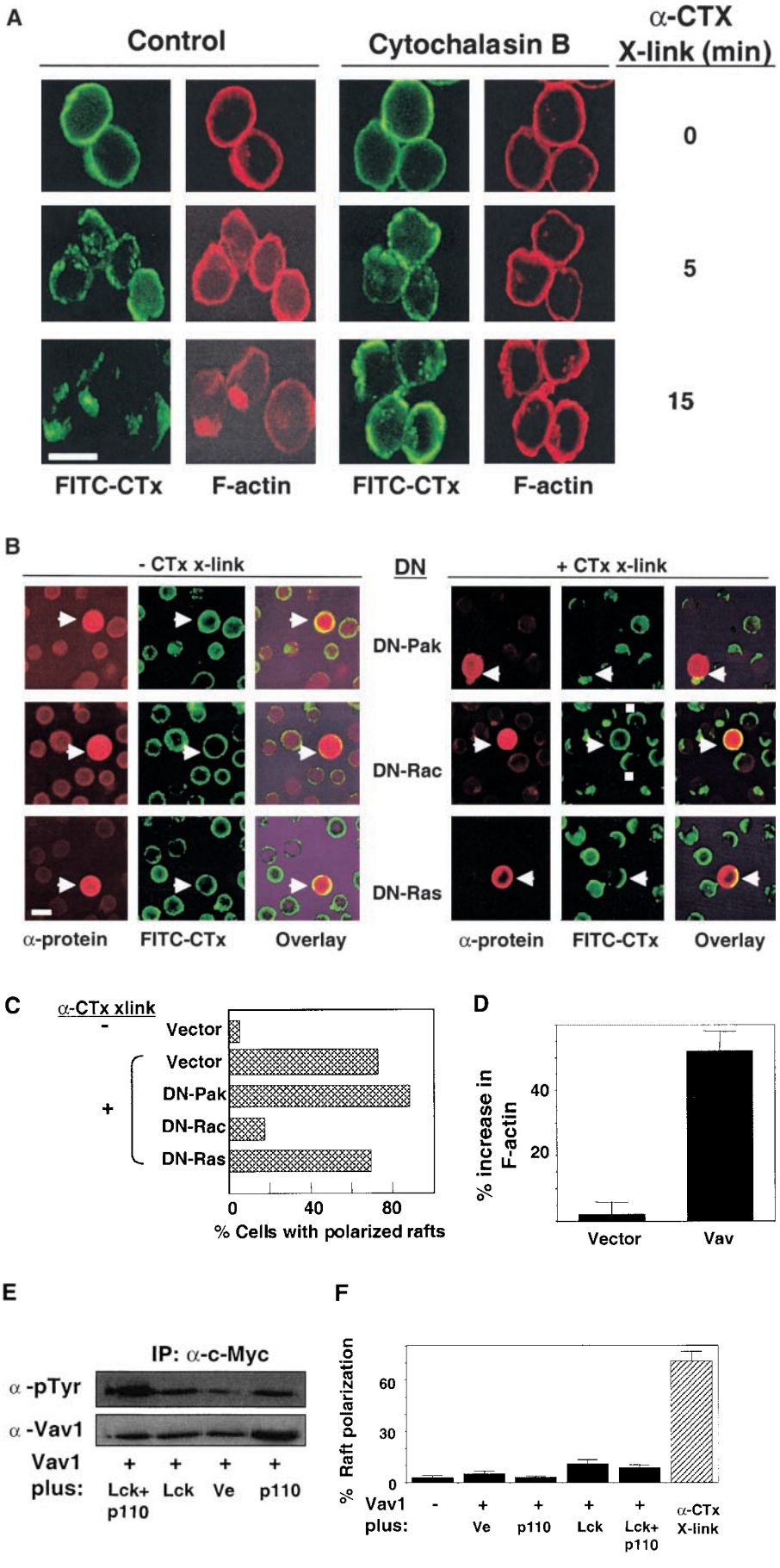

Figure 4. **Lipid raft polarization depends on Vav/Rac function and actin cytoskeleton reorganization, but it is not sufficient**. A. Jurkat E6-1 cells were labeled with FITC-CTx (green) and cross-linked with anti-CTx for the indicated times. Some cells were preincubated for 5 min with cytochalasin B (10 μM) before antibody cross-linking. Cells were fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to visualize F-actin. (B) Jurkat-TAg T cells were transfected with dominant negative (DN) mutants of Pak1, Rac1, or Ras. 2 d later, raft polarization was induced as described in A. The cells were fixed and stained with mAbs specific for the transfected dominant negative proteins (α-protein) to visualize and identify the transfected cells. The arrows indicate the position of transfected T cells. Note two untransfected cells (squares) which display normal raft clustering. (C) Transfected cells displaying raft aggregation were enumerated. At least 50 transfected cells were counted in each sample. (D) Jurkat TAg cells were transfected with 10 μg of empty vector or Vav together with 2.5 μg of GFP. After 36 h, phalloidin was used to stain F-actin, and the percentage of increase over nontransfected cells was calculated. (E) Jurkat TAg cells were transfected with constitutively active Lck (Y505), p110 (CD2-p110), or a combination of both, together with c-Myc-Vav. After 2 d, c-Myc-Vav was immunoprecipitated with an anti-Myc mAb. Samples were subjected to Western blotting analysis with an antiphosphotyrosine mAb (top) and an anti-Vav mAb (bottom). (F) Jurkat TAg cells were transfected as in E. After 2 d, cells were stained with FITC-CTx and anti-Vav mAb and analyzed for raft polarization by confocal microscopy. The percentage of transfected cells that have polarized lipid rafts was calculated. The number is the average of three independent experiments. At least 50 transfected cells were counted each time. Bars: (A) 20 μm; (B) 10 μm.

lipid raft clustering when overexpressed with Vav in the absence of stimulation (Fig. 4 F). These results suggest that a second, possibly extracellular, signal is required for the clustering.

Signal duration is a critical parameter for T cell activation, and recruitment of lipid rafts may stabilize the IS (Dustin and Chan, 2000). Consistent with this view, recruitment of lipid rafts to the interface between T cells and anti-TCR/CD28–coated beads is associated with stabilization of tyrosine phosphorylation events (Viola et al., 1999). Lipid rafts play an important role in T cell activation (Xavier and Seed, 1999; Janes et al., 2000; Bi and Altman, 2001), and several studies suggest a close functional relationship between lipid rafts and the actin cytoskeleton. Thus, similar to the actin cytoskeleton, lipid rafts also localize to the T cell–APC contact area in antigen-stimulated T cells (Bi et al., 2001). Furthermore, polymerized actin is enriched in lipid raft patches induced by CTx-mediated cross-linking of membrane GM1 in T cells (Harder and Simons, 1999). However, little is known regarding the precise functional relationship between these two cellular compartments. In particular, it is not clear whether reorganization of the actin cytoskeleton is required for optimal lipid raft clustering or, conversely, whether lipid raft clustering plays a role in promoting actin cytoskeleton rearrangements.

Here we demonstrate that actin cytoskeleton reorganization, which depends on activation of the Vav1/Rac pathway (Fischer et al., 1998; Holsinger et al., 1998), is required, but not sufficient, for stable lipid raft clustering in T cells induced by antigen stimulation or even by CTx-mediated cross-linking. The latter finding indicates that CTx-mediated cross-linking of membrane GM1 is not a passive but, rather, an active process, consistent with findings that CTx-mediated patching stimulates some signaling events similar to those induced by TCR ligation (Harder and Simons, 1999; Janes et al., 1999). Pharmacological disruption of the actin cytoskeleton by cytochalasin treatment also inhibited lipid raft clustering, although the earlier partial patching of these microdomains still occurred to some extent in the drug-treated cells (Fig. 4 A). One explanation for this finding is that the initial events in lipid raft coalescence may be relatively independent of actin polymerization. Consistent with this notion, treatment of T cells with PP1, a selective inhibitor of Src-family kinases, which are required for actin polymerization in T cells, or with latrunculin, an inhibitor of actin polymerization, did not prevent CTx-induced patch formation, albeit these patches were less condensed than in untreated cells (Harder and Simons, 1999). The finding that disruption of lipid microdomains by cyclodextrin treatment inhibits inducible tyrosine phosphorylation of TCR-ζ chain, and prevents its association with actin (Moran and Miceli, 1998), also supports the notion that lipid raft integrity is required for cytoskeleton-associated signaling events in activated T cells. Lipid raft integrity, or at least membrane structure maintained by cholesterol, is also required for interactions between FcεRI and Lyn in the membrane and for tyrosine phosphorylation of FcεRI in mast cells (Sheets et al., 1999a).

PKCθ translocation to the membrane or lipid rafts is independent of additional signals, and can only be mediated by Vav overexpression alone. The existence of small lipid rafts that coalesce to induce lipid raft clustering has been shown (Janes et al., 1999). Possibly Vav induces PKCθ translocation to the membrane where PKCθ localizes in small rafts. In the presence of a secondary signal, and with the necessary activity of Vav, the small lipid raft cluster, and PKCθ is translocated to the immunological synapse. We suspect that the secondary signal is mediated by crosslinking of receptors (e.g., TCR and CD28) that induce specific changes in the membrane.

On the other hand, other evidence suggests that actin cytoskeleton reorganization is necessary for recruiting or stabilizing lipid rafts (Dustin and Cooper, 2000). Thus, lipid-modified signaling molecules (e.g., Src-family kinases), which are associated with the cytoskeleton, may be colocalized to rafts and thus may function as "handles" to mediate cytoskeleton-driven rearrangement of the rafts (Xavier and Seed, 1999). Our finding that cytochalasin B pretreatment abolished the formation of large membrane patches induced by GM1 cross-linking suggests that PTK-dependent actin cytoskeleton reorganization may help to stabilize membrane patches and promote their further coalescence.

The two sets of findings regarding the relationship between lipid raft clustering and actin cytoskeleton reorganization are not necessarily contradictory. Although the rafts present in resting T cells may be small and contain relatively few associated proteins, receptor-mediated activation of PTKs (e.g., Lck and ZAP-70) could induce actin polymerization, which facilitates coalescence of these GEMs into large membrane patches and stabilize them. Such a mechanism operates in FcεRI-stimulated mast cells (Sheets et al., 1999b; Holowka et al., 2000). Furthermore, some PTK activation may occur before, and independent of, substantial actin polymerization (Miranti et al., 1998; Yan and Berton, 1998; Harder and Simons, 1999). Thus, the relationship between actin cytoskeleton reorganization and lipid raft clustering appears to be complex and multifaceted. This complex relationship is also highlighted by findings that two distinctly regulated raft reorganization steps are required for sustained TCR signal transduction and T cell activation (Patel et al., 2001). Therefore, Vav1/Rac signaling may be particularly important for a later step when lipid rafts coalesce to form large membrane patches and for stabilizing these rafts. Consistent with this view, we find that Vav overexpression lowers the antigen concentration threshold required to induce raft clustering at the IS (unpublished data). At any rate, our findings establish an important role for the Vav1/Rac pathway and for actin cytoskeleton reorganization in stable lipid raft clustering in the context of antigen-specific T cell responses.

Materials and methods

Antibodies, plasmids, and reagents

Anti-PKCθ, -Vav1, -HA, -Xpress, or - CTx antibodies have been described (Villalba et al., 2000a; Bi et al., 2001). The anti-Rac1 antibody (Ab) was from Upstate Biotechnology and the anti-Ras and anti-PKCθ mAbs were from Transduction Laboratories. Alexa 594–conjugated goat anti–rabbit IgG was from Molecular Probes. FITC-conjugated CTx and all other compounds were obtained from Sigma-Aldrich. The Vav1, PKCθ, and dominant negative Ras or Rac1 expression vectors have been described (del Pozo et al., 2000; Villalba et al., 2000a,b; Kaminuma et al., 2001). An HA-

tagged dominant negative Pak1 vector and anti-Pak1 Ab (del Pozo et al., 2000; Villalba et al., 2000a,b; Kaminuma et al., 2001) were obtained from Drs. M. Schwartz and M. del Pozo (The Scripps Research Institute, La Jolla, CA). Synthetic peptides were obtained from Peptidogenic Research and Co. The constitutively active Lck Y591F mutant has been described previously (Liu et al., 2000). A constitutively active CD2-p110 construct was a gift from Dr. D. Cantrell (Imperial Cancer Research Fund, London, UK). $Vav^{-/-}$ mice was obtained from Dr. Victor Tybulewicz (National Institute for Medical Research, London, UK) (Turner et al., 1997).

Cell culture and transfection

Wild-type (E6–1) Jurkat cells, Jurkat-TAg cells, or HA-specific CH7C17 Jurkat cells were maintained and transfected as described recently (Bi et al., 2001). CH7C17 cells were stimulated with mitomycin C–treated LG-2 APCs, which have been pulsed with the specific HA peptide (Villalba et al., 2000a). C57BL/6 ($H\text{-}2^b$) mice were infected with LCMV and killed 7–8 d later (Rodriguez et al., 1997; Slifka et al., 1999). Splenocytes from these infected animals were used as effector cells, and incubated in the presence of relevant LCMV peptides to induce T cell activation. To activate virus-specific $CD8^+$ or $CD4^+$ T cells, splenocytes were incubated with 1 μg/ml of the immunodominant $CD8^+$ peptides GP_{33} (amino acid sequence: KAVYNFATCG) plus NP_{396} (FQPQNGQFI), or with 5 μg/ml of the immunodominant $CD4^+$ peptides GP_{61} (GLKGPDIYKGVYQFKSVEFD) plus NP_{309} (SGEGWPYIACRTSIVGRAWE), respectively.

Subcellular fractionation

Subcellular fractionation was performed as described (Villalba et al., 2000a) to obtain cytosolic (C), membrane (M), and cytoskeletal detergent-insoluble (I) fractions. Detergent-insoluble and -soluble fractions were separated as described previously by detergent lysis and centrifugation on a sucrose step gradient (Bi et al., 2001).

Membrane patching, immunofluorescence, and confocal microscopy

LCMV-primed splenocytes or Jurkat cells were processed and analyzed as described (Bi et al., 2001) in order to determine the cellular localization of lipid rafts, PKCθ, Vav1, or other transfected proteins. For antigen stimulation, peptide-pulsed APCs were settled on poly-L-lysine–coated glass slides and T cells were added for the indicated times. Samples were viewed with a Plan-Apochromat 63× lens on a Nikon microscope. Images were taken using a Bio-Rad Laboratories MRC 1024 laser scanning confocal microscope. Microsoft Powerpoint software was used to prepare digital images of gel scans and micrographs.

F-actin measurement

Measurement of F-actin was performed as described (Villalba et al., 2000a).

Immunoprecipitation and immunoblotting

Cell lysis, immunoprecipitation, and immunoblotting were performed as described (Villalba et al., 2000a; Bi et al., 2001).

We would like to thank J. Hu and S. Hong for technical assistance and N. Weaver for editorial help.

This work was supported by National Institutes of Health Grants GM50819 and CA35299 (A. Altman), and CA91567 to K. Bi. M. Villalba is a special fellow of the Leukemia and Lymphoma Society (formerly the Leukemia Society of America, Inc.). This is publication number 433 from the La Jolla Institute for Allergy and Immunology.

Submitted: 19 July 2001
Revised: 4 September 2001
Accepted: 26 September 2001

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