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Chemokine-Induced Zap70 Kinase-Mediated Dissociation of the Vav1-Talin Complex Activates α4β1 Integrin for T Cell Adhesion

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SUMMARY

Lymphocyte integrins mediate cell arrest on endothelium during immune surveillance after activation by chemokine-stimulated inside-out signals. Here we show that a Vav1-talin complex in T cells is a key target for chemokine-triggered inside-out signaling leading to integrin α4β1 activation. Thus, Vav1 dissociation from talin was required to generate high-affinity α4β1 conformations. Assembly of the Vav1-talin complex required PtdIns(4,5)P_2, which was provided by talin-bound phosphatidylinositol phosphate kinase Iγ. Chemokine-promoted Vav1 dissociation from talin followed an initial increase in talin binding to α4β1. This process was dependent on ZAP-70, which binds to and phosphorylates Vav1 in the complex, leading to further α4β1 activation and cell adhesion strengthening. Moreover, Vav1-talin dissociation was needed for Rac1 activation, thus indicating that α4β1 and Rac1 activation can be coupled by chemokine-stimulated ZAP-70 function. Our data suggest that Vav1 might function as a repressive adaptor of talin that must dissociate from α4β1-talin complexes for efficient integrin activation.

INTRODUCTION

Chemokines promote the migration of immune cells from lymph and blood circulation into lymphoid tissues and sites of inflammation during immune surveillance (Campbell et al., 2003; Charo and Ransohoff, 2006; Rot and von Andrian, 2004). This process is achieved after rapid stimulation of α4β1 and αLβ2 integrin activity on T lymphocytes by chemokines presented on the endothelium. Thus, chemokine binding to G protein-coupled receptors induces rapid activation of effector molecules that lead to integrin-mediated upregulation of cell adhesion, a process called inside-out signaling (Kinashi, 2005; Ley et al., 2007; Luster et al., 2005). Integrin cytoplasmic regions are responsible for sensing and transmitting molecular information originated by this signaling, to finally generate high-affinity conformations of the integrin extracellular domain.

Talin is a homodimer protein that physically links integrin β subunits with the actin cytoskeleton (Calderwood, 2004; Campbell and Ginsberg, 2004; Nayal et al., 2004). The talin N-terminal head domain contains the FERM (protein 4.1, ezrin, radixin, and moesin) region that binds to an NPXY or NPXF membrane-proximal motif in β-subunit cytoplasmic tails (Campbell and Ginsberg, 2004). The FERM region also interacts with the type I phosphatidylinositol phosphate kinase γ90 (PIPKIγ90) (Di Paolo et al., 2002) (also called PIPKIγ-661) (Ling et al., 2002), leading to local accumulation of its product phosphatidylinositol-4, 5-bisphosphate [PtdIns(4,5)P_2)], a phosphoinositide that stimulates talin-β1 integrin interaction (Martel et al., 2001) by disrupting the interaction between the head and the C-terminal rod domain of talin (Goksoy et al., 2008). The talin rod domain interacts with vinculin and actin, providing links to the actin cytoskeleton (Gilmore and Burridge, 1996). Talin is required for affinity regulation of β1 and β3 integrins (Calderwood et al., 1999; Nieswandt et al., 2007; Petrich et al., 2007; Tadokoro et al., 2003; Wegener et al., 2007), and overexpression of the talin head domain promotes separation of α and β subunit cytoplasmic domains (Kim et al., 2003), a key event leading to integrin activation (Carman and Springer, 2003). Moreover, the chemokine CXCL12 also causes spatial separation of these domains in αLβ2 (Kim et al., 2003), suggesting that talin is a true candidate for regulation of integrin activation in response to chemokines.

Earlier work reported that talin associates with the guanine-nucleotide exchange factor (GEF) Vav on mouse T cells (Fischer et al., 1998), but the functional role of this association was not addressed. Vav1 is a key component of the inside-out signaling generated upon binding of CXCL12 to its receptor CXCR4 that leads to stimulation of α4β1 integrin activation on T lymphocytes (Garcia-Bernal et al., 2005). Vav1 is a multidomain protein that functions as GEF predominantly for Rac (Bustelo, 2000; Turner and Billadeau, 2002). Importantly, it has been demonstrated that Vav proteins play crucial roles in T cell activation and development (Tybulewicz, 2005). Activation of Vav GEF activity requires phosphorylation at tyrosine residues located on its acidic domain, whereas the Dbl-homology region is responsible for GEF activity and the SH2 and SH3 domains interact with autophosphorylated tyrosine kinases and with several adaptor proteins (Bustelo, 2000; Turner and Billadeau, 2002). ZAP70,

Lck, Fyn, and Syk have been linked to Vav phosphorylation by activated receptors in lymphocytes, including CXCR4 (Ottoson et al., 2001; Ticchioni et al., 2002; Tybulewicz, 2005).

In the present work we have studied the role of the Vav1-talin association in chemokine-stimulated T lymphocyte adhesion mediated by α4β1. In addition, we have investigated the functional cross-talk between PtdIns(4,5)P_2 and the molecular machinery activated during inside-out signaling triggered by chemokines. We provide evidence that changes in Vav1-talin association, which are controlled by PtdIns(4,5)P_2, are required for regulation of T cell adhesion mediated by α4β1.

RESULTS

CXCL12 Promotes Vav1-Talin Dissociation that Is Linked to Stimulation of Vav1 Phosphorylation

Vav1 and talin were found constitutively associated in nonstimulated human Molt-4 T cells and PBL-T lymphocytes (Figures 1A and 1B). Notably, Vav1-talin association was rapidly reduced upon cell exposure to CXCL12. Dissociation between Vav1 and talin was independent of a potential protease activity on talin (Figure S1A available online), and total talin amounts were unaltered in cells silenced for Vav1 expression by RNA interference (Figure 1B, right), indicating that absence of the Vav1-talin association does not lead to talin cleavage. More than 98% of Vav1 and talin proteins were present in the digitonin- or Triton X-100-soluble fraction after cell lysis (Figure S1B), indicating that changes in dynamic associations between these molecules were detected in a relevant fraction of their total cellular population. In addition, whereas talin showed a cortical distribution independently of cell exposure to CXCL12, Vav1 cortical localization was substantially reduced after incubation with the chemokine (Figure S1C), suggesting a change in Vav1 subcellular localization resulting from CXCL12 actions.

Vinculin coprecipitated with talin, and concomitant with chemokine-promoted Vav1-talin dissociation, vinculin gradually increased its binding to talin (Figure 1B, left). Talin binding to vinculin was independent of Vav1, as shown by the fact that Vav1 silencing did not affect the binding (Figure 1B, right). Also, although Vav1 antibodies coprecipitated talin, no vinculin was detected in these immunoprecipitates, and vinculin antibodies coprecipitated talin but not Vav1 (not shown), suggesting the unlikelihood of Vav1-vinculin interaction.

Vav1 tyrosine phosphorylation was difficult to detect in Vav1-talin complexes in nonstimulated T cells, and only when association decreased because of CXCL12 was a clear Vav1 phosphorylation observed (Figures 1A and 1D). Activation of this phosphorylation was preceded by a gradual increase in Vav1 coprecipitation with ZAP-70 (Figure 1A), a kinase involved in Vav1 phosphorylation (Ottoson et al., 2001; Ticchioni et al., 2002). In addition, we observed that CXCL12 stimulated a transient Vav1-β1 association in Molt-4 and PBL-T cells (Figure 1A).

Interestingly, when we abolished Vav1-talin association by silencing talin expression with i-talin siRNA, we found a defective chemokine-promoted Vav1 tyrosine phosphorylation, as well as impairment in Rac activation (Figures 1C–1E; Figure S2A). To determine whether inhibition of Rac activation was directly dependent on impaired Vav1 activity, we expressed a constitutively active Vav1 form (ΔCH+Ac-GFP) (Lopez-Lago et al., 2000) and analyzed whether Rac activation was restored. With Vav1 ΔCH+Ac-GFP transfection efficiencies ranging 35%–40%, we obtained a partial recovery of Rac activation in talin-silenced cells, as compared to control (WT) GFP transfectants (Figure 1F). These data suggest that impairment in Rac activation after targeting talin involves defective Vav1 phosphorylation, and possibly Vav1-independent mechanisms.

CXCL12 Stimulates Talin-Integrin β1 Association

With similar kinetics as Vav1-talin disassembly, CXCL12 promoted in Molt-4 and PBL-T cells a rapid and transient increase in the association of talin with the β1 subunit of α4β1 (Figure 2A, left and middle). As expected, no talin-β1 complexes were seen in talin-silenced cells (Figure 2A, right), although they had no alterations in α4β1 heterodimer formation or in α4β1 cell surface expression (Figure S2B). Importantly, no talin-β1 complexes were detected in Vav1-silenced cells, and CXCL12 was unable to promote the association (Figure 2B), indicating that talin-β1 binding was dependent on Vav1. Transient Vav1-β1 association triggered by CXCL12 was also confirmed with β1 antibodies, whereas this complex was not formed in Vav1-silenced cells.

We used CCL21 to investigate whether other chemokines can also influence Vav1, talin, and β1 associations. CCL21 induced Vav1 release from talin, which was linked to increased talin-β1 association (Figure 2C), indicating that these dynamic changes might represent common GPCR-mediated T cell responses with functional implications. Together, these data indicate that chemokines regulate associations between Vav1, talin, and β1, which could have important consequences for α4β1-mediated T cell attachment.

Talin Is Required for Chemokine-Promoted α4β1-Dependent T Cell Adhesion

To study whether changes in associations between Vav1, talin, and β1 could influence T cell attachment mediated by α4β1, we silenced talin expression and tested transfectant adhesion to α4β1 ligands. Talin-silenced T cells revealed a partial reduction in CXCL12-stimulated attachment to VCAM-1 and CS-1-FN under static conditions, relative to control and ni-talin 1 siRNA transfectants (Figure S2C).

Flow chamber adhesion assays under shear stress revealed that CXCL12 triggered a rapid firm attachment to VCAM-1 in 75%–85% of Molt-4 and PBL-T cells transfected with control or ni-talin 1 siRNA, while a minor transfectant population (5%–10%) displayed transient arrest (Figure 3A). Control experiments indicated blocking of adhesion by cell pretreatment with pertussis toxin or with α4 mAb (not shown). Instead, i-talin 1 siRNA transfectants predominantly rolled, displaying low stable arrest on VCAM-1 that was associated with increased transient attachment, suggesting that talin is required during initial events of chemokine-triggered α4β1-VCAM-1 interaction. Moreover, control and ni-talin 1 siRNA transfectants developed higher resistance to detachment at increased shear stress than i-talin 1 siRNA counterparts (Figure 3B). The potential involvement of talin at initial steps of adhesion was studied by measuring cell binding of 15/7, a mAb that recognizes a β1 integrin activation epitope on α4β1 (Yednock et al., 1995), as well as by analyzing binding of VCAM-1-Fc. 15/7 mAb and VCAM-1-Fc bound

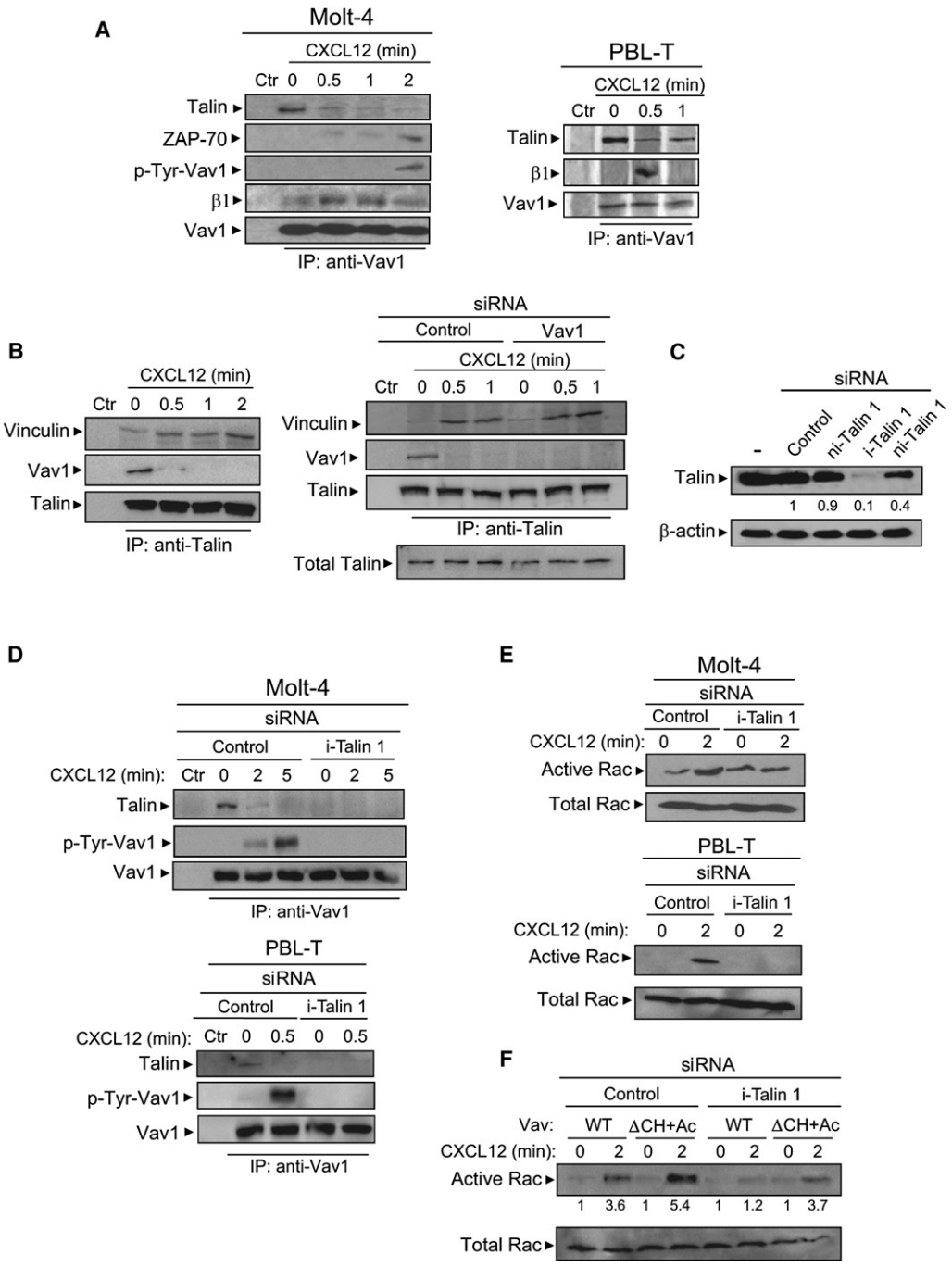

Figure 1. CXCL12 Promotes Vav-Talin Dissociation that Is Linked to Stimulation of Vav1 Phosphorylation

(A) Cells were incubated for the indicated times with CXCL12 (200 ng/ml) and subjected to immunoprecipitation with control (Ctr) or Vav1 antibodies, followed by immunoblotting with antibodies to the stated proteins.

(B) Lysates from nontransfected (left) or control or Vav1 siRNA-transfected (right) Molt-4 cells were subjected to immunoprecipitation and immunoblotting.

(C) Nontransfected (–) or Molt-4 cells transfected with the indicated noninterfering or interfering talin 1 (ni-talin 1 or i-talin 1, respectively) or with control siRNA were analyzed by immunoblotting with talin antibodies. Numbers below gels indicate values from densitometer analyses.

(D and E) Control or i-talin 1 siRNA transfectants were incubated with CXCL12 and tested in immunoprecipitation and immunoblotting assays or subjected to GTPase assays to detect active Rac.

(F) Molt-4 cells were cotransfected with control or i-talin 1 siRNA and wild-type (WT) or constitutively active (ΔCH+Ac) Vav1 forms and tested in Rac GTPase assays.

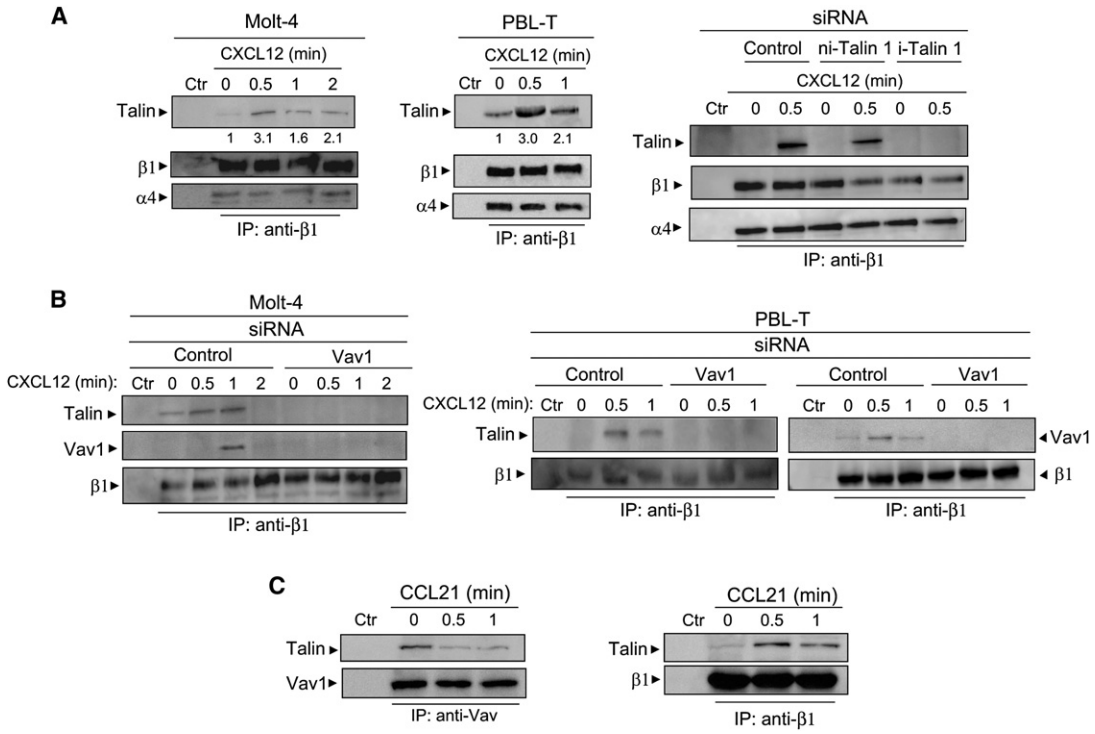

Figure 2. CXCL12 Stimulates Integrin β1-Talin Association on T Cells

(A and B) Molt-4 and PBL-T cells, or the indicated siRNA transfectants, were incubated with CXCL12 and subjected to immunoprecipitation with control (Ctr) or anti-β1 followed by immunoblotting with antibodies to the indicated proteins. Numbers below gels indicate values from densitometer analyses.

(C) PBL-T cells were incubated with CCL21 (200 ng/ml) and subjected to immunoprecipitation with anti-Vav1 or anti-β1 and immunoblotting to the stated proteins.

substantially less to CXCL12-incubated talin knockdown cells than control or ni-talin 1 siRNA transfectants (Figures 3C and 3D). Control experiments showed that all transfectants retained a similar degree of 15/7 mAb or VCAM-1-Fc binding upon exposure to Mn^{2+}, a positive control for integrin affinity regulation (Figures 3C and 3D). These data indicate that activation of α4β1 in response to CXCL12 is defective in talin-silenced cells, which probably accounts for reduced stable T cell tethers and impaired strengthening of α4β1-VCAM-1 interaction.

ZAP-70 Is Essential for Chemokine-Promoted Vav1-Talin Dissociation

CXCL12 induces gradual coprecipitation of Vav1 and ZAP-70 concomitant with weakening of Vav1-talin association and stimulation of Vav1 phosphorylation (Figure 1), so we reasoned that if dissociation involves ZAP-70 function, perhaps by inhibiting its activity or its expression, we could affect the rate of Vav1-talin disassembly. Therefore, we incubated T cells with the ZAP-70 inhibitor piceatannol, or targeted ZAP-70 expression with siRNA, and we analyzed Vav1-talin complexes in coimmunoprecipitation experiments. Whereas cells preincubated with carrier DMSO or control siRNA transfectants displayed Vav1-talin dissociation by CXCL12, those exposed to piceatannol or ZAP-70-silenced cells showed resistance to disassembly of this complex, which was associated to inhibition by piceatannol of Vav1 tyrosine phosphorylation without affecting Vav1-ZAP-70 coprecipitation (Figures 4A and 4B). Furthermore, association between Vav1 and ZAP-70 required a preformed Vav1-talin complex, as indicated by the fact that silencing talin prevented Vav1-ZAP-70 association (Figure 4C). Together with lack of Vav1 phosphorylation in talin-knockdown cells (Figure 1D), these results strongly suggest that ZAP-70-dependent Vav1 phosphorylation, but not the earlier Vav1-ZAP-70 association, is required for Vav1 release from talin. Contrary to defective Vav1-talin dissociation in ZAP-70-silenced cells, talin-β1 or Vav1-β1 associations stimulated by CXCL12 were not affected by ZAP-70 knocking down (Figure 4D), indicating that they do not depend on Vav1-talin disassembly.

Notably, piceatannol-treated or ZAP-70 knockdown T cells showed a blockade of CXCL12-stimulated adhesion to VCAM-1 (Figure 4E), which arose from defective acquisition of α4β1 active conformations leading to reduced adhesion strengthening, as visualized in 15/7 mAb and VCAM-1-Fc binding assays (Figure 4F; Figure S3). These data indicate that CXCL12 promotes ZAP-70 binding to Vav1 in Vav1-talin complexes, followed by stimulation of Vav phosphorylation that leads to reduced Vav1-talin association, which is essential for progression of α4β1-mediated T cell adhesion.

PtdIns(4,5)P_2-Sequestering Probes Impair Both Vav1-Talin-β1 Associations and Cell Adhesion to VCAM-1

PtdIns(4,5)P_2 stimulates talin binding to the β1 integrin cytoplasmic domain (Martel et al., 2001), thereby constituting a candidate molecule regulating cell adhesion by inside-out

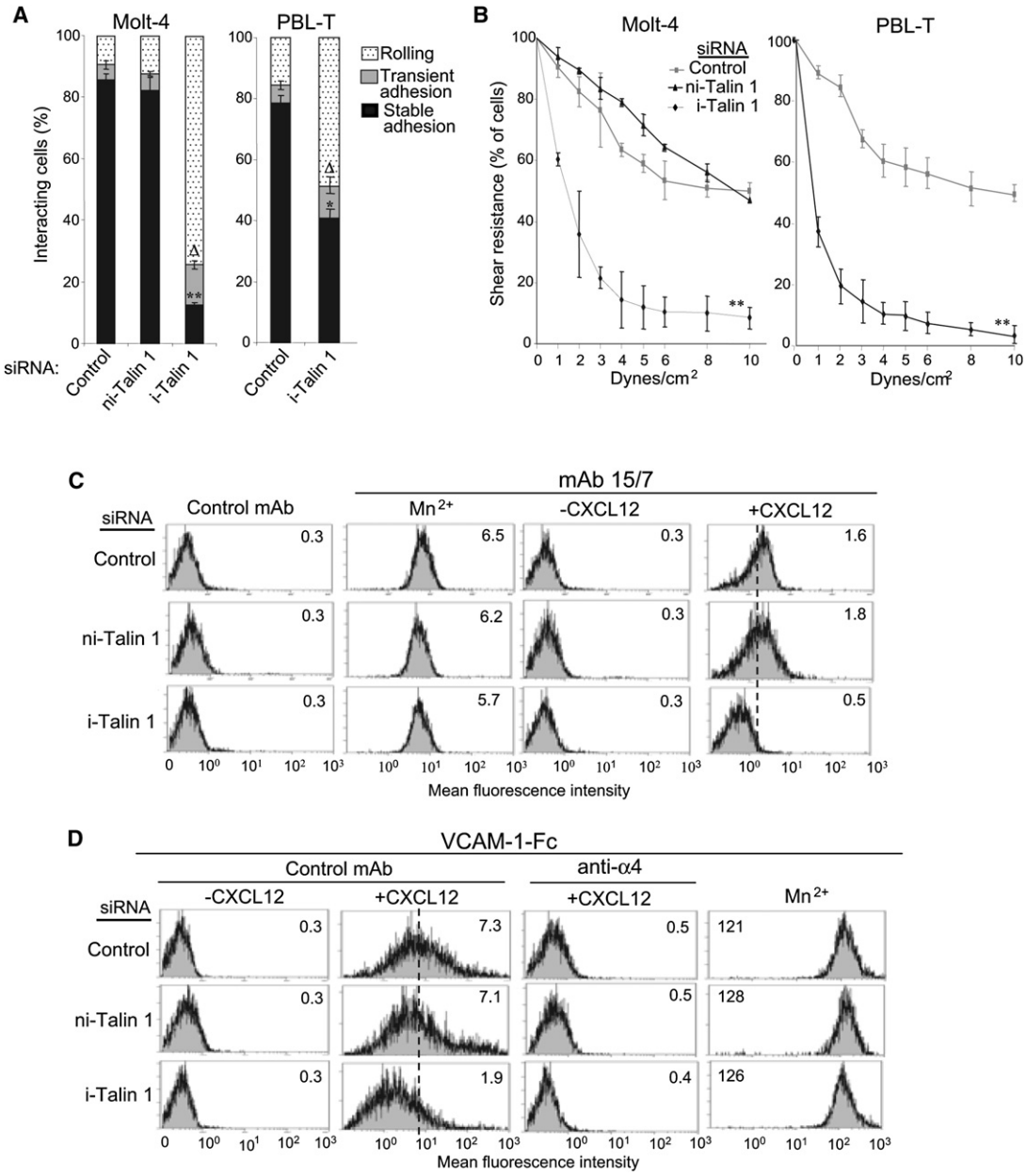

Figure 3. Talin Is Required for Chemokine-Stimulated T Cell Adhesion under Shear Stress Mediated by α4β1

(A and B) Control or talin siRNA transfectants were perfused in flow chambers coated with VCAM-1 immobilized with CXCL12 and analyzed for rolling and transient or stable cell arrest (A; n = 3) or for cell detachment after increasing shear rates (B; n = 3). Data are presented as indicated in Experimental Procedures. **Adhesion was significantly inhibited compared to control siRNA transfectant attachment, $p < 0.01$, or *$p < 0.05$. $^{\Delta}$Transient arrest was significantly increased compared to control siRNA transfectants, $p < 0.05$.

(C) Molt-4 siRNA transfectants were incubated with or without CXCL12 or Mn^{2+}, followed by incubation with 15/7 or control mAb and analysis by flow cytometry.

(D) siRNA transfectants were preincubated with control or α4 mAb and subsequently incubated in the absence or presence of CXCL12 or Mn^{2+}, before addition of VCAM-1-Fc. Cell-bound ligand was detected as indicated in Experimental Procedures. Insert numbers represent mean fluorescence intensity units. Representative results of three independent experiments for (C) and (D) are shown.

signaling. The pleckstrin homology (PH) domain of phospholipase C-δ1 (PLC-δ1) binds PtdIns(4,5)P_2 with high affinity (Watt et al., 2002) and has been used as a GFP-fused form to localize PtdIns(4,5)P_2 and to study the role of this phosphoinositide in cell adhesion (Downes et al., 2005; Martel et al., 2001; Tall et al., 2000). We expressed PH-PLC-δ1-GFP (PH-GFP) in Molt-4 cells, which was predominantly found in the cell membrane fraction, whereas control GFP was localized in the cytoplasm (Figure 5A). Interestingly, PH-GFP expression prevented Vav1-talin assembly and talin-β1 binding promoted by CXCL12 (Figures 5B and 5C). Furthermore, PH-GFP transfectants showed a reduction in chemokine-upregulated attachment

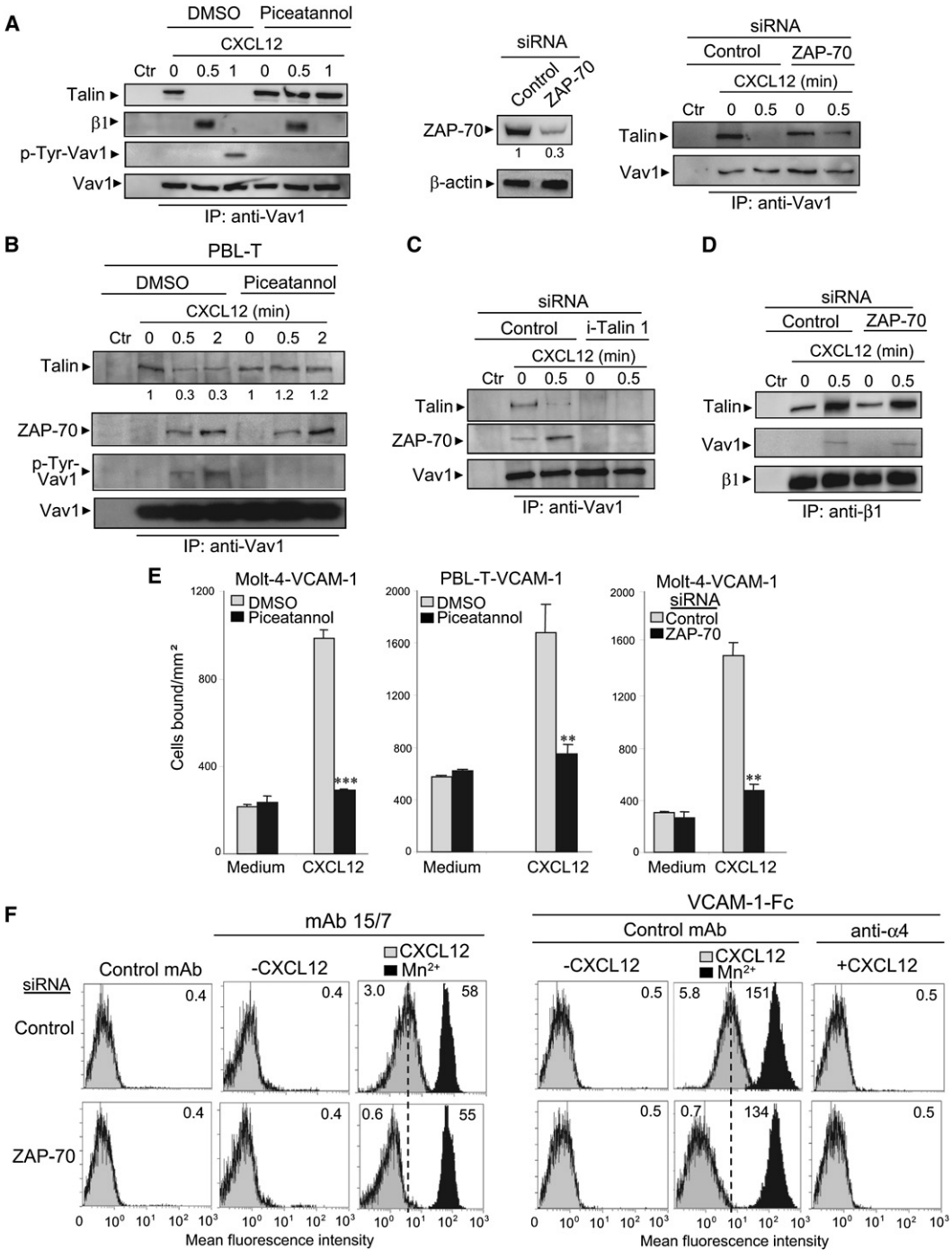

Figure 4. ZAP-70 Is Required for Chemokine-Promoted Vav1-Talin Dissociation and α4β1-VCAM-1 Interaction

(A) Left: Molt-4 cells preincubated in piceatannol (25 μM, 3 hr) or in carrier DMSO were exposed to CXCL12 and subjected to immunoprecipitation and immunoblotting. Lysates from cells transfected with ZAP-70 or control siRNA were analyzed by immunoblotting with anti-ZAP-70 (middle) or subjected to immunoprecipitation and immunoblotting (right).

(B) Piceatannol-treated cells were incubated with CXCL12 and subjected to immunoprecipitation and immunoblotting. Numbers below gels indicate values from densitometer analyses.

(C and D) Lysates from control, talin 1, or ZAP-70 siRNA transfectants were subjected to immunoprecipitation with Vav1 or β1 mAbs, followed by immunoblotting.

(E) Cells preincubated in piceatannol or DMSO, or control or ZAP-70 siRNA transfectants were tested in adhesion assays to VCAM-1 immobilized with or without CXCL12. ***Adhesions were significantly inhibited, $p < 0.001$ or **$p < 0.01$ ($n = 3$).

(F) Control or ZAP-70 siRNA transfectants were tested for 15/7 mAb (left) and VCAM-1-Fc (right) binding as stated in the legend for Figure 3.

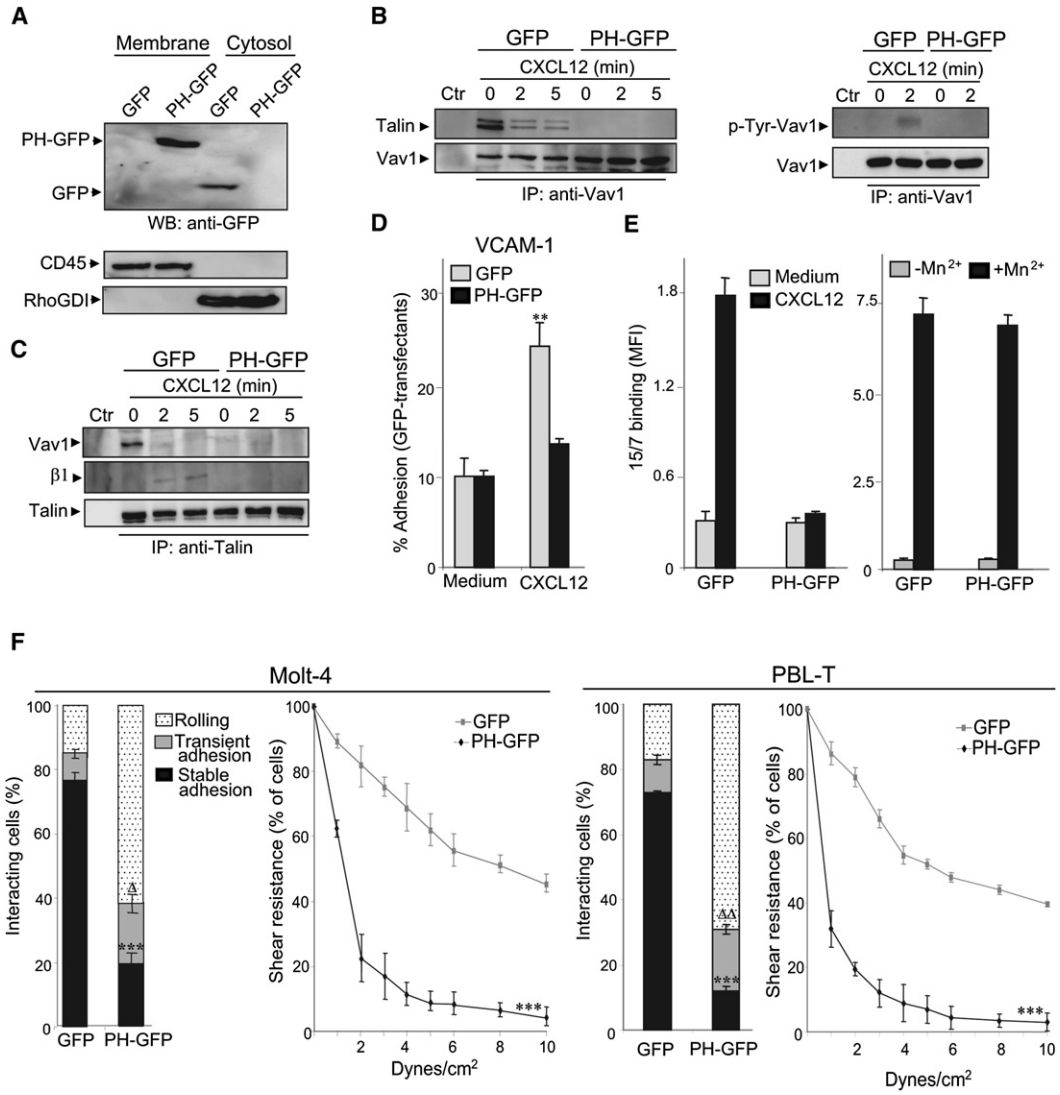

Figure 5. Associations between Vav1, Talin, and β1, and Chemokine-Activated Cell Adhesion Mediated by α4β1 Are Impaired by PtdIns(4,5)P_2-Sequestering Probes

(A) Molt-4 cells transfected with PH-PLC-δ1-GFP (PH-GFP) or GFP vector alone were subjected to cell fractionation assays, and lysates were analyzed by immunoblotting with antibodies to GFP, CD45 (marker for membrane fraction), or RhoGDI (marker for cytosolic fraction).

(B and C) Same transfectants were tested by immunoprecipitation with anti-Vav1 or anti-talin and subsequent immunoblotting.

(D) Transfectants were tested in static adhesion assays to VCAM-1 immobilized with or without CXCL12. **Adhesions were significantly upregulated, $p < 0.01$.

(E) Transfectants were incubated with or without CXCL12 or Mn^{2+}, followed by incubation with 15/7 or control mAb and analysis by flow cytometry. Data represent mean fluorescence intensity values from three independent experiments.

(F) Transfectants were perfused in flow chambers coated with VCAM-1 immobilized with CXCL12 and analyzed as in Figure 3. ***Adhesion was significantly inhibited compared to GFP transfectant attachment, $p < 0.001$. $^{\Delta\Delta}$Transient arrest was significantly increased compared to GFP transfectants, $p < 0.01$, or $^{\Delta}p < 0.05$.

to VCAM-1 under static conditions (Figure 5D), and binding of 15/7 mAb was defective compared to control GFP counterparts, whereas both transfectants retained similar levels of Mn^{2+}-triggered 15/7 mAb binding (Figure 5E). Control flow cytometry experiments indicated that expression of α4 and β1 subunits in PH-GFP transfectants was similar to control GFP counterparts (not shown).

Adhesion assays under shear stress revealed that PH-GFP T cell transfectants had lower stable arrest associated with increased transient adhesion than did GFP transfectants and showed higher detachment at increasing shear rates (Figure 5F). To assess whether integrin-independent cellular functions might be affected by PH-GFP expression, we subjected these transfectants to chemotaxis toward CXCL12 across bare filters. The results revealed that GFP and PH-GFP transfectants achieved comparable chemotaxis (Figure S4), indicating that PH-GFP expression is not exerting global inhibitory effects. Therefore, PH-GFP-dependent alteration of talin association with Vav1 and β1 correlates with impairment in chemokine-stimulated T cell adhesion mediated by α4β1, suggesting that PH-GFP

sequesters PtdIns(4,5)P_2 required for formation of the signaling platform that is essential for adhesion.

Role of PIPKIγ90 in CXCL12-Activated Cell Adhesion Mediated by α4β1 and in Vav1-Talin-β1 Association

Because PtdIns(4,5)P_2 is synthesized by phosphatidylinositol phosphate kinases (Ling et al., 2006), from which the PIPKIγ90 isoform binds to talin (Di Paolo et al., 2002; Ling et al., 2002), we analyzed whether overexpression of this kinase could restore the defective adhesion seen in PH-GFP transfectants. Molt-4 cells express endogenous PIPKIγ90, which associates with talin (Figure 6A), but not with Vav1 (not shown). In addition, we found that extent of PIPKIγ90-talin association was not substantially altered by CXCL12. Transfected PIPKIγ90-GFP (KIγ90-GFP) coprecipitated with talin again independently of CXCL12 action, whereas the mutant KIγ90^{W647F}-GFP form showed large reduction in binding to talin (Figure 6B), as earlier reported with NIH 3T3 cells (Di Paolo et al., 2002). KIγ90-GFP and KIγ90^{W647F}-GFP transfectants had a similar degree of CXCL12-activated attachment to VCAM-1, without further activation relative to control GFP transfectants (Figures 6C and 6D), suggesting that PIPKIγ90 endogenous activity was sufficient to stimulate adhesion of both KIγ90-GFP and KIγ90^{W647F}-GFP transfectants. However, KIγ90-GFP, but not the mutant kinase form, was capable of recovering adhesion that was impaired by PH-GFP cotransfection (Figures 6C and 6D). Constitutive Vav1-talin association and its reduction by CXCL12, as well as stimulation of talin-β1 binding, were not altered in KIγ90-GFP or KIγ90^{W647F}-GFP transfectants, but again only the former showed recovery of these dynamic associations that were abolished by PH-GFP expression (Figures 6E and 6F). Control experiments indicated dominant activity of KIγ90-GFP over PH-GFP in CXCL12-stimulated adhesion to VCAM-1 (Figure S5), suggesting that KIγ90-GFP is probably competing with PH-GFP locally rather than throughout the cell. Therefore, defective associations between Vav1, talin, and β1 resulting from PtdIns(4,5)P_2 sequestering by PH-GFP results in failure to activate cell attachment, but local recovery of phosphoinositide synthesis by talin-bound KIγ90-GFP would allow correct molecular associations leading to rescue of adhesion.

A recent study showed that a RhoA-phospholipase D1 (PLD1)-PIPKIγ90 signaling module regulates LFA-1 activity in human T lymphocytes (Bolomini-Vittori et al., 2009). Because PLD1-derived phosphatidic acid (PA) can regulate PIPKIγ90 activity (Jarquin-Pardo et al., 2007), we studied whether PLD1 could also control chemokine-stimulated T cell adhesion mediated by α4β1. We addressed this question by using n-butanol, a primary alcohol that scavenges PA produced after PLD1 activity (Brown et al., 2007). Although n-butanol blocked CXCL12-promoted, LFA-1-dependent Molt-4 adhesion to ICAM-1, in agreement with previous work (Bolomini-Vittori et al., 2009), it did not affect adhesion to VCAM-1 (Figure S6A). Control experiments showed that the inactive t-butanol did not alter stimulated adhesion to ICAM-1. In addition, binding of 15/7 mAb or VCAM-1-Fc to Molt-4 cells exposed to CXCL12 was not affected by n-butanol (Figure S6B). These results suggest that, contrary to LFA-1, stimulation by chemokines of α4β1-dependent T cell adhesion does not require PLD1 activity.

Finally, CXCL12-stimulated T cell adhesion mediated by α4β1 was not impaired by the PKC inhibitor Gö6850 at concentrations up to 2 μM, whereas it blocked PMA-triggered adhesion (Figure S7A). We did not use higher amounts of Gö6850 because it might influence the activity of other kinases. Furthermore, Molt-4 transfectants overexpressing dominant-negative (DN) PKC α or θ isoforms (DN PKC α K368R or DN PKC θ K409R) (Baier-Bitterlich et al., 1996) attached to VCAM-1 at a similar degree compared to wild-type counterparts upon CXCL12 stimulation (Figure S7B), suggesting that PKC α or θ activity is not needed for chemokine-stimulated T cell adhesion mediated by α4β1. A previous study reported that Gö6850 exerted partial inhibition of CXCL12-activated T cell adhesion to VCAM-1 at concentrations of Gö6850 of 10 μM (Ghandour et al., 2007). The differences in concentrations together with distinct experimental conditions including different time scales might account for the different results.

DISCUSSION

When T lymphocytes become exposed to chemokines, inside-out signals are generated that finally impinge on integrin cytoplasmic domains, leading to integrin activation and stimulation of cell adhesion (Ley et al., 2007). Vav1 is a key component of this signaling because it is required for α4β1 integrin activation (Garcia-Bernal et al., 2005). Talin directly interacts with β subunit integrin cytoplasmic domains and regulates integrin activation (Tadokoro et al., 2003; Wegener et al., 2007), thereby representing a main candidate for transmission of chemokine signals. Indeed, earlier work (Manevich et al., 2007) and our present results demonstrate that talin is essential for chemokine-stimulated T lymphocyte adhesion mediated by α4β1. Here we show that Vav1 and talin constitutively associate in human resting T lymphocytes and that they complex into an essential signaling platform. Thus, when chemokine-triggered signaling converges on this platform, Vav1 and talin gradually dissociate, representing a key event for activation of α4β1-mediated cell adhesion, as shown in the model proposed in Figure S8. The importance of this platform is based on the fact that silencing Vav1 or talin, or expressing PtdIns(4,5)P_2-sequestering probes, leads to failure to assemble the complex and to a subsequent blockade of CXCL12-stimulated T cell attachment to VCAM-1. The molecular properties of Vav1-talin association, whether a direct or indirect interaction takes place, have yet to be studied.

In addition to associate to Vav1, talin has a constitutive low level of binding to β1, which requires Vav1 because it is absent in Vav1-silenced cells. Therefore, a Vav1-talin-β1 complex is formed in nonstimulated T cells, although association between Vav1 and β1 is probably weak and occasionally difficult to detect. Indeed, CXCL12 stimulates Vav1-β1 association with similar kinetics as activation of talin-β1 binding, suggesting that increased talin-β1 association might approach and favor coprecipitation of Vav1 and β1.

PtdIns(4,5)P_2 induces conformational changes on talin, leading to increased talin affinity for β1 integrins (Martel et al., 2001). PtdIns(4,5)P_2 is mainly synthesized by type I phosphatidylinositol phosphate kinases (Ling et al., 2006), from which PIPKIγ90 is able to bind to talin, resulting in increased kinase activity (Di Paolo et al., 2002; Ling et al., 2002). We found that PIPKIγ90 is expressed on T cells and that it associates with talin. Moreover, overexpressed PIPKIγ90-GFP, but not the mutant

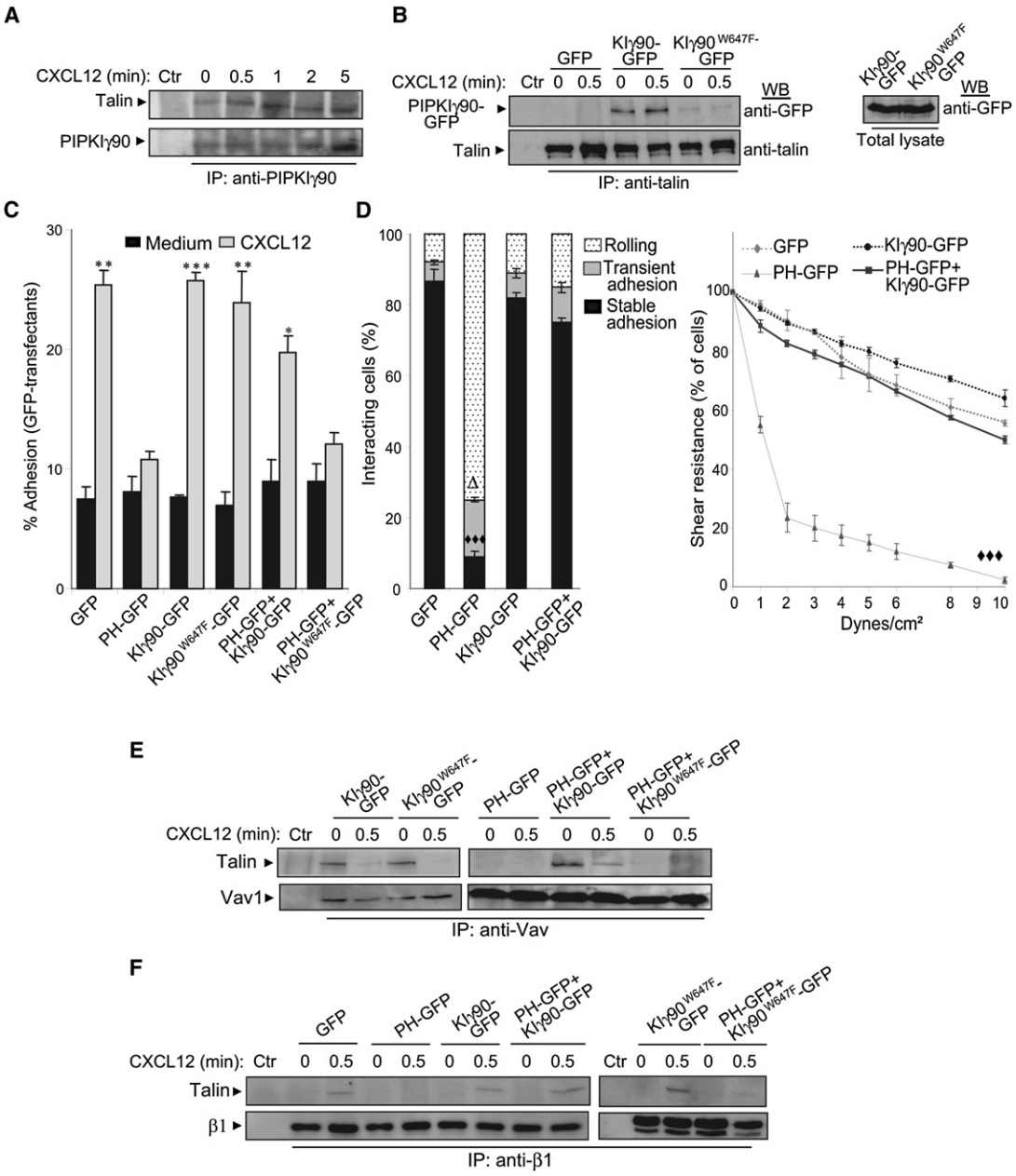

Figure 6. Overexpression of PIPKIγ90 Overcomes PH-GFP-Dependent Blockade of Associations between Vav1, Talin, and β1, as well as α4β1-Mediated T Cell Adhesion

(A and B) Untransfected (A) or Molt-4 cells transfected with PIPKIγ90-GFP, PIPKIγ90^{W647F}-GFP, or GFP vector alone (B) were incubated with CXCL12 and subsequently subjected to immunoprecipitation with anti-PIPKIγ90 or anti-talin and immunoblotting with antibodies to the indicated proteins. Total expression of PIPKIγ90-GFP and PIPKIγ90^{W647F}-GFP was assessed by immunoblotting (B, right).

(C and D) Cells were transfected with PIPKIγ90-GFP or PIPKIγ90^{W647F}-GFP alone or in combination with PH-GFP, and subsequently tested in static adhesion assays to VCAM-1 immobilized with or without CXCL12 (C), or in flow chamber adhesion assays (D) measuring interacting cells (left) or shear resistance (right). ***Adhesions were significantly increased, $p < 0.001$, **$p < 0.01$, or *$p < 0.05$, or inhibited ◆◆◆$p < 0.001$. $^{\Delta}$Transient arrest was significantly increased, $p < 0.05$.

(E and F) Same transfectants were incubated with CXCL12, followed by immunoprecipitation with Vav1 or β1 mAb and immunoblotting with antibodies to the indicated proteins.

PIPKIγ90^{W647F}-GFP form, bound to talin and rescued talin association with Vav1 and β1 from the PH-GFP inhibitory effects. Both PIPKIγ90 and β1 interact with overlapping sites on the F3 subdomain of the FERM talin domain (Barsukov et al., 2003), suggesting that binding is mutually exclusive. PIPKIγ90 associates with talin but not with Vav1 and CXCL12 does not trigger talin release from PIPKIγ90, so we propose that local production of PtdIns(4,5)P_2 by talin-bound PIPKIγ90 promotes assembly and/or stabilization of nearby Vav1-talin complexes, in addition to stimulating constitutive talin-β1 association. Constitutive

β1-associated talin in nonstimulated T cells might function as the ground pool for promoting future tethers in a PtdIns(4,5)P_2-dependent manner. An important role for PIPKIγ90 has also been recently proposed during chemokine-stimulated T cell adhesion mediated by LFA-1 (Bolomini-Vittori et al., 2009).

Increased talin-α4β1 binding and Vav1 dissociation from talin in response to CXCL12 are required dynamic steps for stimulation of aα4bβ1 activation (Figures S8B and S8C). Upregulation of talin-β1 binding needs preformed Vav1-talin complex, but it is independent of Vav1 dissociation from talin, suggesting that it occurs before Vav1-talin disassembly. The molecular events underlying CXCL12-stimulated talin-β1 binding are not known but they might include a better access of the complex to PtdIns(4,5)P_2 resulting from chemokine actions. Stimulation by CXCL12 of talin binding to α4β1 is critical but not sufficient to fully activate the integrin, because it requires ZAP-70-dependent subsequent dissociation of Vav1 from talin for further integrin activation, as detected with 15/7 anti-β1 mAb, a reporter of α4β1 activation. ZAP-70 is recruited and binds to Vav1 in preformed Vav1-talin complexes upon chemokine stimulation, as indicated by the fact that it does not occur in talin-silenced cells. A potential mediator of ZAP-70 recruitment is Lck, which is a target of CXCL12 signaling (Inngjerdingen et al., 2002) and tyrosine phosphorylates ZAP-70. Earlier work demonstrated that ZAP-70 is involved in Vav1 tyrosine phosphorylation in response to CXCL12 (Ottoson et al., 2001). Our results indicate that ZAP-70 binding to Vav1-talin complexes leads to Vav1 phosphorylation, which possibly weakens Vav1-talin association. Therefore, our data strongly suggest that chemokine-promoted, ZAP-70-dependent phosphorylation of Vav1 starts while associated with talin in the signaling platform. This conclusion is based on the following results. Talin silencing or PH-GFP expression blocks both formation of Vav1-talin complex and subsequent Vav1 phosphorylation. Furthermore, talin knockdown impairs Vav1-ZAP-70 binding, and inhibition of ZAP-70 activity abolishes chemokine-dependent Vav1 phosphorylation associated with blocking of Vav1-talin disassembly. Although this phosphorylation is difficult to detect at early time points in Molt-4 cells, results with PBL-T cells together with the above observations indeed indicate that phosphorylation occurs in Vav1-talin complexes, and thus, talin functions as a modulator of Vav1 tyrosine phosphorylation. Together, our data suggest that Vav1 functions as a constitutive repressive adaptor of talin that needs to be phosphorylated in order to be released from α4β1-talin complexes and render talin available for additional β1 integrin activation. It is noteworthy that the talin pool recently dissociated from Vav1 after chemokine-ZAP-70 signals may be involved in α4β1 activation seconds after the first chemokine signal is transduced, and it could also contribute to strengthening of attachment at later times in the adhesion process. According to the present data, it is therefore unlikely that preexisting α4β1 high-affinity molecules, or clustered α4β1 heterodimers after ligand binding that are engaged in outside-in signaling, could be associated to the Vav1-talin platform.

CXCL12-triggered phosphorylation of Vav1 is essential for Rac1 activation and progression of α4β1-mediated T cell adhesion (Garcia-Bernal et al., 2005). An additional consequence of deficient Vav1 phosphorylation in talin-silenced cells was the impairment of Rac1 activation. The kinetics of Rac1 activation in response to CXCL12 indicate that this is a late signaling event mediating stimulation of α4β1-dependent cell adhesion strengthening, rather than involvement in α4β1 activation (Figure S8C). Further adhesion strengthening might come from CXCL12-stimulated binding of vinculin to talin, which might be based on promotion of PtdIns(4,5)P_2-mediated opening of the head-tail vinculin interaction (Gilmore and Burridge, 1996) or talin rod stretching exposing buried vinculin-binding sites (del Rio et al., 2009).

Monocyte adhesion mediated by α4β1 is also tightly controlled by chemokines (Chan et al., 2001). The Syk kinase is expressed in monocytes instead of ZAP-70, which is highly specific of T cells. Syk interacts with and tyrosine phosphorylates Vav proteins (Deckert et al., 1996), thus raising the possibility that a similar pathway of early chemokine-dependent activation of α4β1 involving Syk-Vav1 might occur in monocytes. It is noteworthy that phospholipase C mediates α4β1 activation in response to chemokines in monocytes (Hyduk et al., 2007). Thus, although ZAP-70 and Syk could have parallel roles in Vav activation, the involvement of PLC in α4β1 activation may be restricted to monocytes.

Paxillin binds to the cytoplasmic domain of α4 (Liu et al., 1999), modulating adhesion strengthening to α4β1 ligands (Alon et al., 2005). However, recent data showed that paxillin is not involved in chemokine-stimulated adhesion mediated by α4β1 (Manevich et al., 2007). Instead, Rap1 and kindlin-3 control chemokine-promoted T cell adhesion involving α4β1 and LFA-1 (Moser et al., 2009; Shimonaka et al., 2003). It will be important to investigate potential structural and functional cross-talks between the Vav1-talin signaling platform and Rap1 and kindlin-3 in chemokine-triggered α4β1-dependent adhesion. Because α4β1 function is fundamental for T lymphocyte arrest on endothelium after chemokine stimulation at sites of inflammation (Luster et al., 2005), the present results should contribute to a better definition of the dynamic associations between components of the signaling machinery associated to α4β1 activation.

EXPERIMENTAL PROCEDURES

Cells and Antibodies

Human Molt-4 T cells and peripheral blood T lymphocytes were cultured and prepared as described (Garcia-Bernal et al., 2005). The Consejo Superior de Investigaciones Cientificas Ethics Committee (Madrid, Spain) approved the protocols used to obtain and process the human samples. Control P3X63, α4 HP1/2, β1 TS2/16, and CD45 RP2/21 mAb were gifts from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Polyclonal β1A antibodies and the β1 mAb 15/7 were gifts from G. Tarone and R. Alon (Turin University, Italy; Weizmann Institute of Science, Rehovot, Israel, respectively), whereas PIPKIγ90 antibodies were from P. De Camilli (Yale University, New Haven, CT). CXCR4 mAb was from R&D Systems (Minneapolis, MN); phosphotyrosine, Vav1, RhoGDI, and α4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Rac1 was from BD Biosciences PharMingen (San Diego, CA); anti-GFP was from Molecular Probes (Eugene, OR); and anti-vinculin and anti-talin clone 8D4 were from Sigma-Aldrich (St Louis, MO). Antibodies to ZAP-70 were from J.M. Rojo (Centro de Investigaciones Biológicas, Madrid, Spain).

Transfections and RNA Interference

Vector coding for GFP-fused PH domain of PLC-δ1 was from M.J. Rebecchi (State University of New York, NY), whereas PIPKIγ90-GFP and PIPKIγ90-W647F-GFP vectors were obtained from P. De Camilli. GFP-fused Vav1 wild-type and the mutant Vav1 ΔCH+Ac vectors were from X. Bustelo (Centro

de Investigación del Cáncer, Salamanca, Spain). Three independent siRNA duplexes against human talin 1 were purchased from Ambion (Austin, TX). One of them strongly inhibited talin 1 expression and was labeled as i-talin 1 (interfering-talin 1); the other two showed little or no inhibition of talin 1 expression and they were presented as ni-talin 1 (noninterfering-talin 1) (see Figure 1C). ZAP-70 siRNA was purchased from Dharmacon (Lafayette, CO), and control and Vav1 siRNA (Vav1.3) were as reported (Garcia-Bernal et al., 2005). Vectors and siRNA were nucleofected according to the described procedure (Garcia-Bernal et al., 2005). Molt-4 or PBL-T siRNA transfectants were assayed 16 hr or 6 hr posttransfection, respectively.

Cell Adhesion and Soluble Binding Assays

For static cell adhesion to VCAM-1 alone or immobilized with CXCL12 (R&D Systems), we used the described method (Garcia-Bernal et al., 2005). Extent of adhesion was quantified with a fluorescence analyzer (for siRNA transfectants) or by flow cytometry (GFP transfectants). For flow chamber adhesion assays, we followed the reported protocol (Garcia-Bernal et al., 2006). In brief, transfectants were infused into flow chambers containing coimmobilized VCAM-1 and CXCL12. Rolling cells firmly attaching for at least 20 s were expressed as stable arrest, whereas cells attaching for a maximum of 5 s but resuming rolling were expressed as transient arrest. Otherwise, tethering cells that did not arrest at any moment were expressed as rolling cells. To evaluate shear resistance, cells were allowed to attach, followed by sequential increases of the flow. The number of cells remaining bound was determined as the percentage of total adhered cells after the adhesion step. For soluble binding, transfectants were stimulated for 45 s with CXCL12 or $MnCl_2$ before adding VCAM-1-Fc, which was detected by flow cytometry (Garcia-Bernal et al., 2005). Before addition of the 15/7 mAb, cells were exposed to CXCL12 or $MnCl_2$ for 1 min.

Immunoprecipitation, Immunoblotting, Cell Fractionation, and GTPase Assays

For immunoprecipitation, Molt-4 (2×10^7) and PBL-T (5×10^7) cells were lysed with 1% digitonin as reported (Ticchioni et al., 2002). After preclearing with protein G-sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden), supernatants were incubated with antibodies, followed by coupling to protein G-sepharose beads. Proteins were resolved by SDS-PAGE and transferred to membranes that were sequentially incubated with primary antibodies and with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized with SuperSignal chemiluminiscent substrate (Pierce, Rockford, IL). For cell fractionation, cells (3×10^6) were incubated at 4°C with 100 μl of hypotonic digitonin buffer, as described (Redondo-Munoz et al., 2006). In brief, cytosolic and membrane fractions were separated by centrifugation and the pellet was extracted with NP-40 lysis buffer whereas lysates were clarified by centrifugation. Protein detection was performed as above. For GTPase assays, we followed the method described (Garcia-Bernal et al., 2005). In brief, cells exposed to CXCL12 were lysed and aliquots from extracts were separated for total lysate controls and for incubation with GST-PAK-CD fusion protein (Sander et al., 1998) and glutathione-agarose beads. Bound proteins were eluted and subjected to immunoblotting via Rac1 antibodies.

Statistical Analyses

Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons. In both analyses, the minimum acceptable level of significance was $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include eight figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00499-3.

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