Paxillin Associates with the Microtubule Cytoskeleton and the Immunological Synapse of CTL through Its Leucine-Aspartic Acid Domains and Contributes to Microtubule Organizing Center Reorientation

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Paxillin Associates with the Microtubule Cytoskeleton and the Immunological Synapse of CTL through Its Leucine-Aspartic Acid Domains and Contributes to Microtubule Organizing Center Reorientation

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The cytoskeletal adaptor protein paxillin localizes to the microtubule organizing center (MTOC) in T cells and, upon target cell binding, is recruited to the supramolecular activation complex (SMAC). We mapped the region of paxillin that associates with both the MTOC and SMAC to the leucine-aspartic acid (LD) domains and showed that a protein segment containing LD2–4 was sufficient for MTOC and SMAC recruitment. Examination of the localization of paxillin at the SMAC revealed that paxillin localizes to the peripheral area of the SMAC along with LFA-1, suggesting that LFA-1 may contribute to its recruitment. LFA-1 or CD3 engagement alone was insufficient for paxillin recruitment because there was no paxillin accumulation at the site of CTL contact with anti-LFA-1 or anti-CD3–coated beads. In contrast, paxillin accumulation was detected when beads coated with both anti-CD3 and anti-LFA-1 were bound to CTL, suggesting that signals from both the TCR and LFA-1 are required for paxillin accumulation.

Paxillin was shown to be phosphorylated downstream of ERK, but when we generated a mutation (S83A/S130A) that abolished the mobility shift as a result of phosphorylation, we found that paxillin still bound to the MTOC and was recruited to the SMAC. Furthermore, ERK was not absolutely required for MTOC reorientation in CTL that require ERK for killing. Finally, expression of the LD2–4 region of paxillin substantially reduced MTOC substantially reduced MTOC reorientation. These studies demonstrated that paxillin is recruited, through its LD domains, to sites of integrin engagement and may contribute to MTOC reorientation required for directional degranulation. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: EGFP, enhanced GFP; LD, leucine-aspartic acid; LIM, lin-11 isL-1 mecl-3; MTOC, microtubule organizing center; NP, nucleoprotein; pSMAC, peripheral supramolecular activation complex; SMAC, supramolecular activation complex.

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human NK cells reduced LFA-1–stimulated granule polarization, suggesting that paxillin may contribute to directional degranulation (11). Therefore, many questions remain unanswered regarding how paxillin is recruited to the SMAC and its contribution to T cell activation.

In the current study, we sought to identify the structural requirements for paxillin association with the MTOC and its recruitment to the SMAC. Although it was predicted that the LIM domains would be important for these interactions (9), we established that the N-terminal domain containing the LD domains mediated both binding to the MTOC and SMAC recruitment upon target cell binding. We further defined the requirements for SMAC recruitment and demonstrated that paxillin is preferentially enriched at sites of integrin engagement and that CD3 and LFA-1 coengagement are sufficient for paxillin accumulation, suggesting that LFA-1 and perhaps other integrins are important for paxillin recruitment to the SMAC. Because we have shown that TCR-induced paxillin phosphorylation is ERK dependent, and degranulation is also ERK dependent, we sought to examine the contribution of ERK to paxillin localization and MTOC reorientation. We explored the contribution of paxillin to MTOC reorientation in CTL and found that paxillin does contribute to this process. These findings suggest that paxillin can regulate CTL function, likely through a contribution to integrin engagement leading to MTOC reorientation.

Materials and Methods

Cells

The L1210 (H-2d) and L1210 Kd/Dd target cell lines were a gift from Dr. K.P. Kane (University of Alberta) (12). The nontransformed murine H2-`-allspecific CTL clone lines AB.1 (H-2d) and Clone 11 (H-2b) were described previously (13, 14). The CTL clone line Clone 3/4 is specific for aa 366–374 of the influenza nucleoprotein (NP) in the context of H-2` (15). The CTL clones were cultured in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, 2-ME, and 10 U/ml rIL-2. The clones were stimulated weekly with irradiated allogeneic splenocytes from C57BL/6 mice. In the case of Clone 3/4, the splenocytes were first pulsed for 60 min with 200 μg/ml NP366-374 peptide. All animal studies were approved by the University Animal Policy and Welfare Committee at the University of Alberta and adhered to the guidelines put forward by the Canadian Council on Animal Care.

Abs and reagents

Anti-paxillin was purchased from BD-Transduction Labs (Mississauga, ON, Canada). Anti–LFA-1 (M17/5.2) and anti-CD3ε (145-2C11) were purified from hybridomas, as described previously (16). The rabbit Ab specific for CD28 was purchased from Clontech (Mountain View, CA), as were the mouse anti–CD28 Ab and rabbit polyclonal Abs were purchased from Molecular Probes/Invitrogen (Carlsbad, CA), and the rabbit anti–paxillin was purchased from BD-Transduction Labs (Mississauga, ON, Canada). Anti-LFA-1 (M17/5.2) and anti-CD3ε were obtained from Sigma-Aldrich (Oakville, ON, Canada). Anti-mouse Ig were obtained from Jackson ImmunoResearch (West Grove, PA). Anti–CD3ε and anti–LFA-1 were purchased from BD-Transduction Labs (Mississauga, ON, Canada). Anti–LFA-1 (M17/5.2) and anti-CD3ε were obtained from Sigma-Aldrich (Oakville, ON, Canada). Anti–LFA-1 (M17/5.2) and anti-CD3ε were obtained from Sigma-Aldrich (Oakville, ON, Canada). Anti–LFA-1 (M17/5.2) and anti-CD3ε were obtained from Sigma-Aldrich (Oakville, ON, Canada). Anti–LFA-1 (M17/5.2) and anti-CD3ε were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Cloning of murine paxillin and mutagenesis

Paxillin was cloned from RNA extracted from murine splenocytes and used to generate cDNA that was used as a template for the specific PCR amplification of paxillin, with EcoRI and Sall sites engineered at either end. Full-length paxillin (aa 1–557) was ligated into the pEFGFP-C1 plasmid (Clontech), creating a fusion of enhanced GFP (EGFP) to the N terminal of paxillin. Further constructions of paxillin were made containing the N-terminal half only (pEFGFP-NT, aa 1–323) including the N-terminal 1/2 of the molecule, while the C-terminal half (LD1-D2, LD1-D3, and LD4) were added. All mutations were generated in the pEFGFP-C1 plasmid, and for the purposes of the MTOC reorientation experiments, the LD2–4 segment was also cloned into the pDsRed-Monomer-C1 plasmid (Clontech).

Site-directed mutagenesis of specific amino acids within paxillin was performed by PCR using mutagenic primers and full-length pEFGFP-paxillin as a template. The primers were designed such that they did not overlap completely (overhang of 9 bp), according to the protocol laid out by Zheng et al. (17). Mutations in LIM domains 2 and 3 consisted of cysteine to alanine substitutions (C411/A/C470A), and serine residues Ser83 and Ser130 were also changed to alanines (S83A/S130A). Targeted deletions of LD domains and the proline-rich domain of paxillin were engineered via a step-wise PCR method. The halves of paxillin on either side of the deletion were amplified in separate reactions. The N-terminal segment contained an 11-bp bridge sequence engineered into the primer, which was complementary to the C-terminal side of the deletion to allow for joining later on. After purifying the two fragments, they were combined in a second PCR step with primers to full-length paxillin to create a “joined” product in which both flanking sequences were contiguous, with the desired deletion made. Successful deletion was confirmed by sequencing. This technique was used to delete LD3 (Δ aa 220–226), LD2 (Δ aa 143–154), a larger LD2–4 segment (Δ aa 255–274), and the proline region (Δ aa 45–54). All constructs used for this study were fully sequenced and verified to be correct. All plasmids were prepared using Endo-free Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) for transfection.

Transfection of CTL with plasmid DNA

Transfection of CTL was accomplished by nucleofection using the Amaxa mouse T cell kit, according to the manufacturer’s directions (Lonza, Cologne, Germany). CTL clones were harvested 4 d after spleenocyte stimulation, and 1 × 10^7 cells/sample were dispensed into separate tubes and pelleted at 500 × g for 2 min. The supernatant was removed, and the pellet was resuspended in 100 μl Nucleofection Solution, after which 4 μg plasmid DNA was added, and the sample was nucleofected using the X-01 program on the Amaxa nucleofector. Cells were immediately transferred into 1 ml prewarmed medium and cultured, as recommended by the manufacturer. Transfection efficiency and EGFP expression were assessed by flow cytometry 24 h after nucleofection, and cells were used for experiments.

Preparation of peptide-pulsed target cells

EL4 target cells were first labeled with 7 μM CMAC CellTracker Blue dye (as described below) and then resuspended at a concentration of 1 × 10^7 cells/ml in RPMI 1640. NP366-374 peptide was then added at concentrations of 100 nM or 1 or 10 μM. After incubation for 1 h at 37ºC, the cells were washed four times in cold RPMI 1640 with 4% FCS prior to mixing with CTL.

Preparation of CTL for confocal microscopy

In preparation for microscopy, L1210Kd/Dd target cells were labeled with 7 μM CMAC CellTracker Blue dye (Molecular Probes/Invitrogen) for 20 min at 37 ºC in DMEM media. Following a 30-min incubation in fresh medium, the cells were washed twice in PBS and resuspended at the desired concentration. In the case of transfected cells, 24 h after transfection, 4–5 × 10^5 cells were combined with an equal volume of 4 × 10^5 L1210 Kd/Dd target cells in PBS. In the case of nontransfected CTL, 6 × 10^5 CTL were mixed with 3 × 10^5 L1210 Kd/Dd for an E:T ratio of 2:1. To encourage conjugation, the cells were centrifuged briefly for 1 min at 100 × g and then incubated at 37ºC for 10 or 20 min. Conjugates were gently vortexed and transferred using a wide-bore pipette tip into poly-t-lysine-coated coverslips and allowed to settle and adhere for 7 min at room temperature. Fixation and permeabilization were then performed by treatment with 4% paraformaldehyde in PBS for 10 min and 0.2% Nonidet P-40 in PBS for 5 min, respectively. The cells were then washed twice with 2% FCS in PBS. Cells were stained with the appropriate primary (45 min) and fluorescently-coupled secondary Abs (30 min) and resuspended at a concentration of 1 × 10^6 cells/ml at room temperature in a dark chamber, with washes in between. Stained coverslips were mounted on microscope slides in a droplet of Mowiol-based mounting medium. Images were acquired using a Zeiss LSM 510 confocal microscope with an X40 oil-immersion objective (N.A. 1.3).

Dozens of conjugates were viewed for any given experimental condition, images were collected over at least three separate experiments, and a representative image was chosen. With respect to the use of coengagement, when the paxillin mutant clearly localized to both the MTOC and the SMAC, as was the case for most of the constructs examined, this occurred in all cells examined with low to medium expression levels of
fusion protein. For some mutants, there was obvious accumulation in the nucleus in all cells examined. The constructs that did not show obvious MTOC or nuclear enrichment were difficult to score, because there was a range of phenotypes that would vary with expression levels. For experiments in which MTOC reorientation was quantified, the identities of the slides were blinded prior to imaging, and large, randomly chosen fields were captured to prevent bias. Only those conjugates that were unobstructed and in which the MTOC (stained by anti-α-tubulin) and target cell interface could be clearly observed were counted. Cells expressing the DsRed constructs could be easily identified and were compared with untransfected cells on the same slides as a control. An MTOC was considered to be reoriented only when it was in close contact with the membrane at the target interface. Quantification was performed across at least three experiments, and 50–100 transfected and 100–200 untransfected conjugates were scored for each experiment. The results were analyzed by a two-tailed Student *t* test.

Cell stimulation with immobilized anti-CD3 and Western blotting

Immobilization of anti-CD3 was achieved by incubating 96-well plastic plates with 10 μg/ml 145-2C11 Ab overnight at 4°C, washing, and blocking with PBS containing 2% BSA. A total of 4.5 × 10^5 transfected CTL in 40 μL PBS was added to the anti-CD3–coated wells and incubated at 37°C for 25 min. The cells were lysed by the addition of 40 μL 2× Laemmlili reducing sample buffer, boiled, and loaded onto 7.5 or 8.5% acrylamide SDS-PAGE gels. The separated proteins were then transferred to polyvinylidene fluoride membranes for Western blot analysis. The membranes were probed with the indicated Abs and detected with the ECL system (Perkin Elmer, Life Science Products, Boston, MA).

Results

Paxillin localizes to the microtubule cytoskeleton and the CTL immunological synapse

As we reported previously (7), paxillin in CTL colocalizes with tubulin at both the MTOC and the microtubules in both resting and activated CTL, and it is enriched at the interface between a CTL and its target (Fig. 1A). In these experiments, nontransformed CTL clones were mixed with target cells bearing a chimeric allogeneic MHC class I molecule (L1210 Kb/Dd) and imaged by confocal microscopy. To address the contributions of the paxillin LD and LIM interaction domains in recruitment to the MTOC and SMAC, the N terminus of full-length mouse paxillin (aa 1–557) was fused to EGFP as a platform for mutational analysis. First, we assessed EGFP-paxillin to ensure that it behaved similarly to the endogenous protein. As we described previously, paxillin undergoes a mobility shift on SDS-PAGE upon TCR activation as a result of extensive phosphorylation (5). To test whether EGFP-paxillin could be phosphorylated after TCR signaling, CTL were transfected with pEGFP-paxillin and activated with anti-CD3. Analysis of the cell lysates showed that EGFP-paxillin underwent a mobility shift, although the increased size of the fusion protein resulted in a smaller visible shift on the gel relative to that of the endogenous protein (Fig. 1B). The subcellular distribution of EGFP-paxillin was then visualized by transfecting CTL with either empty pEGFP plasmid or pEGFP-paxillin, mixing them with L1210 Kβ/Dd target cells, and staining for EGFP and α-tubulin. Although uncoupled EGFP displayed diffuse cellular staining, the EGFP-paxillin fusion protein localized to the microtubule cytoskeleton and was recruited to the target cell interface, similarly to the endogenous protein (Fig. 1C, 1D). It is also of importance for the experiments described below that no endogenous paxillin was detected in immunoprecipitates of EGFP-paxillin (data not shown), eliminating the possibility of paxillin dimerization, which would have complicated the interpretation of the results. Therefore, we considered EGFP-paxillin to be an appropriate tool for further localization studies, and this construct was subsequently altered by a series of deletion and truncation mutations.

![FIGURE 1. Paxillin is targeted to the MTOC and CTL–target cell interface via its LD-containing N-terminal segment. A, AB.1 CTL clones were conjugated with allogeneic L1210 Kβ/Dd target cells (labeled with CellTracker Blue) and stained for paxillin (detected by anti-mouse rhodamine) and α-tubulin (detected by anti-rabbit Alexa Fluor 488). Note that the images have been pseudocolored for the sake of consistency with subsequent images. B, Clone 11 CTL clones were transfected with pEGFP-paxillin and either activated with plate-bound anti-CD3 or left unstimulated. Total cell lysates were sequentially immunoblotted for EGFP (top panel) and paxillin (bottom panel). C–H, Clone 11 CTL were transfected with the indicated plasmids, conjugated to L1210 Kβ/Dd target cells, and stained for EGFP (detected with anti-mouse Alexa Fluor 488) and α-tubulin (detected with anti-rabbit Alexa Fluor 594). All images are single confocal planes and are representative of >100 conjugates observed over at least three independent experiments.](http://www.jimmunol.org/)

Localization of paxillin in CTL is mediated by the N-terminal LD domains

The LIM2 and LIM3 domains were capable of binding to α-tubulin when segments of paxillin were fused to GST and used to pull down binding partners from CHO cell lysates (9). Each LIM domain contains cysteine- or histidine-based coordination sites for two zinc atoms, and mutation of any of these residues causes structural disruption of the domain (18). Mutation of cysteine residues in LIM2 and LIM3 (C411A/C470A) abolished binding to α-tubulin (9); therefore, we determined whether similar mutations
would disrupt paxillin recruitment to the MTOC and microtubules. Two EGFP-tagged constructs were created for this purpose: one containing a segment encompassing LIM2 and LIM3 only (aa 376–503) and the other containing full-length paxillin bearing the C411A and C470A point mutations. Microscopic analysis revealed that the removal of critical LIM2/3 cysteine residues had no effect upon paxillin binding to the MTOC and microtubules (Fig. 1E). Reciprocally, the LIM2 and LIM3 domains alone were not sufficient to effect recruitment to the microtubule cytoskeleton, and, in fact, this fragment was collected mainly in the nucleus (Fig. 1F).

We then addressed whether microtubule binding was mediated by the N-terminal or C-terminal region of the paxillin molecule. The N-terminal (aa 1–323) and C-terminal (aa 319–557) halves of paxillin, containing the five LD domains and the four LIM domains, respectively, were each fused to EGFP and transfected into CTL. The paxillin N-terminal localized normally (Fig. 1G), whereas the paxillin C-terminal did not and, like the LIM2–LIM3 segment, was concentrated primarily in the nucleus of the cell (Fig. 1H). We concluded that the domain(s) responsible for paxillin localization was located in the LD domain region and performed a more detailed analysis of this area by step-wise deletion and truncation mutagenesis (Fig. 2). Note that the images shown for these and further colocalization experiments were selected based on whether the cells had a clearly distinguishable MTOC and target interface, to facilitate an analysis of paxillin localization. In many cases, this was best achieved by showing cells in which the MTOC had not fully reoriented toward the target cell membrane, as can be seen in many of the images shown. Therefore, no conclusions can be drawn with respect to the impact of the various constructs on MTOC reorientation based on these images.

A segment containing LD domains 2, 3, and 4 is sufficient for paxillin localization

Successive truncations were made of the N-terminal portion of paxillin, resulting in the following segments, which contained no LIM domains: LD1–4 (aa 1–296), LD1–3 (aa 1–264), and LD1–2 (aa 1–219). Truncation of LD5 and LD4 had no effect upon paxillin localization (Fig. 3A, 3B), although truncation of LD3 partially disrupted MTOC and interface recruitment (Fig. 3C). The segment containing LD1–2, although able to localize to the MTOC and SMAC, also displayed a diffuse cytoplasmic staining pattern (Fig. 3C) not observed for the other segments (Fig. 3A, 3B). This result suggested a partial weakening of protein interactions, either because other LD domains were required to enhance and support binding or because a tertiary structural disruption caused by the extensive truncation reduced the ability of LD1 and 2 to interact with a target protein. Past studies of FAK and Pyk2 interactions with paxillin showed that, although the LD2 and LD4 domain alone is each capable of binding FAK or Pyk2, maximal binding is achieved with both sites present (19, 20). We postulated that a similar phenomenon was responsible for the partial disruption of MTOC binding in this case; therefore, a further analysis of the LD domains was conducted.

Truncation of the N-terminal LD domains from full-length paxillin resulted in EGFP fusion proteins lacking LD1 (LD1trunc, aa 136–557) and LD1–2 (LD1–2trunc, aa 214–557). Neither mutation disrupted paxillin localization in CTL (Fig. 3D, 3E). To further examine the contribution of each LD domain, we deleted individual LD domains and the proline-rich region from full-length paxillin. Deletion of the proline domain (∆Pro, Δ45–54) had no effect on localization (Fig. 4A). Likewise, removal of the individual LD2 (∆LD2, Δaa 143–154) and LD3 (∆LD3, Δaa 220–226) domains did not disrupt paxillin recruitment (Fig. 4B, 4C), further implicating the involvement of more than one LD domain. We next deleted a larger segment encompassing the LD2, 3, and 4 regions (∆LD2–4, Δaa 55–274). This protein fragment, although capable of limited colocalization with the MTOC and target interface, was clearly not enriched at these locations because this fragment displayed diffuse staining within the cell and...

**FIGURE 2.** Diagram of paxillin mutants used for this study. The N terminus of full-length paxillin was fused to EGFP and then further modified by mutagenesis, as described in Materials and Methods.

**FIGURE 3.** Truncation of LD3, LD4, and LD5 results in more diffuse localization of paxillin, whereas truncation of LD1 and LD2 has no effect on localization. A–E, Clone 11 CTL were transfected with the paxillin constructs indicated, conjugated with L1210 Kb/Dd target cells (labeled with CellTracker Blue), and stained for EGFP (detected with anti-mouse Alexa Fluor 488) and α-tubulin (detected with anti-rabbit Alexa Fluor 594). All images are single confocal planes and are representative of >100 conjugates observed over at least three independent experiments.
some nuclear staining (Fig. 4D). This is illustrated by the staining-intensity profiles for tubulin and EGFP, which were compared with those of the ΔLD3 construct (Fig. 4E). The intensity profile revealed that, although there is some localization of the ΔLD2–4 protein at the MTOC and SMAC, there is limited enrichment at these sites, and staining is distributed throughout the cell. This suggests that, although the LD2, 3, and 4 domains are not individually required, deletion of all three domains strongly disrupts MTOC and SMAC recruitment.

To determine whether the deleted LD2–4 segment was sufficient for paxillin localization, we created an EGFP fusion protein containing only the LD2–4 region (aa 136–296) and assessed its cellular distribution. The resulting protein was bound to the MTOC and microtubules and recruited to the target interface (Fig. 4F), demonstrating that the region of paxillin encompassing the LD2, 3, and 4 motifs is sufficient for normal localization. These results revealed that no single domain mediates binding but that multiple domains together are capable of reconstituting recruitment to the MTOC and SMAC.

Paxillin is recruited to the peripheral SMAC

We observed that for all mutants tested, microtubule and SMAC localization was always seen concurrently, and mutations that disrupted paxillin binding at one site also disrupted the other. This suggested that the two populations may be functionally linked, and we set out to further investigate the conditions required for paxillin recruitment to the membrane of CTL. The immune synapse formed between CTL and target cells was shown to change over time, such that in mature synapses the membrane-proximal proteins are separated into two phases: the central SMAC, to which are segregated the TCR and associated signaling molecules, and the peripheral SMAC (pSMAC), which contains integrins, such as LFA-1 (21–23). Given the association of paxillin with integrins and focal adhesions in adherent cells and its presence at the immune synapse in CTL, we examined whether paxillin segregated with LFA-1 into the pSMAC. CTL conjugates were stained for the β2 subunit of LFA-1 and paxillin, and those with mature SMAC were imaged by a series of optical sections and reconstructed in three dimensions. When the SMAC was rotated into a head-on projection, it was clear that paxillin cosegregated with LFA-1 in the outer ring of the synapse (Fig. 5). The colocalization of paxillin with LFA-1 was not complete; however, in all conjugates examined where the pSMAC was clearly visible, as defined by LFA-1 staining, paxillin segregated to the pSMAC.

LFA-1 and TCR coengagement is sufficient for paxillin recruitment to the contact

That paxillin segregates into the pSMAC suggests that it may be recruited to the site by adhesion molecules, such as LFA-1. We wished to address whether paxillin was recruited to the membrane by adhesion events or by TCR-signaling events. As a first step, we mixed CTL clones with beads coated with either anti–LFA-1 or anti-CD3 alone and detected no paxillin accumulation at either of the beads (data not shown), suggesting that neither TCR nor LFA-1 engagement alone is sufficient for paxillin recruitment to the contact zone. In the absence of TCR binding, adhesion proteins on the CTL interact with the target to form a conjugate, although the stability of this conjugate is reduced (7), and MTOC reorientation

**FIGURE 4.** Deletion of a segment containing LD2, LD3, and LD4 partially disrupts paxillin localization, and this segment is sufficient for paxillin targeting. A–D, Clone 11 CTL were transfected with the indicated deletion mutant of paxillin, conjugated with L1210 K/Dd target cells (labeled with CellTracker Blue), and stained for EGFP (detected with anti-mouse Alexa Fluor 488) and α-tubulin (detected with anti-rabbit Alexa Fluor 594). For D, images of the two extreme phenotypes are shown, rather than a representative image. E, Colocalization staining profile for CTL transfected with pEGFP-ΔLD3 (top panel) and pEGFP-ΔLD2–4 (bottom panel). Red and blue markers on the microscopy images provide orientation to the staining-intensity profiles on the right, with the blue line marking the MTOC. The green profile represents EGFP intensity, and the red profile represents tubulin intensity. F, Cellular distribution of EGFP-LD2–4. CTL were transfected and treated as in A–D. All images are single confocal planes and are representative of >100 conjugates observed over at least three independent experiments.
does not occur (24). In our control experiments, we noticed that when a CTL was bound to an Ag-independent target cell, there was paxillin accumulation at those contacts (data not shown), suggesting that LFA-1 or other integrins might be responsible for paxillin recruitment. We next performed an experiment in which we presented the TCR signal and the “adhesion signal” on separate surfaces to determine to which paxillin is recruited. CTL clones were mixed with beads coated with anti-CD3 and with L1210 targets expressing no allogeneic MHC recognizable to this CTL clone and then were imaged for paxillin and tubulin. We used anti–LFA-1–coated beads as negative control because we knew that paxillin was not recruited to these beads, a finding that was confirmed in these experiments (Fig. 6 A). We observed that paxillin appeared to be preferentially enriched at the cell contact point, rather than at the anti-CD3 bead (Fig. 6 B). Despite the fact that paxillin associates with TCR signaling molecules, such as Pyk2 and Src kinases, it appears that its enrichment at the membrane is favored by cell adhesion rather than TCR engagement.

These Ag-independent conjugates are primarily LFA-1 mediated, although other proteins likely contribute to these interactions, and low levels of TCR engagement cannot be eliminated, raising the possibility that TCR and LFA-1 coengagement at the same location are important for paxillin recruitment. To address this question, we immobilized anti-CD3 and anti–LFA-1 on the same bead and examined the cells for paxillin recruitment. These two proteins together are clearly sufficient for paxillin recruitment (Fig. 6 C). These results demonstrated that coimmobilized anti-CD3 and anti–LFA-1 are sufficient for paxillin recruitment, whereas either alone is unable to recruit paxillin to the SMAC.

Phosphorylation by ERK is not required for MTOC or SMAC recruitment of paxillin

We showed that TCR-triggered serine phosphorylation of paxillin and the concomitant m.w. shift require ERK activity (7), and paxillin was demonstrated to be a direct target of ERK phosphorylation (6). Given that ERK is necessary for CTL degranulation (25), we addressed whether ERK phosphorylation of paxillin influences MTOC association, SMAC recruitment, or MTOC reorientation. It was published that the extensive paxillin serine/threonine phosphorylation cascade is primed by ERK phosphorylation of serine 130, followed by glycogen synthase kinase 3 phosphorylation of serine 126 (26). Serine residue 83 was also identified as an ERK target (27). Based on these findings, we engineered mutations replacing serines 83 and 130 with alanine residues. We created two mutants: one bearing a single S83A substitution and one with a double S83A/S130A substitution. CTL transfected with the EGFP fusion proteins were either left unstimulated or activated through the TCR with plate-bound anti-CD3. Using the m.w. shift of paxillin as an indicator of serine/threonine phosphorylation, immunoblots demonstrated that, although EGFP-paxillin was phosphorylated in response to anti-CD3 (Fig. 7 A, lane 2), the S83A mutation partly reduced the m.w. shift (lane 4). When S130 was mutated in tandem with S83, paxillin phosphorylation was almost completely prevented (lane 6). The m.w. shift of endogenous paxillin was not affected by any of the expressed constructs.

Knowing that the S83A and S130A mutants are poorly phosphorylated by ERK enabled us to examine the requirement for serine phosphorylation in paxillin subcellular localization. CTL transfected with either EGFP-S83A or EGFP-S83A/S130A were conjugated to targets and imaged for EGFP and α-tubulin (Fig. 7 B). Both fusion proteins displayed normal recruitment to the MTOC and SMAC, suggesting that serine/threonine phosphorylation events initiated by ERK are not necessary to localize pax-
Serine phosphorylation of paxillin by ERK does not affect localization, and ERK is not required for MTOC reorientation in CTL. A. CTL were transfected with either pEGFP-paxillin (lanes 1 and 2) or the serine mutants pEGFP-S83A (lanes 3 and 4) and pEGFP-S83A/S130A (lanes 5 and 6). CTL were either activated with plate-bound anti-CD3 or left unstimulated, and whole-cell lysates were sequentially immunoblotted for GFP (top panel) and paxillin (bottom panel). B. CTL were transfected, as in A, conjugated with L1210 K\(^2/D^1\) target cells (labeled with CellTracker Blue), and stained for EGFP (detected with anti-mouse Alexa Fluor 488) and \(\alpha\)-tubulin (detected with anti-rabbit Alexa Fluor 594). The images represent a single confocal plane. C. CTL were pretreated with either 10 \(\mu\)M of the MEK inhibitor U0126 or an equivalent amount of DMSO carrier control and then conjugated with L1210 K\(^2/D^1\) target cells for 10 or 20 min and stained for paxillin and \(\alpha\)-tubulin, as in B. Slides were randomized before imaging and then conjugates were scored for MTOC reorientation as described in the Materials and Methods. The data represent five independent repeats, with 50–150 conjugates scored per experiment. 

**FIGURE 7.** Serine phosphorylation of paxillin by ERK does not affect localization, and ERK is not required for MTOC reorientation in CTL. A. CTL were transfected with either pEGFP-paxillin (lanes 1 and 2) or the serine mutants pEGFP-S83A (lanes 3 and 4) and pEGFP-S83A/S130A (lanes 5 and 6). CTL were either activated with plate-bound anti-CD3 or left unstimulated, and whole-cell lysates were sequentially immunoblotted for EGFP (top panel) and paxillin (bottom panel). B. CTL were transfected, as in A, conjugated with L1210 K\(^2/D^1\) target cells (labeled with CellTracker Blue), and stained for EGFP (detected with anti-mouse Alexa Fluor 488) and \(\alpha\)-tubulin (detected with anti-rabbit Alexa Fluor 594). The images represent a single confocal plane. C. CTL were pretreated with either 10 \(\mu\)M of the MEK inhibitor U0126 or an equivalent amount of DMSO carrier control and then conjugated with L1210 K\(^2/D^1\) target cells for 10 or 20 min and stained for paxillin and \(\alpha\)-tubulin, as in B. Slides were randomized before imaging and then conjugates were scored for MTOC reorientation as described in the Materials and Methods. The data represent five independent repeats, with 50–150 conjugates scored per experiment. 

**Discussion**

We showed that paxillin in CTL is localized to the MTOC and the pSMAC and that this recruitment is effected by the LD domains. In particular, a region encompassing LD2–4 was sufficient for paxillin subcellular localization. This segment, when fused with DsRed and expressed in CTL, partly impaired MTOC reorientation in response to target cells. MTOC translocation was particularly sensitive to the LD2–4 construct at lower Ag concentrations (Fig. 8D). This result suggested that this construct has the potential to act as a dominant negative mutant that interferes with endogenous paxillin function, and it indicates that paxillin contributes to MTOC movement. This possibility has been raised before, in a publication by Li et al. (10), in which they reported concluded that MEK-dependent ERK activation is not absolutely required for targeting of the MTOC in the CTL clones analyzed.

**Expression of a paxillin segment containing LD2, LD3, and LD4 impairs MTOC reorientation**

The fact that EGFP-LD2–4 localizes normally but has limited protein-binding domains suggested that it might function as a dominant negative mutant to inhibit endogenous paxillin function. The function of paxillin in CTL is unknown, but the presence of paxillin at the microtubule cytoskeleton and the pSMAC, given that the MTOC anchors to the pSMAC (28), raises the possibility of the involvement of paxillin in MTOC reorientation. We found that expression of GFP alone can reduce or delay MTOC reorientation in CTL clones. A comparison of Clone 11 left untransfected and transfected with pEGFP (empty vector) showed that EGFP alone significantly reduced MTOC translocation, from 80% (after a 10-minute incubation with targets) to 44%. The reason for this is unknown; however, the nucleofection process alone does not influence MTOC reorientation. We found that monomeric DsRed did not impact MTOC reorientation, so this was used as a tag for our functional studies.

To evaluate the dominant negative potential of the LD2–4 segment, we transfected Clone 11 CTL with either empty pDsRed (monomer) plasmid or pDsRed-LD2–4 and then mixed them with target cells and stained for DsRed and \(\alpha\)-tubulin (Fig. 8A). Conjugates positively stained for DsRed were evaluated for MTOC polarization and compared with untransfected CTL conjugates on the same slide (Fig. 8B). MTOC reorientation was observed in 83% of untransfected CTL and 81% of pDsRed-transfected CTL but in only 63% of CTL expressing pDsRed-LD2–4 (a reduction of 22% of the pDsRed vector, \(p = 0.0038\)). This outcome has two implications: first, that LD2–4 may indeed interfere with endogenous paxillin and, second, that paxillin may function in the translocation of the MTOC. It is worth noting that the LD2–4 segment is not significantly overexpressed at the population level (Fig. 8C), which may limit its potency as a dominant negative mutant. Also, the level of surface allogeneic MHC on the target cells is high and provides an unusually strong stimulus that may reduce the sensitivity of the assay. Therefore, we repeated the experiment using a CTL clone (Clone 3/4), which recognizes influenza NP366–374 peptide in the context of H-2\(^b\), allowing us to titrate the amount of Ag expressed on the EL4 target cells (Fig. 8D). For this experiment, we present the data as percent inhibition, because the degree of MTOC reorientation varies with peptide concentration. At the lowest peptide concentration (100 nM), MTOC reorientation after expression of DsRed-LD2–4 was reduced almost by half (48% inhibition). As the peptide level was increased, the effect of DsRed-LD2–4 lessened, indicating that as the Ag signal becomes weakened, MTOC translocation becomes more sensitive and more dependent upon paxillin involvement.
that paxillin was required for NK cell killing. No further functional studies were done, but we can speculate that their results may have been due to a defect in MTOC polarization or subsequent granule targeting. Our findings are consistent with a recent study using human NK cells that found that paxillin contributed to LFA-1–stimulated granule polarization (11).

Our data demonstrated that paxillin exists in at least two distinct locations within the cell: the MTOC and the pSMAC. It is not clear whether the paxillin at the two locations is also distinguished by other factors, such as serine or tyrosine phosphorylation. We do know that neither serine phosphorylation at the two major sites (Fig. 7) or tyrosine phosphorylation (data not shown) is required for MTOC or pSMAC localization. Although paxillin resides constitutively at the MTOC and microtubules, it is inducibly recruited to the immune synapse. It is likely, then, that paxillin is mobilized to sites of adhesion from either the microtubule pool or the cytoplasm, and it may be that localization is sequential and that an event at one site allows recruitment to the other. Because MTOC and synapse localization were always seen coincidentally for all paxillin mutants tested, it appears that the same region of the protein is responsible for its recruitment to both sites, and it is possible that paxillin recruitment is being effected by the same binding partner. However, the flexibility of binding inherent in the LD regions makes that uncertain. It is difficult to speculate about the dynamics of paxillin recruitment, regulation, and cycling given that it is not known what protein-binding partners are involved in effecting localization.

Our results suggest a model in which paxillin LD domains cooperate to bind to target proteins and in which no one LD domain is essential. Deletion of the segment encompassing LD2–4 substantially reduced the recruitment of paxillin to the MTOC and SMAC and resulted in a greater distribution in the cytoplasm and nucleus (Fig. 4D). A similar binding pattern to that reported in this article was seen when the interaction between paxillin and the α_{4} integrin subunit was examined (29). All individual LD domains were able to bind α_{4} integrin, although in some cases at much reduced levels. A fragment containing LD3–4 was sufficient, yet not completely necessary, for the interaction, much as we found a segment containing LD2–4 to be sufficient, but not absolutely required, for paxillin localization to the MTOC and SMAC. The LD3–4 segment (Ala176–Asp275) was found to act as a dominant negative mutant for α_{4}-paxillin function (29), and the LD2–4 segment that we produced appears to behave in a similar way in CTL. In light of the findings relating to α_{4} integrin, it is perhaps not surprising that we were unable to fully disrupt paxillin localization by deleting combinations of LD domains.

A certain level of redundancy has also been observed in the binding of the LD to other target proteins: vinculin is able to bind to LD1, LD2, and LD4; actopaxin is able to bind to LD1 and LD4; and FAK and Pyk2 are able to bind to LD2 and LD4 (3). However, other paxillin-binding partners are very specific to one LD domain or another, indicating that the LDs are not fully interchangeable. The flexibility of the LD interactions may be advantageous in allowing the amalgamation of larger protein complexes when binding-partner proteins are competing for docking sites on paxillin. Our results suggested that although all LD domains may contribute to localization there is likely a preferred configuration. LD domains with an amino acid sequence more suited to bind the relevant target proteins will make a greater contribution to localization, and consequently their removal will disrupt paxillin recruitment to a greater degree. Analysis is complicated by the fact that more than one LD domain may interact with a target protein at once, such as in the case of Pyk2 and FAK (19, 20). A more detailed dissection of LD domain-binding preferences can be made once the protein(s) responsible for retaining paxillin at the microtubules and target interface are identified.

We previously showed that paxillin becomes associated with Lck, either directly or through the tyrosine kinase Pyk2, upon TCR stimulation (5). The association of paxillin with the MTOC likely does not occur through Lck, because this interaction is inducible, and we cannot detect Lck at the MTOC (data not shown). It is possible that Lck recruits paxillin to the SMAC; however, it would have to be in conjunction with LFA-1 or other integrins, because TCR stimulation alone does not support paxillin recruitment to the contact zone (Fig. 6). We have been unable to find a direct association between LFA-1 and paxillin, so more indirect associations of integrins with paxillin are likely required in CTL.
Paxillin is constitutively associated with Fyn (data not shown). Despite the fact that Fyn localizes to the microtubules in T cells (30), we ruled out the possibility that Fyn is required to recruit paxillin to the microtubules. In Fyn-deficient CTL, EGFP-paxillin displayed a normal localization pattern (data not shown). Finally, we showed that paxillin constitutively interacts with the tyrosine kinase Pyk2 in CTL (5). This interaction was shown to occur through LD2/LD4 (2), and we confirmed that a paxillin mutant lacking both LD2 and LD4 has ∼80% reduction in association with Pyk2 in CTL (data not shown). We also recently showed that Pyk2 that is in association with paxillin preferentially localizes to the MTOC, suggesting that the two proteins are in a complex at the MTOC (31). Pyk2 is likely not associated with paxillin at the SMAC because we cannot detect strong colocalization of Pyk2 with paxillin using an Ab to Pyk2 that preferentially binds to Pyk2 bound to paxillin (31). Therefore, Pyk2 is a strong candidate for recruiting paxillin to the MTOC but not the SMAC. We are currently trying to identify additional proteins in CTL that might interact with the LD2–4 region of paxillin.

The immune synapse of CTL, with its adhesive and signaling functions, may seem analogous to focal adhesions, although in adhesive cells the LIM3 domain, not the LD region, is required for focal adhesion localization of paxillin (19). The mechanism of paxillin incorporation into focal adhesions has never been demonstrated, although paxillin may interact directly with some integrins, as it does with α4 and, possibly β1, integrins (32). Our data suggested that LFA-1 contributes to paxillin recruitment, along with the TCR complex (Fig. 6). However the contribution of LFA-1 is likely not exclusive because recruitment to the beads is never as strong as that seen with target cells. The α4 β1 integrin is recruited to the Ag-specific synapse between either human or mouse T cells and APC (33). Given that the α4 tail directly binds paxillin (34), this integrin would appear to be a strong candidate for mediating paxillin recruitment to the SMAC. However, we showed that CTL clones do not express α4 β1 integrin on the cell surface (35), a finding we confirmed using in vitro-activated CTL (data not shown), thereby making this integrin an unlikely candidate for recruitment of paxillin to the immune synapse in the cells used for this study.

It is not clear how integrins contribute to paxillin recruitment to the SMAC. Consistent with the inability of anti-CD3 alone to induce paxillin accumulation, expression of the LD2–4 construct had no effect on MTOC reorientation toward beads bearing anti-CD3 (data not shown). This would suggest that there are CD3-specific pathways for induction of MTOC reorientation independent of paxillin. That we saw stronger inhibition of MTOC reorientation with LD2–4 at lower peptide concentrations (Fig. 8) suggested that the contribution of paxillin to MTOC reorientation dominates when a greater dependence on the contribution of adhesion molecules is required.

ERK is required for degranulation for the CTL clones used for this study (25), a rapid process that is independent of transcription, and it would stand to reason then that ERK is likely involved through a cytoplasmic-phosphorylation target, rather than a nuclear target. Paxillin is a known cytoplasmic target of ERK, and we speculated that paxillin may form a link between ERK activity and the degranulation process in CTL. However, we showed that ERK is not required for MTOC reorientation, in apparent conflict with a previous report that a CTL clone that required ERK for killing also required ERK for MTOC reorientation (36). However, another clone used in this same study showed no inhibition of MTOC inhibition or killing in the presence of a MEK inhibitor (36), suggesting that there may not be an absolute requirement for ERK activation for MTOC reorientation and that there may be some heterogeneity in CTL with respect to the contribution of ERK to MTOC reorientation. Although paxillin is phosphorylated by ERK, paxillin phosphorylation, as assessed by the S83A/S130A mutant, is not required for its association at the MTOC or SMAC. These data suggested that, although paxillin contributes to MTOC reorientation, ERK is not absolutely required for this process and that the contribution of ERK to degranulation is likely not through the control of MTOC reorientation. Interestingly, paxillin was not required for degranulation of human NK cells when stimulated through the CD16 receptor, but it did contribute to granule polarization induced with an LFA-1 ligand (11), suggesting that paxillin is required for polarization of the killer cells during specific circumstances.

A greater mystery is presented by the association of paxillin with microtubules and how paxillin might influence microtubule dynamics. Regulation of the microtubule network and the MTOC are especially influential in CTL function, because the microtubules themselves are used as “guide rails” for cytolytic granules moving toward the target cell, as directed by the reoriented MTOC (37). Paxillin is likely recruiting other proteins important for microtubule regulation, but it is unclear what those might be. A recent publication defined a requirement for Fyn and Lck in the translocation and membrane docking of the MTOC, respectively, but it is not known which downstream targets of the kinases are relevant (38). Because Lck and Fyn are known to associate with paxillin, and phosphorylation of paxillin is dependent on Src-family kinase activity (5), our data suggested paxillin as a potential Lck or Fyn substrate that could contribute to MTOC reorientation downstream of this recently identified signaling cascade leading to MTOC reorientation.

It is not known whether the paxillin localized to the MTOC or to the pSMAC is relevant to MTOC reorientation. The association of paxillin with the MTOC is constitutive; however, because paxillin is phosphorylated in response to TCR stimulation, the inducible phosphorylation of paxillin at the MTOC could be important. We know that phosphorylation of paxillin is not required for its MTOC localization (Fig. 7); however, it is possible that phosphorylation is required for its function, a possibility that needs to be tested in paxillin-deficient T cells. It is also possible that the inducible association with the pSMAC contributes to MTOC reorientation or the anchoring of the MTOC in position, because the microtubules were shown to anchor at the pSMAC in CTL (28). Future studies will aim to fill in details relating to paxillin localization, as well as to build upon the functional implications of the data to answer larger questions about the role of paxillin in cytotoxic T cell function.

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References
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