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A Role for Phosphatidylinositol 3-Kinase in TCR-Stimulated ERK Activation Leading to Paxillin Phosphorylation and CTL Degranulation

Leslie K. Robertson, Laura R. Mireau, and Hanne L. Ostergaard

PI3K is an important regulator of a number of cellular processes. We examined the contribution of PI3K to mouse CTL signaling, leading to degranulation. We show that TCR-triggered, but not phorbol ester and calcium ionophore-induced, CTL degranulation is dependent on PI3K activity. Although PI3K activity is required for optimal LFA-1-mediated adhesion and cell spreading, this most likely does not account for its full contribution to degranulation. We demonstrate that PI3K is required for TCR-stimulated ERK activation in CTL, which we have shown previously to be required for CTL degranulation. We thus define a pathway through which PI3K most likely regulates degranulation and in which ERK appears to be a key signaling molecule. Furthermore, we identified the cytoskeletal adaptor paxillin as a target of ERK downstream of TCR stimulation. Consistent with a role in degranulation, we demonstrate that paxillin is localized to the microtubule organizing center in resting cells and upon target cell binding is recruited to the contact point with the target cell. These studies demonstrate that PI3K regulates ERK activity leading to CTL degranulation, and identify paxillin as a target of ERK downstream of the TCR. That paxillin is independently phosphorylated by both tyrosine kinase(s) and ERK downstream of the TCR and localized both at the microtubule organizing center and at the target cell contact point suggests an important role for paxillin in CTL-mediated killing. The Journal of Immunology, 2005, 175: 8138–8145.

When CTL encounter a target cell bearing the appropriate Ag recognized by the clonotypic TCR, they exocytose specialized lysosomally derived granules containing cytolytic molecules, including perforin and various granzymes, that lead to death of the target cell (see Refs. 1 and 2 for review). The signals that regulate the process of CTL degranulation have not been fully established. Triggering CTL through the TCR leads to initiation of many of the same signaling pathways that have been studied in other T cells. However, because CTL degranulation does not require new gene transcription and can occur within 30–60 min of stimulation, the downstream targets of these signaling pathways are not transcriptional regulators, but rather proteins that lead to microtubule organizing center (MTOC) reorientation, movement of the cytolytic granules to the contact point with the target cell, and fusion of the granules with the plasma membrane.

PI3K is a lipid kinase that phosphorylates the 3-hydroxy position of the inositol ring of various phosphorylated forms of phosphatidylinositol (3). PI3K is not a single enzyme, but is a family of proteins that are categorized as class I, II, or III (reviewed in Ref. 4). The class Ia PI3K are heterodimers consisting of regulatory and catalytic subunits and are thought to be important downstream of the TCR (5). The phosphorylated phosphatidylinositol products of PI3K serve as important lipid signaling mediators by directly binding to certain plekstrin homology (PH) domains of many proteins, thus recruiting them to membranes or regulating their activity (4, 5). There are numerous PH domain-containing proteins, but some that are relevant in T cells include the cytoskeletal regulator Vav, the Tec family tyrosine kinases, phospholipase C-γ, and the serine/threonine kinase AKT (also known as protein kinase B) (4, 5). PI3K can regulate a number of pathways downstream of the TCR, including the expression of genes required for cell survival and IL-2 expression (4, 5). PI3K has been shown to be important for degranulation, but not for Fas-mediated cytolytic pathways in CTL (6). It is not yet known which targets downstream of PI3K are important for CTL degranulation. PI3K has been shown to regulate ERK activity in T cells (7–9), although the mechanism of this regulation is not resolved. We have shown that ERK activation is required for CTL degranulation, suggesting that there are targets of ERK phosphorylation that are essential for this process (10). In NK cells, PI3K has been shown to regulate the ERK pathway, which is required for killing and granule reorientation triggered through an unknown NK cell receptor (11).

Paxillin is a complex cytoskeletal adaptor protein (12, 13) that is phosphorylated in an ERK-dependent fashion after PMA treatment of the EL4 thymoma (14). Paxillin knockout mice are embryonic lethal; however, cells from these embryos display reduced or delayed cell spreading on extracellular matrix (15, 16). We have shown that paxillin associates with the tyrosine kinase Pyk2 in T cells and becomes tyrosine phosphorylated upon TCR stimulation (17). Although paxillin plays an important role in cell adhesion and motility in adherent cells (15, 16), it is not clear what role paxillin plays in T cells.
In the current study, we sought to determine the role that PI3K plays in CTL activation leading to degranulation. Our studies indicate that PI3K is not required for the process of degranulation per se, but rather for signaling leading to degranulation. We show that regulation of ERK activation is a means by which PI3K regulates CTL degranulation. Furthermore, we identify paxillin as a target of the PI3K to ERK pathway downstream of the TCR in CTL. Finally, we demonstrate that paxillin localizes to the MTOC and the contact point with the target cell, supporting an important role for this molecule in CTL degranulation.

Materials and Methods

Cells

The mouse Ag and IL-2-dependent CTL clone AB.1 (H-2 d anti-H-2 b), which has been described previously (18), was maintained by weekly stimulation with irradiated C57BL/6 spleen cells in medium supplemented with IL-2. All experiments were performed 4–6 days after stimulation. All animal studies have been approved by the University Animal Policy and Welfare Committee at the University of Alberta. The L1210 lymphoma cell line expressing chimeric class I MHC was a generous gift from K. Kane (University of Alberta, Edmonton, Canada). Briefly, L1210 cells were transfected with class I containing the D d (19). L1210 cells and the transfectant were grown in DMEM supplemented with 8% heat-inactivated defined calf serum.

Abs and reagents

mAbs 145-2C11 (anti-CD3), M17/5.2 (anti-LFA-1), and PY72 were purified, as previously described (10). The mAbs for paxillin and Vav were purchased from BD Transduction Laboratories and from Upstate Biotechnology, respectively. Polyclonal Abs specific for AKT and for phospho-AKT (Ser473) were obtained from Cell Signaling Technology. HRP-coupled goat anti-mouse and rhodamine anti-mouse Abs were purchased from Jackson Immunologics, and protein A from Pierce. Anti-tubulin was purchased from Abcam, and anti-rabbit Alexa 488 from Molecular Probes. Ionomycin was acquired from Calbiochem, and PMA from Sigma-Aldrich. Wortmannin, an irreversible PI3K inhibitor, and LY-294002, a reversible PI3K inhibitor, were purchased from Calbiochem.

Ab immobilization and CTL stimulation

For Ab immobilization, wells of 96-well Falcon 3912 plates (BD Biosciences) were incubated with 750 ng of 145-2C11 in PBS overnight at 4°C. Each well was then washed three times with PBS and blocked with 2% BSA in PBS at 37°C for 45 min. Wells were washed three times with PBS and immediately used for assay. For experiments requiring immunoprecipitation, 5-cm suspension plastic dishes were coated with 50 μg of 145-2C11 in 5 ml of PBS overnight at 4°C and blocked as for 96-well plates. CTL clones were pretreated with various concentrations of wortmannin for 30 min at 37°C. Depending on the experiment, the drug was either washed out or left in during stimulation.

Degranulation assay

Degranulation, as measured by the release of serine esterase, was assayed, as previously described (10). AB.1 cells were washed three times with centrifugation in PBS and added at a 1:5 × 10^7 cells/well directly to wells of 96-well plates previously coated with 145-2C11 (anti-CD3 Ab) or mixed at a 1:1 ratio with target cells in a total volume of 150 μl of 2% newborn calf serum in RPMI 1640. In some cases, CTL were stimulated with 150 ng/ml PMA and/or 2 μM ionomycin. Plates were then incubated at 37°C for 4–5 h, after which 25 μl of supernatant was assayed for benzoyloxycarbonyl-L-lysine thiobenzyl ester-esterase activity (20). All points were obtained in triplicate, and the SD was calculated and shown as error bars. The OD of reactions from supernatants harvested from untreated cells were determined and subtracted from all experimental measurements, and therefore represented as ΔOD. All experiments were completed at least three times with identical results.

SDS-PAGE, immunoblotting, and immunoprecipitation

For stimulation of CTL with anti-CD3, 2 × 10^5 CTL in PBS (Invitrogen Life Technologies) were directly added to Ab-coated wells. After incubation at 37°C for the indicated time, the cells were lysed by addition of 2× Laemmli reducing sample buffer and the lysates were separated on 8.5% SDS-PAGE gels. Immunoblotting was performed using protein-specific primary Abs, followed with rabbit anti-mouse Ab or protein A coupled to HRP. Blots were developed by ECL.

For stimulation of CTL signals with target cells, CTL and target cells were washed as above and resuspended in PBS. CTL were then either pretreated with inhibitor or left untreated at 37°C for 30 min, followed by washing before addition to the assay. A total of 10^7 AB.1 was mixed with 2 × 10^5 target cells on ice and centrifuged for 4 min at 100 × g to optimize conjugation. Conjugates were incubated at 37°C for the indicated time, after which cells were lysed in 1% Nonidet P-40 lysis buffer. Post-nuclear lysates were combined with 2× Laemmli reducing sample buffer, boiled, subjected to SDS-PAGE, and blotted as above.

For large-scale stimulation of T cells required for immunoprecipitations, 10^7 AB.1 were added to 145-2C11-coated or control 5-cm dishes. After incubation at 37°C for 20 min, the cells were lysed on ice with 1% Nonidet P-40, 20 mM Tris (pH 7.4), 150 mM NaCl, 0.1 M m sodium vanadate. After clarification of the lysates, paxillin was recovered by immunoprecipitation after addition of anti-paxillin, followed by rabbit secondary Ab and protein A coupled to Sepharose 4B. The immunoprecipitates were extensively washed and subjected to reducing SDS-PAGE. All experiments were performed a minimum of three times with similar results.

Conjugate assay

CTL and target cells were washed with PBS and stained with green PKH67 and red PKH26 dyes, respectively (Sigma-Aldrich). The cells were then washed three times with 5% calf serum in PBS, and incubated at 37°C for a 1- to 2-h rest period to prevent dye transfer during the assay. During the last 30 min of the rest period, CTL were treated as indicated with 50 nM wortmannin, followed by one wash in 5% calf serum in PBS. AB.1 and target cells were resuspended at 1 × 10^6 cells/ml and 2 × 10^6 cells/ml, respectively, in 5% calf serum in PBS. Equal volumes of AB.1 and targets were mixed at 4°C; however, in some cases, 10 μg/ml M17/5.2 (anti-LFA-1) was added to the CTL 20 min before mixing. The cell mixtures were centrifuged at 100 × g for 3 min at 4°C. At time zero, cell mixtures were placed in a 37°C water bath and incubated for the indicated time, followed by gentle vortexing and fixation in 4% formaldehyde. Conjugate formation was measured by FACScan analysis, and conjugates were defined as pairs of cells fluorescing both red and green. The percentage of conjugation is the number of AB.1 found in conjugates compared with the total number of AB.1 in the assay. All conjugate experiments were performed a minimum of four times with identical results.

Immunofluorescence microscopy

L1210 K/Dd target cells were stained with 7 μM CMAC Cell Tracker Blue (Molecular Probes) for 20 min, washed, incubated for 30 additional min, and mixed with AB.1 CTL clones at an E:T ratio of 2:1. To encourage conjugation, the cells were centrifuged for 1 min at 100 × g and incubated at 37°C for 5 min. Approximately 9 × 10^5 total cells were then adhered to a poly(t-lysine) coverslip for 10 min at room temperature. The conjugates were fixed with 4% formaldehyde for 10 min and permeabilized with 0.2% Nonidet P-40 for 5 min. Staining was then performed with 9 μg/ml anti-paxillin, followed by 13 μg/ml anti-mouse rhodamine and 1 μg/ml anti-tubulin, followed by 10 μg/ml anti-rabbit Alexa 488. Primary Abs were incubated for 1 h, and secondary Abs for 45 min at room temperature. All Abs were diluted in PBS with 2% FCS. Coverslips were then adhered to slides and visualized by a Zeiss LSM 510 confocal microscope with a ×40 oil-immersion objective (N.A. 1.3). Alexa 488 and rhodamine were excited using 488-nm argon and 543-nm HeNe lasers, respectively. Targets and CTL were distinguished by visualizing CMAC Cell Tracker with a 535-nm UV laser.

Results

PI3K activity is required for CTL degranulation

It has been reported that PI3K activity is required for degranulation-dependent effector mechanisms by CTL (6). We first wanted to confirm that this is the case in our CTL clones as well as to determine the optimal wortmannin concentrations for inhibition. The CTL clone AB.1 was pretreated with various concentrations of wortmannin to irreversibly inhibit PI3K activity and then stimulated with plate-bound anti-CD3 Ab, or with Ag-bearing target cells (L1210-K/Dd). For experiments involving target cells, excess wortmannin was washed away from CTL before assay to minimize potential effects on the target cells. After 4.5 h, the supernatant was assayed for serine esterase activity as a measure of...
degranulation. CTL degranulation stimulated by either anti-CD3 or Ag-bearing target cells was inhibited in a concentration-dependent manner by wortmannin (Fig. 1A). To ensure that we were indeed inhibiting PI3K activity, we indirectly measured its activity by determining the phosphorylation of the Ser/Thr kinase AKT, also referred to as protein kinase B (21, 22). Inhibition of AKT phosphorylation was observed at concentrations of drug similar to those required for inhibition of degranulation (Fig. 1B). Although there was complete blocking of AKT phosphorylation at 10 nM (Fig. 1A), there was only partial inhibition of degranulation at this concentration (Fig. 1A). The differences at the 10 nM concentration of wortmannin could be because AKT phosphorylation was measured 20 min after stimulation, whereas the degranulation was measured 4.5 h after stimulation in the presence of serum, and the effects may not be as complete at this concentration over the entire assay period. Similar degranulation results were observed with the reversible, but highly specific, PI3K inhibitor LY-294002 (Fig. 1C). Taken together, these results confirm the previous report that PI3K plays a critical role in CTL degranulation (6).

PI3K appears to be involved in many cellular processes in many different cell lines. It has been implicated in the translocation of glucose transporters to the cell surface in response to insulin (23, 24), suggesting that vesicle fusion can be regulated by this enzyme. It is therefore conceivable that PI3K is required for either signaling leading to CTL degranulation or the physical process of granule exocytosis. To distinguish these possibilities, we examined the effects of wortmannin on CTL stimulated to degranulate by the phorbol ester PMA and the calcium ionophore ionomycin, thus bypassing membrane-proximal signaling events. Degranulation induced by the combination of PMA and ionomycin was completely resistant to the effects of wortmannin, in contrast to degranulation stimulated with anti-CD3 (Fig. 2). This suggests that PI3K is not required for the process of membrane fusion for degranulation per se, but is most likely required for the signaling events leading to degranulation by CTL, or perhaps for the directional degranulation triggered with target cells and plate-bound anti-CD3.

**Wortmannin attenuates the kinetics of conjugation between CTL and target cells**

O’Rourke et al. (25) have previously shown that wortmannin can significantly decrease TCR-triggered binding of CD4+ T cells to purified ICAM-1. It is possible that PI3K could block adhesion of the cells, thereby preventing efficient stimulation through the TCR and inhibiting downstream degranulation. To examine this possibility, we compared the degree of conjugation between the AB.1 CTL clone and L1210-Kb/Dd target cells in the presence and absence of wortmannin. We found that 50 nM wortmannin decreased the rate of conjugate formation (Fig. 3). However, the maximum percentage of conjugation was reduced by only 25–30% at 30 min. We are able to completely block Ag-specific conjugation with anti-LFA-1 in this system, suggesting that wortmannin only partially inhibits TCR-triggered LFA-1-mediated adhesion by CTL (Fig. 3), as was observed with CD4+ T cells adhering to ICAM-1 (25).

These data suggest that although wortmannin does not completely inhibit conjugate formation, PI3K is required for optimal conjugation, particularly at the earlier time points. Of note, we also observed delayed, but not inhibited, cell spreading of the CTL added to solid-phase anti-CD3 in the presence of PI3K inhibitors.

**FIGURE 1.** PI3K activity is required for optimal CTL degranulation. A, CTL clone AB.1 cells were preincubated with various concentrations of wortmannin for 30 min or left untreated. For those cells that had been treated with wortmannin, the drug was either present throughout the assay (145-2C11 stimulated) or washed out before assay (target cell stimulated). CTL were then stimulated with immobilized 145-2C11 or target cells bearing the relevant MHC (L1210-Kb/Dd) and assayed for serine esterase release. B, AB.1 CTL clones were pretreated with wortmannin for 30 min at the indicated concentration, or left untreated. CTL were stimulated with immobilized 145-2C11 for 20 min after lysis and probed with anti-phospho-AKT (p-AKT, top panel) by immunoblotting. The blot was re-probed with anti-AKT (bottom panel). C, AB.1 CTL clones were pretreated with various concentrations of LY-294002 for 30 min or left untreated. The inhibitor was not washed out before assay, and CTL were then stimulated to degranulate with plate-bound 145-2C11, as in A. Error bars indicate SD. Note that in A, the error bars are smaller than the symbols.

**FIGURE 2.** PI3K is not required for PMA and ionomycin-stimulated CTL degranulation. AB.1 CTL clones were preincubated with varying concentrations of wortmannin for 30 min, followed by stimulation with either plate-bound 145-2C11, 150 ng/ml PMA, 2 μM ionomycin, a combination of PMA and ionomycin, or no stimulus. After 4.5 h, supernatants were assayed for serine esterase activity. Error bars indicating SD are smaller than the symbols.
This is representative of four different experiments.

PI3K inhibition does not significantly impact induction of tyrosine phosphorylation

It is possible that the impact on the kinetics of conjugation could have a profound impact on the induction of degrauination by reducing the ability of cells to signal appropriately because of the lack of early contact between CTL and the target cell or between CTL and Abs on the plate. We therefore determined whether inhibition of PI3K activity impacted the induction of tyrosine phosphorylation at 20 min after stimulation, as this is when we detect peak tyrosine phosphorylation after stimulation with plate-bound Ab (10). In general, wortmannin had no discernible impact on the overall tyrosine phosphorylation pattern observed after T cell activation stimulated with solid-phase anti-CD3 (Fig. 4, top panel). This result suggests that although the cells have not spread on the Ab (data not shown), they are still making sufficient contact for optimal tyrosine phosphorylation. Furthermore, identical kinetics of tyrosine phosphorylation were also observed in the presence and absence of PI3K inhibitors (data not shown), suggesting that the delayed cell spreading did not impact the kinetics of tyrosine phosphorylation. There was also no inhibition of overall tyrosine phosphorylation induced with target cells in the presence of wortmannin; however, this is more difficult to detect because of the high levels of background tyrosine phosphorylation (data not shown).

PI3K regulates paxillin phosphorylation during CTL activation

Although we detected no major impact of wortmannin on the overall tyrosine phosphorylation, there was a reduction in the tyrosine phosphorylation of proteins between 58 and 84 kDa with increasing concentrations of wortmannin (Fig. 4, top panel). From our previous studies on paxillin, we recognized that this is the region of the gel in which phosphorylated paxillin migrates (17). We therefore probed this blot with Ab specific for paxillin and found that the anti-CD3-induced shift of paxillin from a faster to a slower migrating species is inhibited with wortmannin treatment (Fig. 4, middle panel). Blots were probed with Abs to Vav as a loading control (Fig. 4, bottom panel). The migratory shift of paxillin is indeed due to phosphorylation, as alkaline phosphatase treatment compresses paxillin to a single 58-kDa protein band (data not shown).

Paxillin can be phosphorylated on multiple tyrosine and serine residues (12). The altered detection of paxillin on the anti-phosphotyrosine blot could be due either to decreased tyrosine phosphorylation or decreased migration of paxillin due to decreased serine phosphorylation with no change in tyrosine phosphorylation. To distinguish these possibilities, we examined paxillin phosphorylation after immunoprecipitation from CTL stimulated with solid-phase anti-CD3 Ab in the presence or absence of wortmannin. Paxillin was highly tyrosine phosphorylated upon anti-CD3 stimulation, and this tyrosine phosphorylation was not detectably inhibited with wortmannin, even though the migration was substantially impaired (Fig. 5A). This implies that the PI3K regulates serine, but not tyrosine, phosphorylation of paxillin downstream of TCR stimulation. Identical results were observed using the LY-294002 inhibitor (data not shown).

It is possible that wortmannin either inhibits the initiation of paxillin serine phosphorylation or perhaps allows for the more rapid down-regulation of paxillin phosphorylation after stimulation with anti-CD3. The kinetics of paxillin phosphorylation in the presence and absence of 50 nM wortmannin (Fig. 5B) suggested that wortmannin inhibits the initial phosphorylation of paxillin. There was still a small migratory shift of paxillin in the presence of wortmannin; however, the majority of paxillin remained in the most rapidly migrating form. PI3K-dependent phosphorylation of paxillin was also observed after stimulation of ex vivo CD8+ T cells with immobilized anti-phosphotyrosine Abs (18). We therefore determined whether inhibition of PI3K activity impacted the induction of tyrosine phosphorylation of proteins between 58 and 84 kDa with increasing concentrations of wortmannin (Fig. 4, top panel). From our previous studies on paxillin, we recognized that this is the region of the gel in which phosphorylated paxillin migrates (17). We therefore probed this blot with Ab specific for paxillin and found that the anti-CD3-induced shift of paxillin from a faster to a slower migrating species is inhibited with wortmannin treatment (Fig. 4, middle panel). Blots were probed with Abs to Vav as a loading control (Fig. 4, bottom panel). The migratory shift of paxillin is indeed due to phosphorylation, as alkaline phosphatase treatment compresses paxillin to a single 58-kDa protein band (data not shown).

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PI3K functions upstream of ERK for CTL degranulation

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PI3K-dependent phosphorylation of paxillin is mediated through ERK

It has been demonstrated in the EL4 T lymphoma that PMA-activated ERK phosphorylates paxillin (14) and in epithelial cells that hepatocyte growth factor-induced paxillin phosphorylation is ERK dependent (26). These studies further showed that paxillin is phosphorylated by ERK in in vitro kinase assays (14, 26). Furthermore, inhibition of PI3K activity has been shown to diminish TCR-stimulated paxillin phosphorylation in mouse lymph node T cells (8) and human peripheral T cells (9). We therefore determined whether PI3K activity is required for TCR-stimulated ERK activation in the mouse CTL clones used in this study. Cells were stimulated with plate-bound anti-CD3 in the presence or absence of 50 or 100 nM wortmannin, and the activation of ERK was indirectly assessed by probing with an Ab recognizing the phosphorylated and activated form of ERK. At both concentrations, wortmannin substantially blocked both isoforms of ERK (ERK1 and ERK2) activation (Fig. 6A).

Similar results were seen using LY-294002 to inhibit PI3K (data not shown). It is unlikely that wortmannin or LY-294002 nonspecifically blocks ERK activation, as PMA-stimulated ERK activation in these cells was not inhibited by either PI3K inhibitor, but even seems to be enhanced (Fig. 6B). This provides one possible explanation for why PI3K inhibition has no impact on PMA-stimulated CTL degranulation (Fig. 2). Taken together, these experiments suggest that TCR-stimulated ERK activation in CTL is largely PI3K dependent.

Because ERK activation is substantially dependent on PI3K activity in these CTL clones, we determined whether ERK activity accounts for all paxillin phosphorylation. Stimulation of AB.1 in the presence of the MEK inhibitor U0126 inhibits all detectable ERK phosphorylation, as expected, but has no significant impact on overall tyrosine phosphorylation levels, with the exception of paxillin and ERK phosphorylation, confirming that the inhibitor is not globally inhibiting signaling in these cells (Fig. 7A). As predicted, if paxillin is indeed a substrate of ERK, TCR-stimulated paxillin phosphorylation, as measured by the migratory shift, was completely inhibited in the presence of the MEK inhibitor (Fig. 7A). As published previously (14), the AB.1 CTL clone also exhibited PMA-inducible paxillin phosphorylation; however, this was not PI3K dependent (Fig. 7B). These results demonstrate that anti-CD3-stimulated paxillin serine phosphorylation in CTL is entirely MEK/ERK dependent. Inhibition of MEK/ERK activity has no significant impact on the tyrosine phosphorylation of paxillin downstream of the TCR (Fig. 7C), suggesting that tyrosine phosphorylation of paxillin is not dependent on the serine phosphorylation, as would be predicted from the PI3K inhibition of phosphorylation (Fig. 5).
Paxillin localizes to the MTOC and the contact point in which a CTL binds to its target cell

Paxillin has been shown to localize to focal adhesions in adherent cells; however, T cells do not have typical focal adhesions. To gain insight into the potential role of paxillin in CTL degranulation, we have performed immunocolocalization studies by confocal microscopy. In unstimulated T cells, the paxillin is localized, at least in part, to the MTOC (Fig. 8A), as previously reported (27). However, when a CTL binds to its cognate target cell, in addition to remaining associated with the MTOC that has reoriented in the direction of the target cell, the paxillin also becomes highly localized to the contact point with the target cell (Fig. 8B). In all cases in which we detect MTOC reorientation, as a measure of tight conjugation and specific recognition of the target cell, we detect significant staining of paxillin at the interface with the target cell.

We do not think that paxillin serine phosphorylation is required for the MTOC reorientation because wortmannin has only a limited impact on the initiation MTOC reorientation. At 20 min after the initiation of conjugation, 84% of the CTL conjugates had their MTOC reoriented to the target cell. In the presence of wortmannin, although we detect only about one-half of the number of conjugates, of those CTL in conjugates 72% had their MTOC reoriented to the target cell. These data suggest that the PI3K to ERK pathway is not required to signal MTOC reorientation, but is most likely important for a step downstream of this event.

Discussion

We have clearly demonstrated that PI3K is important for CTL degranulation, consistent with previous findings showing a role for PI3K in degranulation, but not Fas ligand-mediated killing (6). A number of studies have implicated PI3K in the activation of LFA-1-mediated adhesion (25, 28–31), perhaps through the action of the PH domain-containing protein cytohesin-1 (28, 30). The adhesion between the CTL clones and target cells that we use for our studies is mediated primarily by LFA-1 (Fig. 3), implying that PI3K does play a role in regulating the activation of LFA-1 adhesion. However, we find that PI3K is important for optimal kinetics of conjugation between the CTL and its target cell, suggesting that PI3K regulates TCR-triggered adhesive events, but is not obligatory.

It is unlikely that the delayed LFA-1-mediated adhesion that we observe in the presence of PI3K inhibitors is significant enough to impact downstream degranulation, for a number of reasons. First, although adhesion is delayed in the presence of PI3K inhibitors, it is only minimally impacted at 30 min and we would expect to detect substantial degranulation over a 4-h assay. Second, we observe inhibition of degranulation stimulated by anti-CD3, which is not dependent on LFA-1 adhesion. Third, we do not detect any significant impact of PI3K inhibition on the ability of anti-CD3 (Fig. 4) or target cells (data not shown) to induce tyrosine phosphorylation, which would be predicted to be blocked by substantially impaired adhesion. Because degranulation triggered with PMA and ionomycin is not inhibited by wortmannin (Fig. 2), we conclude that PI3K is not required for degranulation per se, but is most likely required for a signaling event leading to CTL degranulation. These results have led us to speculate that there is a signaling event downstream of the TCR and adhesion that requires PI3K to trigger degranulation.

In the present study, we have shown that PI3K activity is important for TCR-induced activation of ERK, as inhibition of PI3K activity substantially inhibited both the duration and degree of ERK phosphorylation (Fig. 6). We have previously shown that ERK activity is required for CTL degranulation (10), suggesting that an ERK substrate(s) mediates critical steps in degranulation. In this study, we have identified paxillin as a downstream target of PI3K that we think might be the ERK substrate required for degranulation (10), suggesting that an ERK substrate(s) mediates critical steps in degranulation. In this study, we have identified paxillin as a downstream target of PI3K that we think might be the ERK substrate required for degranulation. In this study, we have identified paxillin as a downstream target of PI3K that we think might be the ERK substrate required for degranulation.
that MEK activity was regulated by PI3K, suggesting that PI3K is acting on the Ras to ERK pathway (8). Stimulation of degranulation of NK cells through an unknown receptor appears to occur through a pathway from PI3K to Rac1 to p21-activated kinase 1, which phosphorylates MEK and regulates ERK (11). We do not think this pathway is operational in these CTL clones, as we saw no impact on Rac1 activation by TCR stimulation in the presence of wortmannin (data not shown). We have previously shown that Erk activation stimulated with solid-phase anti-CD3 is entirely dependent on diacylglycerol binding, and that the late phase of ERK activation requires protein kinase C (PKC) activity (32). Interestingly, it has very recently been suggested that PKCθ can regulate the diacylglycerol-binding Ras exchange factor RasGRP1 in T cells upstream of the Ras to ERK pathway (33). Furthermore, we recently demonstrated that there is a PI3K requirement for PKCθ activation in CTL (34). Taken together, we favor a pathway whereby PI3K regulates PKCθ, which in turn regulates the Ras-GRP and the Ras to ERK pathway in T cells.

We have previously shown that ERK activity is required for CTL degranulation (10). Because CTL degranulation occurs rapidly and does not require any transcriptional activity, we have been interested in identifying downstream targets of ERK in CTL that regulate nontranscriptional targets leading to degranulation. In this study, we show that paxillin is a downstream direct or indirect target of MEK/ERK in CTL. Direct phosphorylation of paxillin by ERK has been demonstrated in PMA-stimulated EL4 thymoma cells and hepatocyte growth factor-treated epithelial cells (14, 26), and we have extended this by demonstrating that phosphorylation of paxillin by ERK occurs downstream of the TCR. It is possible that ERK mediates all of the serine phosphorylation of paxillin; however, we find this possibility unlikely, particularly because paxillin has also been suggested to be phosphorylated downstream of PKC (35) and p38MAPK (36, 37). We prefer a model whereby ERK phosphorylation of a specific site(s) is obligatory and this phosphorylation in turn permits the phosphorylation of additional serine residues by other kinases. The initial phosphorylation of paxillin by ERK could perhaps regulate accessibility of additional serine residues or allow for recruitment or localization with other kinases that mediate the phosphorylation of other sites.

Our studies indicate that, in the absence of ERK activity, there is still tyrosine phosphorylation of paxillin. It has been suggested that tyrosine phosphorylation of paxillin after stimulation of cells with hepatocyte growth factor results in an association with inactive ERK (38). This complex also contains the upstream ERK regulators Raf and MEK, and activation of this complex results in localized phosphorylation of paxillin after hepatocyte growth factor stimulation of epithelial cells (38). Although we do observe TCR-stimulated ERK phosphorylation of paxillin, we have to date been unable to detect an association between ERK and paxillin by coimmunoprecipitation (data not shown). Our previous study on paxillin clearly showed that it is phosphorylated on tyrosine in a src family kinase-dependent pathway downstream of the TCR (17). We therefore propose that src family-dependent tyrosine phosphorylation of paxillin downstream of the TCR localizes the paxillin, where it can be phosphorylated by ERK after its activation. Once ERK phosphorylates paxillin, this allows it to be phosphorylated by other serine kinases, leading to fully phosphorylated paxillin. This phosphorylated paxillin can then serve as an adaptor protein to bring together molecules at the contact point in which the CTL binds the target cell, as suggested by our evidence that paxillin is localized to this contact point. Paxillin is therefore a candidate protein for an obligatory, nontranscriptional target of ERK leading to CTL degranulation.

Paxillin is a complex adaptor protein that appears to link various signaling molecules at the cell surface with the actin cytoskeleton (12, 13). Interestingly, paxillin is localized to the site of focal adhesions, and tyrosine phosphorylation of this protein appears to be required for fibroblast cell spreading (39). Paxillin knockout mice are embryonic lethal; however, cells from these embryos display reduced or delayed cell spreading on extracellular matrix (15, 16). Thus, this interesting adaptor molecule plays a very important role in cell adhesion and motility during development. It is not clear what role paxillin plays in T cell activation. In nontransformed T cells, it is one of the major proteins detected on an anti-phosphotyrosine blot after TCR stimulation (17) (Fig. 4 and data not shown). In these studies, we show that there is localization of paxillin with the MTOC, as has been shown previously (27). However, we also detect strong localization of paxillin at the contact point between the CTL and target cell, suggesting that it may play an important role at a later stage in some aspect of CTL degranulation. One possibility is that it helps to maintain tight contact with the target cell until the granule contents are delivered. Given the localization with both the contact point and the MTOC, it is also possible that it holds the MTOC and/or the associated
granules in place to facilitate the directional secretion of the granules. Experiments are currently underway to identify a specific function for this interesting molecule in T cells.

Further studies are required to identify other relevant substrates of ERK for CTL degranulation and to determine the precise role of the ERK-mediated phosphorylation of paxillin in T cells. That paxillin is independently phosphorylated by both tyrosine kinases and ERK downstream of the TCR and that it is localized both at the MTOC and contact point with a target cell implicates an important role for paxillin in CTL-mediated killing.

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