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Protein Kinase C δ Localizes to Secretory Lysosomes in CD8⁺ CTL and Directly Mediates TCR Signals Leading to Granule Exocytosis-Mediated Cytotoxicity

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Lytic granule exocytosis is the major effector function used by CD8⁺ CTL in response to intracellular pathogens and tumors. Despite recent progress in the field, two important aspects of this cytotoxic mechanism remain poorly understood. First, TCR-signaling pathway(s) that selectively induces granule exocytosis in CTL has not been defined to date. Second, it is unclear how Ag receptor-induced signals are converted into mobilization of lytic granules. We recently demonstrated that protein kinase C δ (PKC δ) selectively regulates TCR-induced lytic granule polarization in mouse CD8⁺ CTL. To better understand how PKC δ facilitates granule movement, here we studied dynamics of intracellular localization of PKC δ in living CD8⁺ CTL. Strikingly, we found that PKC δ localizes to the secretory lysosomes and polarizes toward immunological synapse during the process of target cell killing. Also, biochemical and structure-function studies demonstrated that upon TCR ligation, PKC δ becomes rapidly phosphorylated on the activation loop and regulates granule exocytosis in a kinase-dependent manner. Altogether, our current studies provide new insights concerning the regulation of TCR-induced lytic granule exocytosis by revealing novel intracellular localization of PKC δ, providing the first example of colocalization of a kinase with secretory lysosomes in CD8⁺ CTL and demonstrating that PKC δ directly transduces TCR signals leading to polarized granule secretion. The Journal of Immunology, 2008, 181: 4716–4722.

Granal exocytosis-mediated cytotoxicity by CD8⁺ T cells is one of the major mechanisms of adaptive immunity to intracellular pathogens and tumors. Lytic granule exocytosis is a multistep process: upon recognition of the cognate peptide in the context of MHC class I, the microtubule-organizing center (MTOC) reorients to the target cell contact site, lytic granules polarize toward the MTOC, followed by docking and fusion of the granules with the plasma membrane at the contact site. Release of the granule contents in the cleft formed between CTL and the target cell initiates target cell death. CTL granules have been characterized as secretory lysosomes as, in addition to the cytolytic proteins (i.e., perforin and several serine proteases or granzymes), they contain lysosomal proteins (1). A recent study demonstrated that in human CD8⁺ CTL fusion of two types of granules, designated “lytic” and “exocytic” are necessary for productive granule exocytosis-mediated killing (2). The existence of a similar maturation step in mouse CD8⁺ CTL has not been reported to date.

Target cell-induced MTOC reorientation is a prerequisite not only for lytic granule polarization but also for other aspects of T cell function, including vectorial secretion of cytokines and recruitment of molecules into the immunological synapse (3). TCR-induced MTOC reorientation is regulated by Fyn, Zap, Lat, SLP-76, and Erk1/2 (3–5), all of which are the components of different signaling pathways downstream of TCR triggering. MTOC reorientation has been shown to be regulated by molecules also involved in reorganization of the actin cytoskeleton, including formins FMNL1 and DIA1 (4) and GTP-ases Rac1 (4), Cdc42 (3), and its effector IQGAP1 (6).

Several molecules are known to regulate specific distal stages of lytic granule exocytosis in mouse CD8⁺ CTL. The α subunit of Rab geranylgeranyltransferase regulates polarization of lytic granules, most likely in an indirect manner, by prenylating an unknown rab protein involved in regulation of the granule movement (7). Rab27a controls detachment of granules from microtubules and/or docking of the granules to the plasma membrane (7), while synaptotagmin VII (8) regulates docking/fusion of lytic granules with the plasma membrane. In human CD8⁺ CTL, AP-3 regulates lytic granule polarization (9), while Rab27a and its effectors Munc13-4 and syntaxin 11 are involved in regulation of more distal stages of granule exocytosis [i.e., granule docking and membrane fusion (10, 11)]. Although mouse homologues of some of these genes (i.e., Munc13-4 and syntaxin 11) have also been shown to play a role in regulation of granule exocytosis-mediated cytotoxicity (12, 13), the exact stages of lytic granule exocytosis that are regulated by these molecules in mouse CD8⁺ CTL have not been reported to date.

Although notable progress has been made in recent years with respect to understanding the regulation of lytic granule exocytosis in CD8⁺ CTL, our knowledge concerning this T cell effector function remains limited. This is particularly evident for regulation of lytic granule polarization. Similar to Rab geranylgeranyltransferase in mouse CD8⁺ CTL, the role of AP-3 in regulation of lytic granule polarization in human CD8⁺ CTL is most likely indirect as
it was proposed that AP-3 regulates lysosomal sorting of a molecule(s) that regulates granule polarization (9). Thus, a molecular link between TCR engagement and induction of lytic granule movement in CTL is missing. Also, although there is evidence that TCR engagement can selectively induce lytic activity in T cells (14, 15), components of the TCR signaling pathway that specifically mediates this effector function have not been identified to date.

By studying the function of mouse protein kinase C (PKC) δ-deficient CD8+ CTLs, we have recently shown that PKC δ is required for lytic granule exocytosis but is dispensable for activation, cytokine production, and expression of cytolytic molecules in response to TCR reorientation (16). We also showed that PKC δ does not regulate MTOC reorientation but is required for TCR-induced lytic granule polarization. Those studies demonstrated that PKC δ is involved in selective regulation of lytic granule movement in response to TCR engagement on CD8+ CTL. To better understand the role of PKC δ in the regulation of granule polarization, here we investigated intracellular location of PKC δ in CD8+ CTL. Surprisingly, time-lapse analysis of living CD8+ CTL showed that PKC δ localizes to the secretory lysosomes and PKC δ comigrates with the granules and accumulates at the immunological synapse upon target cell recognition.

Materials and Methods

Mice and cells

C57BL/6 and BALB/c mice were purchased from Taconic Farms. PKC δ-deficient mice on a C57BL/6 background were used for the studies. 2C11 hybridoma, L1210 cells (from ATCC), and IL-2-producing cell line IL2P (obtained from Dr. S. Vakamanovic, Children’s Hospital, Washington, DC) were cultured in RPMI 1640 containing 10% FBS. Experiments using mice were performed with the permission of the Children’s National Medical Center Institutional Animal Care and Use Committee. All experiments on mice were performed in accordance with the institutional and national guidelines and regulations.

Abs and reagents

Splenocytes were stimulated in vitro with culture supernatants containing anti-CD3 Ab produced by 2C11 hybridoma and, where indicated, additionally cultured in the presence of 5 v/v% culture supernatants containing mouse IL-2 produced by the IL-2-producing cell line. CD8+ spleen T cells were purified using the magnetic bead-coupled Ab MACS system (Miltenyi Biotec). The following Abs were used: Anti-CD3ε Ab, clone 2C11 (BD Pharmingen), and the isotype control hamster IgG1 Ab (BD Pharmingen) were used in re-directed Chromium release assays. The same anti-CD3ε Ab and the secondary goat anti-hamster IgG (Caltag Laboratories) were used for the CD8+ T cell crosslinking in vitro. Intracellular staining: PE-conjugated anti-human granzyme B and mouse IgG1-PE isotype control Ab (both from Caltag Laboratories), rabbit anti-PKC δ and the secondary FITC-anti-rabbit Ab (both from Santa Cruz Biotechnology). Immunoblotting: rabbit anti-PKC δ, rabbit anti-phospho-PKC δ (Thr505) and rabbit anti-GAPDH Ab (all from Cell Signaling Technology), HRP-conjugated anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories). The Lysotracker Red was used for labeling of lysosomes in T cells, while Cell Tracker Blue (both from Molecular Probes) was used for labeling L1210 cells. [35S]Cr nuclear- nucleotide used in the redirected cytoxotic assay was from PerkinElmer.

In vitro T cell stimulation and CD8+ T cell purification

To generate T cell blasts, 4 × 106 total splenocytes in 4 ml of complete RPMI 1640 per well of a 6-well plate were cultured for 36–48 h in the presence of 1% v/v% of supernatants containing anti-CD3 Ab produced by 2C11 hybridoma. Where indicated, the cells were additionally cultured in the presence of 5% v/v% of supernatants containing IL-2. CD8+ T cells were purified by magnetic immunobeads and used as described in the Results section. Purity of T cells was ≥95%, as determined by flow cytometry.

Granzyme A release

Purified CD8+ CTL were stimulated with either anti-CD3 or hamster IgG Ab immobilized on plastic (10 µg/ml Ab in 100 µl of PBS per well) of a 96-well plate was incubated for 60 min or longer at 37°C and granzyme A release was assessed by benzoyloxycarbonyl-L-lysine thiobenzyl ester serine esterase assay. A total of 2 × 106 cells/well were plated in 100 µl of RPMI 1640 containing 10% FCS in 96-well plates and incubated for 4 h at 37°C. A total of 20 µl of the supernatant was mixed with 180 µl of substrate (PBS, 0.2 mM N-benzoxycarbonyl-L-lysine-thiobenzylester, and 0.2 mM diithiobis-nitrobenzoic acid) for 30 min, and the absorbance was read at 415 nm. Maximal release from cells was determined by treatment of cells with 1% Triton X-100, while spontaneous release was determined from supernatants of cells incubated with medium only. The supernatant enzymatic activity was expressed as a percentage of the total enzyme in Triton X-100 cell lysates.

Chromium release assay

Re-directed chromium release assay was performed as previously described (16). Anti-CD3 or hamster IgG isotype control Ab was added to Fas-resistant L1210 target cells at a final concentration of 1 µg/ml and were present during the assay.

Generation of expression vectors and site-directed mutagenesis

To create PKC δ-GFP expression vector, mouse PKC δ gene was amplified by PCR from cDNA generated from naive CD8+ mouse (C57BL/6) mice using the primers that introduced HindIII restriction site at the 5’ end and BamHI site after the stop codon at the 3’ end of the gene. The PCR product was cloned into HindIII-BamHI sites of pMaxFP-Green-N (pMax-GFP) plasmid vector (Amaza Biosystems). The identity and the absence of mutations in the cloned PKC δ gene were confirmed by DNA sequencing. Kinase-negative (KN) PKC δ-GFP mutant was generated by substituting lysine residue (K376) in the ATP-binding site of the kinase domain of PKC δ with methionine, using the QuikChange XL site-directed mutagenesis kit (Stratagene). The presence of the mutation was confirmed by DNA sequencing. The PKCδ-ires-GFP plasmid, generated as previously described (16), and pMaxFP-Green-N plasmid were used as GFP expression vectors.

Transfection

Total resting splenocytes were stimulated in the presence of anti-CD3 Ab for 36 h, re-plated in the absence of anti-CD3 Ab, and cultured for an additional 8–10 h in the presence of IL-2 and then transfected with indicated plasmid DNA using nucleofection kit for primary mouse T cells according to the manufacturer’s protocol (Amaza Biosystems). After nuclofection, the cells were cultured in RPMI 1640 medium containing 10% FCS in the absence of anti-CD3 Ab and in the presence of IL-2 for an additional 16–24 h and then either analyzed by flow cytometry or CD8+ T cells were purified by magnetic immunobeads and used in re-directed Chromium release assays. Untransfected activated splenic cells were cultured with IL-2 for the same periods of time as the transfected cells, followed by purification of CD8+ T cells.

Activation of CD8+ T cells by CD3 crosslinking in vitro: immunoblotting

Total resting splenocytes were stimulated in the presence of anti-CD3 Ab for 36 h, re-plated and cultured for an additional 24 h in the presence of IL-2, followed by purification of CD8+ T cells. The cells in complete RPMI 1640 (106 cells/ml) were incubated for 30 min on ice in the presence of 10 µg/ml of purified anti-CD3 Ab followed by addition of 25 µg/ml of goat anti-hamster IgG at 37°C for indicated periods of time. Cells were immediately lysed with ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% glycerol, 1% nonidet P-40, 2 mM EDTA, 1 mM PMSF, 2 mM Na3VO4, 10 mM NaF, and protease inhibitor mixture (Sigma-Aldrich)) for 30 min on ice and subjected to SDS-PAGE followed by immunoblotting, as previously described (16).

Intracellular staining, cell labeling, and microscopy

Purified CD8+ T cells were transferred to cover slips coated with Poly-l-lysine. After 5 min of incubation at room temperature, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.01% Triton X-100 and 0.5% BSA, stained intracellularly for PKC δ (direct staining using rabbit anti-PKC δ Ab followed by secondary FITC-anti-rabbit Ab staining) or granzyme B (direct staining using PE-conjugate anti-human granzyme B Ab), and analyzed by grid-based optical sectioning microscopy with a ×60 1.3 n.a. oil/DIC objective (Carl Zeiss Axiovert 200M microscope with Carl Zeiss Apotome).

Live imaging

CD8+ T cells transfected with the indicated expression vectors were analyzed in the absence of target cells, whereas the transfected cells loaded
FIGURE 1. PKC δ is organized in granular pattern in CD8⁺ CTL. In vitro activated PKC δ-deficient or WT splenic T cells were either left untransfected (i.e., cultured in the presence of IL-2) or were transfected with the indicated plasmids as described in Materials and Methods. A. The cells were analyzed by flow cytometry: control, non-GFP coding, pcDNA3.1 plasmid vector (shaded histogram); pmax-GFP expression vector (full lines in the histogram); pmax-PKC δ-GFP expression vector (dotted lines in the histogram). The same results were obtained with PKC δ-deficient and WT splenic T cells. The results with the PKC δ-deficient cells are shown. A representative of three independent experiments giving the similar results is shown. B, CD8⁺ T cells were purified by magnetic immunobeads, followed by re-directed 4-h chromium release assay against L1210 cells. Each E:T ratio was done in triplicate samples. A representative of three independent experiments giving the similar results is shown. Error bars represent SD. C, Live CD8⁺ CTL transfected either with pmax GFP or with pmax-PKCδ-GFP, as described in A, or CD8⁺ CTL stained intracellularly for PKC δ, as described in Materials and Methods section, were analyzed by two-photon microscopy or grid-based optical sectioning microscopy, respectively. Representative images from three independent experiments giving similar results are shown. At least 10 and 20 cells were observed in each experiment during the imaging of live cells and intracellularly stained cells, respectively.

with LysoTracker Red were analyzed in the absence or in the presence of target cells. LysoTracker Red and Cell-Tracker Blue loading was performed by incubating CD8⁺ T cells or L1210 cells, respectively, at 37°C for 60 min with the 60 nM dye in serum-free RPMI 1640 medium, followed by two washes with 1 ml of the medium. A total of 2 × 10⁵ of LysoTracker Red-labeled CD8⁺ T cells were mixed with the equal number of CellTracker Blue-labeled target L1210 cells in 200 μl of complete RPMI 1640 in the presence of 1 μg/ml anti-CD3 Ab. Cells were placed in a temperature-controlled chamber [heated open supfuser chamber (RC-25F; Warner Instruments)]. Preheated serum-free medium was pumped over the slices for the length of the imaging experiment, and the chamber and perfusate temperature was maintained at 37°C. Sequential confocal images were acquired every 4 s for 10–15 min with a Zeiss LSM 510 Meta NLO system equipped with an Axiovert 200M microscope (Zeiss), with 488-nm epifluorescence and Nomarski differential interference contrast for the transmitted light. A ×40 1.3 n.a. oil/DIC objective was used. The images were processed with Zeiss LSM version 3.2 software.

Online supplemental material
Video1 shows comigration of PKC δ-GFP (green) and secretory lysosomes (red) in live CD8⁺ CTL interacting with multiple target cells (blue). Video2 shows polarization and accumulation at the target cell-contact site of PKC δ-GFP (green) and secretory lysosomes (red) in live CD8⁺ CTL interacting with a single target cell (blue).

Results
PKC δ fused to GFP efficiently mediates lytic granule exocytosis in CD8⁺ CTL
To gain insights into the site of action of PKC δ, we studied the intracellular localization of PKC δ in live CD8⁺ CTL. For this purpose, we generated an expression vector encoding a fusion protein between mouse PKC δ and GFP. To test whether PKC δ-GFP is physiologically relevant for the studies concerning the role of PKC δ in regulation of lytic granule exocytosis, we ectopically expressed the fusion protein in PKC δ-deficient CD8⁺ CTL and assessed its ability to reverse the cytolytic defect characteristic of PKC δ-deficient CTL. The PKC δ-GFP expression vector was introduced into polyclonally activated primary mouse CD8⁺ T cells by nucleofection, followed by re-directed chromium release assay using Fas-resistant L1210 cells as targets to specifically assess granule exocytosis-mediated cytotoxicity (16). We have previously used this transfection system to achieve high frequency of ectopic gene expression in polyclonal population of highly potent CD8⁺ CTL (16). As shown in Fig. 1, ectopic expression of GFP-tagged PKC δ (Fig. 1A) reversed defective granule exocytosis-mediated lytic function in PKC δ-deficient CD8⁺ CTL (Fig. 1B), demonstrating that PKC δ fused to GFP retained its functional properties and was able to mediate lytic granule release.

PKC δ is organized in a granular pattern in CD8⁺ CTL
The intracellular localization of PKC δ-GFP fusion expressed in PKC δ-deficient CD8⁺ CTL was monitored by live imaging. Surprisingly, we observed that in resting CD8⁺ CTL (cultured in the absence of TCR stimulation for 24 h) GFP-tagged PKC δ was organized in a distinct granular pattern (Fig. 1C). In contrast, PKC δ-deficient CD8⁺ CTL transfected with the expression vector encoding either GFP only (Fig. 1C), or the full-length PKC δ and IRES-regulated GFP (i.e., where GFP is not
PKC δ colocalizes with lytic granules in CD8\(^+\) CTL

The observation that PKC δ is organized in a granular pattern, together with our previous finding that PKC δ regulates TCR-induced lytic granule polarization (16), suggested that PKC δ might associate with lysosomal/lytic granules in CD8\(^+\) CTL. To test this we labeled live CD8\(^+\) CTL with Lysotracker, an acidotropic dye that selectively accumulates in lysosomal granules in living cells and is used as a marker for secretory lysosomes in CTL (9). PKC δ-deficient CD8\(^+\) CTL expressing GFP-tagged PKC δ were loaded with Lysotracker Red and the localization of PKC δ-GFP with respect to lysosomal granules in CTL before and after recognition of target cells was assessed by time-lapse multiphoton imaging. In resting CTL (cultured in the absence of TCR stimulation for 24 h), PKC δ-GFP fusion colocalized with lysosomal granules (Fig. 2, A). Upon target cell recognition by CD8\(^+\) CTL, PKC δ comigrated with the granules toward the target cell contact site (video1.avi and Fig. 2D-video2.avi) and accumulated at the immunological synapse (Fig. 2E-video2.avi).

To determine specifically whether PKC associates with lytic granules, CD8\(^+\) CTL expressing GFP-tagged PKC δ were stained intracellularly for granzyme B and analyzed by microscopy. These experiments showed that the majority of PKC δ-positive granules colocalized with granzyme B-containing granules (Fig. 2C). However, a subpopulation of PKC δ-positive granules did not colocalize with granzyme B granules, indicating that in addition to lytic granules, PKC δ colocalizes with a distinct, nonlytic compartment of secretory lysosomes.

PKC δ regulates granule exocytosis-mediated cytotoxicity in a kinase-dependent manner

The finding that PKC δ constitutively colocalizes with lysosomal granules in CD8\(^+\) CTL suggested that PKC δ might not actively transduce TCR-initiated signals via its kinase activity but rather may serve as a scaffold for other regulators of lytic granule exocytosis. In fact, there is increasing evidence in the literature that PKC δ regulates various cellular functions in a kinase-independent manner (17). This prompted us to determine whether the kinase activity of PKC δ is required for regulation of granule exocytosis-mediated cytotoxicity. For this purpose, we generated a KN mutant by substituting methionine for lysine residue (K376) in the ATP-binding site of the kinase domain of PKC δ. This mutation has been demonstrated to completely abolish the kinase activity of PKC δ and PKC δ-GFP fusion protein containing the K376 mutation has been used for study of kinase-dependent PKC δ function in different cell types (18, 19). Wild-type (WT) or KN PKC δ mutant were expressed in PKC δ-deficient CD8\(^+\) CTL and colocalization of the mutant with secretory lysosomes and its ability to reverse the lytic defect was assessed. As determined by flow cytometry, the level of the ectopically expressed KN PKC δ mutant was equivalent to that of WT PKC δ (Fig. 3A). Also, viability of CD8\(^+\) CTL expressing the mutant or WT PKC δ was similar, as determined by forward vs side scatter (Fig. 3A) and trypan blue exclusion (data not shown). The KN PKC δ mutant associated with secretory lysosomes in live CTL (Fig. 3B), but the mutant could not reverse lytic defect in PKC δ-deficient CTL (Fig. 3C). We have previously shown that PKC δ is dispensable for conjugate formation but is required for lytic granule exocytosis, specifically, for lytic granule polarization (16). Consistent with this,
PKC δ-deficient CTL reconstituted with the KN PKC δ mutant, did not release lytic granule contents (Fig. 4A), and did not polarize lytic granules in response to TCR engagement (Fig. 4B). Collectively, these results show that kinase activity of PKC δ is not necessary for its association with secretory lysosomes in CTL but is required for regulation of downstream effectors of lytic granule exocytosis.

**PKC δ is activated in CD8+ CTL upon TCR engagement**

The phenotype of the KN PKC δ mutant shows that upon TCR engagement, PKC δ kinase activity is required for lytic granule exocytosis. To determine whether in CD8+ CTL PKC δ becomes activated by TCR-initiated signals, we analyzed phosphorylation of the PKC δ activation loop (i.e., Thr505). Stimulus-induced phosphorylation of this site has been used as an indicator of PKC δ activation in different cell types, including human CD4+ T cells (17, 20). Induction of activation loop phosphorylation in PKC δ is generally accepted to result in the enhancement of catalytic activity (17), although a recent study suggested that in Jurkat cells it instead modulates substrate specificity of the enzyme (20). Thus, we assessed TCR-induced phosphorylation of the PKC δ activation loop in CD8+ CTL by immunoblotting using the Ab that specifically recognizes phosphorylated form of Thr505. We found that Thr505 was phosphorylated in resting CD8+ CTL and that Thr505 phosphorylation was further increased upon brief TCR ligation (Fig. 4C), indicating that PKC δ is activated in CD8+ CTL as a consequence of TCR engagement.

**Discussion**

In sum, we show in this work that PKC δ localizes to secretory lysosomes in CD8+ CTL and polarizes toward the target cell synapse upon TCR engagement. Following TCR ligation, PKC
δ is rapidly phosphorylated on the activation loop (Thr505) and via its kinase activity transduces signal(s) that results in lytic granule exocytosis. Together with our previous finding that PKC δ regulates lytic granule polarization in CD8\(^+\) CTL, results of the current study suggest that secretory lysosome-associated PKC δ directly mediates selective mobilization of the granules in response to TCR signals.

The finding that PKC δ localizes to secretory lysosomes in CD8\(^+\) CTL is striking. Functional PKC δ has been found in different intracellular locations, including nucleus, mitochondria, endoplasmic reticulum, Golgi, and cytoskeleton (17, 21, 22). Despite the evidence that PKC δ regulates trafficking and function of endosomal and lysosomal vesicles (23, 24), localization of PKC δ to these subcellular compartments had not been reported before our studies. Importantly, PKC δ is now the first kinase reported to colocalize with lytic granules in CTLs and to regulate their movement. The molecular basis for PKC δ localization to secretory granules in CD8\(^+\) CTL is not clear at present. Since PKC δ contains diacylglycerol- and phosphatidylycerine-binding C1 domains (17), it is possible that its interaction with the membranes of lysosomal granules is mediated through these determinants. However, mutation of the C1B domain in PKC δ, which is responsible for its binding to diacylglycerol mimetics (25), does not prevent colocalization of PKC δ with the granules in live CTL (our unpublished observation). This suggests that PKC δ might interact with granules indirectly, possibly by binding to a molecule located on the granule membranes. One possibility is that PKC δ interacts with the granules via receptors for activated C kinases, which are membrane-associated anchoring proteins that recruit specific PKC isoforms to distinct subcellular compartments (26). Based on the use of peptide inhibitors and activators of PKC δ-specific interactions in cells, a receptor for activated C kinase specific for PKC δ has been proposed to exist (26) but has not been identified to date. Since our studies indicate that PKC δ associates with granules in resting CD8\(^+\) CTL before TCR-engagement, one possibility is that PKC δ associates with the granules through interaction with a receptor for inactive C-kinases (26).

Based on both results of our work and increasing evidence that compartmentalized signaling has an important role in regulation of cellular functions, it is tempting to speculate that PKC δ is involved in signaling that takes place on secretory lysosomes in CTLs. Signaling on different intracellular endomembranes, including endosomes, Golgi, endoplasmic reticulum, and mitochondria, has been reported (27). In Jurkat cells and in Ag-unexperienced primary T cells, N-Ras and H-Ras become rapidly activated on Golgi upon TCR engagement (28, 29). Of note, the intracellular location of Ras activation in effector T cells, including CD8\(^+\) CTL, has not been reported. Also, endosomal adaptor protein p14, which confines MAPK signaling to late endosomes/lysosomes, has been shown to be required for lytic function of human T cells (30). Thus, PKC δ might be a component of TCR-induced signaling pathway (potentially involving the Ras-MAPK cascade) that occurs on lytic granules in CTL and which leads to lytic granule exocytosis. TCR-induced phosphorylation of the PKC δ activation loop in CD8\(^+\) CTL that we observed in our studies might play a role in modifying the substrate specificity of the enzyme, as suggested for Jurkat cells (20), such that regulator(s) of granule exocytosis are selectively activated.

Regardless of the exact molecular mechanism of PKC δ function in lytic granule exocytosis, our studies now identify PKC δ as an active constituent of TCR signaling pathway that selectively induces movement of lytic granules in CTLs. Future studies aimed at understanding PKC δ association with granules and determining the nature of this interaction as well as identification of upstream and downstream effectors of PKC δ in CD8\(^+\) CTL will add to our knowledge concerning specific TCR signals that induce lytic granule exocytosis. This will potentially allow selective manipulation of granule exocytosis-mediated cytotoxicity in CD8\(^+\) CTL in disease.

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