The lymphocyte-oriented kinase (LOK), also called serine threonine kinase 10 (STK10), is synthesized mainly in lymphocytes. It is involved in lymphocyte migration and polarization and can phosphorylate ezrin, radixin, and moesin (the ERM proteins). In a T lymphocyte cell line and in purified human lymphocytes, we found LOK to be cleaved by caspases during apoptosis. The first cleavage occurs at aspartic residue 332, located between the kinase domain and the coiled-coil regulation domain. This cleavage generates an N-terminal fragment, p50 N-LOK, containing the kinase domain and a C-terminal fragment, which is further cleaved during apoptosis. Although these cleavages preserve the entire kinase domain, p50 N-LOK displays no kinase activity. In apoptotic lymphocytes, caspase cleavages of LOK are concomitant with a decrease in ERM phosphorylation. When non-apoptotic lymphocytes from mice with homozygous and heterozygous LOK knockout were compared, the latter showed a higher level of ERM phosphorylation, but when apoptosis was induced, LOK−/− and LOK+/− lymphocytes showed the same low level, confirming in vivo that LOK-induced ERM phosphorylation is prevented during lymphocyte apoptosis. Our results demonstrate that cleavage of LOK during apoptosis abolishes its kinase activity, causing a decrease in ERM phosphorylation, crucial to the role of the ERM proteins in linking the plasma membrane to actin filaments.

The lymphocyte-oriented kinase (LOK),² also called serine threonine kinase 10 (STK10), is a serine/threonine kinase predominantly produced in lymphoid organs such as the spleen, thymus, and bone marrow. The N-terminal catalytic domain of LOK displays homology to the STE20 family members, involved notably in regulating the MAPK cascades. LOK also displays a central proline-rich region and a large C-terminal coiled-coil structure not conserved in the STE20 family (1, 2). Although the upstream mechanisms regulating LOK kinase activity remain to be elucidated, the LOK kinase domain is known to undergo dimerization, leading to trans-phosphorylation and activation of LOK catalytic activity (3).

Several potential targets of LOK have been described. First, LOK can phosphorylate polo-like kinase 1, which suggests a role in regulating the cell cycle (4). Furthermore, in vitro peptide specificity analyses have identified an optimal LOK substrate sequence similar to the ezrin, radixin, and moesin (ERM) phosphorylation sites. Genetic evidence confirms that ERM are LOK substrates in lymphocytes because LOK knockout mice display strongly reduced ERM phosphorylation at a C-terminal site (5). The major function of ERM is to create links between the plasma membrane and cortical actin filaments. Their N-terminal FERM domain binds the plasma membrane through interaction with phospholipids and transmembrane proteins such as CD44 and intracellular adhesion molecule (ICAM), whereas their C-terminal domain associates with actin. ERM binding to membrane lipids and subsequent phosphorylation of a conserved C-terminal threonine residue are thought to disrupt the intramolecular association between the FERM domain and the C-terminal domain, unmasking sites required for other interactions. Besides LOK, other kinases can phosphorylate ERM proteins, including PKC isoforms, Rho-associated protein kinase, Nck-interacting kinase (6), MST4 (7), and STE20-like serine, threonine-protein kinase (SLK) (8). Last, the unique LOK/SLK homolog of Drosophila, called the Slik kinase, has been shown to phosphorylate its sole ERM protein, moesin (9).

In lymphocytes, ERM phosphorylation contributes to cell rigidity and to the maintenance of microvilli. In response to chemokines, dynamic dephosphorylation of ERM participates in a transition to a flexible, migrating cell form (10). Increased migration efficiency in LOK-deficient lymphocytes is consistent with rearrangements of the actin cytoskeleton resulting
from ERM dephosphorylation (5). LOK deficiency also leads to increased LFA-1-mediated lymphocyte adhesion (11). In epithelial cells, localized ezrin phosphorylation by LOK contributes to restricted formation of microvilli at the apical surface (12).

Although the involvement of LOK deregulation in pathology is still elusive, LOK has been found mutated in its kinase domain in human testicular germ cell tumors and in the coiled-coil region in aggressive lymphoma (13, 14). Interestingly, these LOK mutations were shown to inhibit apoptosis of dexamethasone-treated cells, suggesting that LOK may act as a tumor suppressor (14). In contrast, high-throughput siRNA screening in Ewing sarcoma cell lines identified LOK as favoring cell survival and growth (15). LOK might thus exert a pro- or anti-apoptotic action according to the cell type.

Members of the STE20 family are targeted by caspases during apoptosis. For instance, SLK, highly similar to LOK, is cleaved by caspase 3 to generate an activated N-terminal kinase fragment that can promote cell death (16, 17). Similarly, caspase 3 cleavage of MST1 generates an active kinase fragment that can translocate to the nucleus, phosphorylate histone H2AX, and promote DNA fragmentation (18, 19).

We demonstrate here that LOK targeting by caspases during apoptosis causes a decrease in the level of full-length LOK and the appearance of an N- and a C-terminal fragment. Although the N-terminal fragment encompasses the entire kinase domain, it is devoid of kinase activity. In vitro and in vivo evidence shows that caspase cleavages of LOK prevent ezrin, radixin, and moesin phosphorylation in lymphocytes undergoing apoptosis.

Experimental Procedures

Cell Culture and Mice—Human peripheral blood mononuclear cells were separated from peripheral blood from healthy donors by gradient centrifugation on Ficoll (GE Healthcare) at room temperature. Jurkat human T leukemia cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and 50 μg/ml gentamycin. HEK-293 cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS and 50 μg/ml gentamycin. A murine strain with the LOK gene locus modified by insertion of the FRT-loxP flanking neomycin cassette between exons 2 and 5 was generated in a mixed C57BL/129 background. After backcross-breeding to C57BL/6, these mice were mated with β-actin-Flp recombinase transgenic mice to obtain a strain with preconditional floxed alleles (lok flox/flox). A complete LOK knockout strain was then generated by breeding the floxed mice with Cre transgenic mice. All mice used in this study were housed in a specific pathogen-free facility and cared for in accordance with National Institutes of Health guidelines, and all protocols were approved by the NCI/National Institutes of Health Animal Care and Use Committee. Single-cell suspensions of mouse spleen were prepared and cultured in RPMI 1640 medium (Invitrogen) containing l-glutamine, 25 mM Hepes, 10% FBS (HyClone), and 50 μM β-mercaptoethanol.

Cytokines and Drugs—Staurosporine, anisomycin, and the ezrin inhibitor NSC668394 were purchased from Calbiochem (San Diego, CA). Etoposide and camptothecin were purchased from Sigma-Aldrich (St. Louis, MO). To inhibit caspase activity, cells were preincubated for 30 min with 20 μM Z-VAD-fmk (Calbiochem) or Q-VD-OPH (SM Biochemicals, Anaheim, CA) before treatment with an apoptosis inducer.

Plasmid Constructs—LOK cDNA was provided by Dr. Karasuyma, digested with the restriction enzymes EcoRV and NotI (Invitrogen), and subcloned in-frame in the pcDNA3 FLAG and V5 vectors. FLAG-LOK DAVN, in which aspartic acid 332 was replaced with an asparagine, was created using the QuikChange site-directed mutagenesis system of Stratagene using pcDNA3 FLAG-LOK as a template and the oligonucleotides 5’-GAGGAGGATCTGTGAATGTGGTCCGCC-CTG-3’ and 5’-CAGGGGCGGAACAGCATCAGCATCCTCCTC-3’. FLAG-LOK KD (kinase-dead, mutated at the DFG site) was created by site-directed mutagenesis using pcDNA3 FLAG-LOK as a template and the oligonucleotides 5’-GACATCACGCTGCTGATAATTGCTGTGGTCCA-CTGCAG-3’ and 5’-CTGATGCTGCCAGACACAGCATCAGCATCCTCCTC-3’. LOK DAVN-V5 and LOK KD-V5 were created by site-directed mutagenesis using pcDNA3 V5-LOK as a template and the oligonucleotides described above. LOK-332-V5 was obtained by PCR amplification from LOK cDNA and subcloned in pcDNA3 V5-LOK between the EcoRI and BamHI restriction sites. LOK-332-KD-V5 was created by site-directed mutagenesis using pcDNA3 V5-332-V5 as a template and the oligonucleotides described above. GFP-LOK-332 and GFP-LOK-332 KD were obtained by PCR amplification with the oligonucleotides 5’-CGAATTCTAGGCTTTCGGCCAATTTC, introducing the EcoRI restriction site, and 5’-TGATCCCAGCATCCTCCTCCTCCTCCTCC, introducing the BamHI restriction site from LOK cDNA or LOK KD cDNA, respectively. It was subcloned in the pmaxFP Green-N vector (Lonza). GFP-LOK-422 was a gift from Dr. Avery August (Cornell University). The kinase domain was cloned into pSET-b (Invitrogen) between the Ncol and EcoRI sites using naturally occurring Ncol and EcoRI sites within the N terminus of human LOK. The resulting clone was then cut with Nhel and HindIII and cloned between the Nhel and HindIII sites of pEGFP-N3 (Clontech).

Antibodies—Polyclonal antibody directed against the C-terminal region of LOK between residues 900 and 968 was purchased from Bethyl Laboratories (Montgomery, TX). The rabbit polyclonal antibody recognizing mouse LOK was developed in the Park laboratory at the Experimental Immunology Branch of the NCI/National Institutes of Health and generated by ProSci Inc. The peptides used for immunization (epitopes) correspond to the unique murine LOK part of the sequence, amino acids 353–372 (DSNKLQDSSTPLPPSOPQPE) and 378–400 (CSQPSGDPQLTTPSADGLSNK) as in the mouse STK10 gene (Uniprot no. O55098). Rabbit polyclonal antibodies directed against the C-terminal domain of human PARP-1 (H250) and the antibody against ERK2 (C14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibody against actin and the rabbit polyclonal antibody against FLAG were purchased from Sigma.
Caspase Cleavages of LOK

clonal antibody against the V5 epitope was purchased from Invitrogen. Secondary antibodies conjugated with horseradish peroxidase were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The polyclonal antibody against the C-terminal region of ezrin was purchased from Upstate Biotechnology (Billerica, MA), and the rabbit polyclonal antibody against human moesin 473–486 was purchased from Abnoba. The monoclonal antibody against carboxy-phosphorylated ERM Thr(P)-567 (phospho-ERM), whose reactivity includes moesin Thr(P)-558, ezrin Thr(P)-567, and radixin Thr(P)-564, was purchased from BD Life Sciences. It was conjugated with Alexa Fluor 647 (Invitrogen). The rabbit monoclonal phycerythrin (PE)-conjugated antibody against cleaved caspase 3 (C92-605), rat monoclonal FITC-conjugated anti-CD4 (GK1.5 or RM4.4), and anti-CD8α (53–6.7) antibodies were obtained from BD Life Sciences.

Immunoblotting—Jurkat cells (2.10⁵ cells/well) or HEK-293 cells (2.10⁵ cells/well) were grown in 6-well plates in RPMI 10% FBS or DMEM 10% FBS, respectively. The cells were suspended in PY lysis buffer with 2.5 μg/mL leupeptin, 20 μg/mL aprotinin, 20 μM β-glycerophosphate, and 1 mM sodium orthovanadate. Lysates were clarified by centrifugation at 4 °C, and the protein concentration was determined by BCA protein assay (Pierce). Western blotting was performed by centrifugation at 4 °C, and the protein concentration was determined by BCA protein assay (Pierce). Western blotting was performed as described previously (20). For quantification of protein expression, luminescence was captured by digital imaging using a cooled charge-coupled device camera (LAS 3000, Fuji, Tokyo, Japan), and quantification was performed using Multi gauge V3.0 software. The background-adjusted volume was normalized to an empty well.

Caspase 3 Activity—Jurkat cells were lysed in PY buffer without protease inhibitor. 50 μL of cell lysate adjusted to protein content was transferred to a 96-well plate, and 50 μL of assay buffer (100 mM Hepes, 1% sucrose, 10 mM DTT, and 500 μM EDTA) was added. After 30 min of incubation at 37 °C, the caspase substrate DEVD-AFC (Calbiochem) was added to each well, and the fluorescence was monitored every 10 min for 3 h (excitation, 395 nm; emission, 510 nm) with a fluorimeter (excitation, 395 nm; emission, 510 nm) with a fluorimeter. The reactivities of phosphorylated proteins were resolved by SDS-PAGE and subjected to Western blotting (20). Purified active caspases were provided by Dr. G. S. Salvesen (The Burnham Institute, La Jolla, CA).

Flow Cytometric Analysis—A protocol described previously was used for lymphocyte immunofluorescence staining (10). Murine and Jurkat cells were fixed in suspension with 4% paraformaldehyde for 15 min at 37 °C and then permeabilized in buffer containing PBS with 0.2% BSA, 2.5 μM EDTA, 0.1% saponin, and 10 μM Hoesp (15 min at room temperature). The cells were then washed and stained with fluorescein or Alexa Fluor 647-conjugated antibodies for 1 h at room temperature or overnight at 4 °C. This was followed by three washes with permeabilization buffer. The fluorescence intensity of the cells was measured on an LSR Fortessa, on a FACSCalibur, or on a Canto II (BD Biosciences). For apoptosis analysis, cells were labeled with Alexa Fluor 488-Annexin V and propidium iodide with the dead cell apoptosis kit for flow cytometry (Invitrogen). Data were recorded with BD FACSDiva™ software and analyzed with FlowJo software.

In Vitro Kinase Assays—HEK-293 cells (1.10⁸ cells/well) were transfected with an expression vector encoding V5 epitope-tagged, full-length LOK or LOK332 (the fragment extending from the N terminus of LOK to the caspase site DAVID32)). Proteins were immunoprecipitated in lysis buffer with V5 antibody. Immune complexes were washed four times with ice-cold PY lysis buffer and once with kinase buffer (20 mM MOPS (pH 7.2), 7.5 mM MgCl₂, 25 mM glycerophosphate, 5 mM EGTA, 1 mM Na₃VO₄, and 1 mM DTT). Each immunoprecipitate was incubated for 30 min at 30 °C with 20 μL of kinase reaction buffer (kinase buffer supplemented with 50 μM ATP and 10 μM (10 μCi) [γ-³²P]ATP (2000 Ci/mmol) (PerkinElmer Life Sciences). As substrate, human recombinant histone H2A was purchased from Biolabs (Ipswich, MA). C-terminal moesin (502–577) was expressed and purified as a GST fusion protein according to the GST Gene Fusion System Handbook (Amersham Biosciences, Arlington Heights, IL). Kinase reactions were stopped by addition of 10 μL of Laemmli sample buffer (5×). The phosphorylated proteins were resolved by 12% SDS-PAGE and dried, and incorporation of [γ-³²P] was visualized by autoradiography on hyperfilm-MP (Amersham Biosciences).

Results

LOK Is Targeted by Caspases—To investigate the fate of LOK during apoptosis, Jurkat T leukemia cells, which endogenously express the LOK gene, were treated with the apoptotic inducer anisomycin (Fig. 1A), staurosporine (Fig. 1B), and camptothecin (Fig. 1C). Upon treatment with anisomycin, the cells showed, from 2 h, a decreased level of full-length LOK that became lightly detectable after 6 h of treatment. Concomitantly, two smaller fragments were detected with antibody directed against the C-terminal region of LOK. Because the apparent molecular masses of these C-terminal fragments were, respectively, about 115 and 65 kDa, the fragments are henceforth referred to as p115 C-LOK and p65 C-LOK. The...
former was detected after 1 h of treatment and disappeared after 6 h, whereas p65 C-LOK appeared later and was still detected after 8 h of treatment (Fig. 1A). Camptothecin treatment also caused the level of full-length kinase to decrease, but only one C-terminal fragment, p65 C-LOK, was generated (Fig. 1B). Apoptosis was detectable on the basis of PARP cleavage after 1 h of anisomycin treatment or 4 h of camptothecin treatment (Fig. 1, A–C). Treatment with the pan-caspase inhibitor Z-VAD-fmk led to the absence of p65 C-LOK after 8 h of anisomycin treatment while increasing the level of p115 C-LOK. Treatment with Q-VD-Oph, a pan-caspase inhibitor with a broader action spectrum, inhibited generation of both LOK fragments (Fig. 2, A and C). In peripheral blood mononuclear cells treated with staurosporine, the level of full-length LOK was drastically decreased, and p65 C-LOK appeared. As in the Jurkat cell line, Z-VAD-fmk treatment prevented p65 C-LOK generation while favoring p115 C-LOK (Fig. 2B). Quantification of full-length LOK showed that Z-VAD-fmk treatment rescued LOK expression only partially, whereas rescue was complete with Q-VD-Oph (Fig. 2C).

FIGURE 1. Caspase-dependent cleavage of LOK along apoptosis. A–C, Jurkat cells were treated with anisomycin (50 μM) (A), staurosporine (1 μM) (B) or camptothecin (20 μM) (C) for the indicated time. Cell lysates were analyzed by Western blotting (WB) with an antibody directed against the C-terminal (C-Term) domain of LOK. The membrane was stripped and reprobed sequentially with an anti-PARP antibody and finally with an anti-ERK2 antibody as a loading control. Molecular masses are added, and arrowheads indicate the positions of full-length LOK and LOK fragments, PARP, cleaved PARP, and ERK2. The Western blot are representative of at least three experiments. Sizes of molecular weight marker are shown on the left of each blot.
It is worth noticing that, according to the partial inhibition of caspase cleavage of LOK by Z-VAD-fmk inhibitor, PARP cleavage is not affected and only weakly inhibited in Jurkat and peripheral blood mononuclear cells, respectively. In contrast, the broader spectrum inhibitor Q-VD-Oph induced efficient PARP cleavage inhibition in Jurkat cell. As expected, both inhibitors abolished caspase 3 activity in Jurkat cells, demonstrating Z-VAD-fmk efficiency at least on this caspase (Fig. 2D). These results suggest that LOK is cleaved by caspases during apoptosis to generate two C-terminal fragments, p115 C-LOK and p65 C-LOK.

**Caspase Cleavages of LOK**

![Caspase Cleavages of LOK](image-url)

**A**

- **JURKAT**
  - ZVAD + Anisomycin
  - QVD + Anisomycin

**WB LOK C-Term**

- LOK
- p115 C-LOK
- p65 C-LOK

**WB PARP**

- PARP
- cleaved PARP

**WB ERK2**

- ERK2

**B**

- **PBMC**
  - ZVAD + Staurosporine

**WB LOK C-Term**

- LOK
- p115 C-LOK
- p65 C-LOK

**WB PARP**

- PARP
- cleaved PARP

**WB ERK2**

- ERK2

**C**

- c, Ani, ZVAD, Ani + ZVAD, QVD, Ani + QVD

**WB LOK C-Term**

- LOK
- p115 C-LOK
- p65 C-LOK

**D**

- Control
- Ani
- ZVAD
- ZVAD + Ani
- QVD
- QVD + Ani

**Relative expression of LOK (%)**

- c, Ani, ZVAD, Ani + ZVAD, QVD, Ani + QVD

**Time in minutes**

- 0, 20, 40, 60, 80, 100, 120, 140, 160
Next, the caspase cleavages of LOK were examined in vitro with various recombinant caspases and with C-terminally V5-tagged or N-terminally FLAG-tagged LOK exogenously produced in HEK-293 cells (Fig. 3A). From C-terminally V5-tagged LOK, caspase 3 and, to a lesser extent, caspases 8 and 9 were found to generate only p115 C-LOK (Fig. 3A). In contrast, caspase 7 was able to generate both p115 and p65 C-LOK. From N-terminally FLAG-tagged LOK, the effector caspases 3 and 7 and, to a lesser extent, the initiator caspases 8 and 9 were found to generate an N-terminal fragment of about 50 kDa, henceforth referred to as p50 N-LOK (Fig. 3B).

In conclusion, the ability of the broad-spectrum caspase inhibitor Q-VD-OPh to prevent formation of both p115 C-LOK and p65 C-LOK suggests that they are both generated by caspase cleavages. The additional observation that the caspase inhibitor Z-VAD-fmk causes the disappearance of p65 C-LOK in favor of p115 C-LOK suggests that p65 C-LOK is generated later during apoptosis than p115 C-LOK, apparently at the expense of the latter. Taken together, our results suggest that LOK is first cleaved by several caspases to generate p115 C-LOK, which is then cleaved, likely by caspase 7, to generate p65 C-LOK. The N-terminal counterpart of the p115 C-LOK fragment could be p50 N-LOK, detected with the FLAG-tagged protein.

Caspase Cleavage of LOK at the DAVD332 Site—In the mouse and human LOK sequences, we identified a consensual caspase site (DAVD332) between the kinase and coiled-coil domains. Cleavage at this site should generate a 50-kDa N-terminal fragment and a 115-kDa C-terminal fragment (Fig. 4A). To assess the functional role of this site, aspartic residue 332 was replaced with asparagine (DAVN332). Wild-type LOK (LOK WT), DAVN332 LOK (LOK DAVN), and kinase-dead LOK (LOK KD, mutated at the conserved DFG site) were produced in HEK-293 cells, and lysates were exposed to active caspase 3 and analyzed by Western blotting. As expected, V5-tagged LOK WT and LOK KD yielded p115 C-LOK and, to a lesser extent, p65 C-LOK when incubated with active caspase 3. This treatment failed to yield either C-terminal fragment when applied to V5-tagged LOK DAVN, which remained detectable as a full-

FIGURE 3. In vitro caspase cleavage of LOK. A and B, HEK-293 cells were transfected with a vector expressing C-terminally V5-tagged LOK (A) or N-terminally FLAG-tagged LOK (B). Total cell lysates were incubated in the absence or presence of different recombinant active caspases as indicated. Cell lysates were analyzed by Western blotting (WB) with an antibody directed against V5 (C-terminal end) or FLAG (N-terminal end). The membrane was stripped and reprobed with an antibody against ERK2 as a loading control.

Caspase Cleavages of LOK

FIGURE 2. Caspase-dependent cleavage of LOK during apoptosis. A, Jurkat cells were treated with 50 μM anisomycin (Aini) for 8 h. Cells were treated or not with the pan-caspase inhibitor Z-VAD-fmk (ZVAD, 20 μM) or Q-VD-OPh (QVD, 20 μM) for 30 min before apoptosis treatment. C-term, C-terminal; WB, Western blotting. B, peripheral blood mononuclear cells (PBMC) were treated with staurosporine (1 μM) for 8 h with or without the pan-caspase inhibitor Z-VAD-fmk (20 μM). A and B, cell lysates were analyzed by Western blotting with an antibody directed against the C-terminal domain of LOK. The membrane was stripped and reprobed sequentially with an anti-PARP antibody and finally with an anti-ERK2 antibody as a loading control. Molecular masses are added, and arrowheads indicate the positions of full-length LOK and LOK fragments, PARP, cleaved PARP, and ERK2. The Western blot are representative of at least three experiments. The sizes of molecular weight markers are shown on the left of each blot. C, Jurkat cells were treated or not with the pan-caspase inhibitor z-VAD-FMK (20 μM) or Q-VD-OPh (20 μM) for 30 min before apoptosis induction with 50 μM anisomycin for 8 h. Cell lysates were analyzed by Western blotting with an antibody directed against the C-terminal domain of LOK. Molecular masses are added, and arrowheads indicate the positions of full-length LOK and LOK fragments. Luminescence from the Western blot was captured with a charge-coupled device camera, and the expression levels of the full-length LOK were quantified. The percentage of expression was calculated using the untreated control as reference (n = 3, mean ± S.D.). D, for each condition, caspase 3 activity was measured and expressed as relative fluorescent units (RFU) of degraded substrate.
length protein (Fig. 4B). Consistently, caspase 3 treatment of FLAG-tagged LOK DAVN failed to generate p50 N-LOK (Fig. 4C). It is worth noticing that N-terminal FLAG-tagged LOK is detected at the apparent molecular size of about 130 kDa, whereas endogenous or C-terminal V5 tagged version are detected at about 170 kDa, suggesting that the N-terminal tag modifies conformation and migration of the protein.

We next examined whether LOK is processed at this site in HEK-293 cells undergoing apoptosis. HEK-293 cells exogenously producing FLAG-tagged LOK WT or LOK DAVN were incubated with staurosporine, and cell lysates were analyzed by Western blotting (Fig. 4D). FLAG-tagged LOK WT, but not FLAG-tagged LOK DAVN, was found to yield the p50 N-LOK fragment under apoptotic conditions. It is worth noting that LOK DAVN gave rise to a weak signal at 90 kDa both in vitro (Fig. 4C) and in apoptotic HEK-293 cells (Fig. 4D). Although p65 C-LOK, generated by the second caspase cleavage of LOK, was not detected when the DAVD site was mutated, this 90-kDa fragment is probably the N-terminal counterpart of p65 C-LOK. In conclusion, our results indicate that the main caspase cleavage of LOK occurs between the kinase domain and the coiled-coil domain, at the DAVD332 caspase site.

The p50 N-LOK Fragment Generated during Apoptosis Is an Inactive Kinase—To evaluate the kinase activity of the p50 N-terminal fragment generated upon LOK cleavage, which contains the entire kinase domain (Fig. 5), we expressed, in Jurkat cells, constructs encoding the following GFP-fused fragments: the fragment extending from the N terminus to the DAVD332 caspase site (GFP-LOK 332), a kinase-dead version of this fragment mutated at the DFG site (GFP-LOK 332 KD), and

FIGURE 4. Identification of caspase cleavage sites in LOK. A, Schematic of the LOK protein showing the putative caspase cleavage site DAVD332 located between the kinase and the coiled-coil mouse (mo) and human (hu) domains. B, HEK-293 cells were transfected with a vector expressing a C-terminally V5-tagged LOK (LOK WT, LOK DAVN, or LOK KD, kinase-dead). Total cell lysates were incubated in the absence or presence of recombinant active caspase 3. C, control; WB, Western blotting. C, HEK-293 cells were transfected with a vector expressing an N-terminally FLAG-tagged LOK (LOK WT or LOK DAVN332). Total cell lysates were incubated in the absence or presence of recombinant active caspase 3. D, HEK-293 cells were transfected with a vector expressing an N-terminally FLAG-tagged LOK (LOK WT or LOK DAVN332). HEK-293 cells were treated with staurosporine for 6 h. B—D, cell lysates were analyzed by Western blotting with an antibody directed against V5 (C terminus) or FLAG (N terminus). The membrane was stripped and reprobed with an antibody against ERK2 as a loading control.
a longer fragment extending from the N terminus to Ile^{442} (GFP-LOK 422). Analysis of GFP expression by flow cytometry showed that all three constructs were expressed similarly (Fig. 6A). Flow cytometry with anti-phospho-ERM antibody was used to estimate the ability of these constructs to phosphorylate ERM proteins. LOK substrate phosphorylation was higher in cells expressing the GFP-LOK 432 construct than in control cells expressing the GFP construct. In contrast, neither GFP-LOK 332 nor GFP-LOK 332 KD were found to increase ERM phosphorylation, suggesting that both of these fragments are inactive kinases. When the cells were distributed into four groups corresponding to increasing ranges of GFP fluorescence intensity (and, hence, GFP construct expression), the level of ERM phosphorylation was found to depend on the level of GFP-LOK 422 construct expression but to remain unaltered whatever the level of GFP-LOK 332 or GFP-LOK 332 KD expression (Fig. 6A). Next we performed in vitro kinase assays on HEK-293 cells exogenously expressing V5-tagged LOK WT, LOK 332, or LOK 332 KD. Full-length LOK efficiently phosphorylated both histone H2A and the C-terminal domain of moesin, and serial dilution of the cell extract led to decreased substrate phosphorylation. In contrast, LOK 332 WT and KD showed no or little ability to phosphorylate these substrates, despite being present at levels comparable that of full-length LOK in the cell lysates diluted 1/8 and 1/16 (Fig. 6B). This again suggests that, although caspase cleavage conserves the integrity of the kinase domain, the p50 N-LOK fragment is devoid of kinase activity.

Decreased ERM Phosphorylation during Apoptosis—Because caspase cleavages of LOK decrease the level of full-length LOK and generate a kinase-inactive fragment, we evaluated ERM phosphorylation during lymphocyte apoptosis. In a time course experiment where anisomycin was used to induce apoptosis in Jurkat cells, neither the ezrin nor the moesin level decreased during apoptosis, whereas, as expected, both LOK and PARP were cleaved (Fig. 7A). Under similar conditions, flow cytometry analysis revealed that phosphorylated ERM decreased during apoptosis, the effect being measurable at 4 h and almost total after 8 h of treatment (Fig. 7B). In culture treated for 2 h with ionomycin, isolation by cell sorting of the annexin V-positive population but negative for PI revealed that ERM phosphorylation was found in about 50% of the cells at the early stage of apoptosis compared with 80% in untreated cells. In the late state of apoptosis (both Annexin V- and PI-positive), the percentage of cells displaying ERM phosphorylation dropped to 20% (Fig. 7C). Accordingly, co-staining with anti-phospho-
ERM and anti-cleaved caspase 3 antibodies revealed, with increasing treatment time, an increasing population of cells where caspase 3 was activated and phosphorylated ERM was no longer present (Fig. 7D).

To investigate the link between LOK cleavage and ERM phosphorylation during apoptosis, we used mice where LOK was either homozygously or heterozygously knocked out (Fig. 8, A and B). Absence of LOK expression in LOK knockout mice was assessed by Western blotting (Fig. 8C). Purified splenic lymphocytes from these mice were treated for 3 h with etoposide to induce apoptosis. When living cells displaying no caspase 3 activation were compared, lymphocytes from heterozygous mice showed, as expected, a higher level of ERM phosphorylation than lymphocytes from LOK−/− mice, confirming that LOK is involved in vivo in ERM phosphorylation (Fig. 8D).

When cells undergoing apoptosis and thus displaying active caspase 3 staining were compared, lymphocytes from heterozygous and homozygous LOK knockout mice showed the same drastically reduced level of ERM phosphorylation. This demonstrates that, in apoptotic cells, in contrast to living cells, LOK is no longer involved in ERM phosphorylation. Results were similar for T cells and B cells. It is worth noting that apoptotic cells from both LOK−/− and heterozygous mice showed a lower level of ERM phosphorylation than non-apoptotic LOK−/− lymphocytes. This suggests that other kinases involved in ERM phosphorylation are also inactivated during apoptosis.

To assess the involvement of ERM phosphorylation during T cell apoptosis, we took advantage of a cell-permeable ezrin inhibitor developed recently that is able to bind directly to ezrin and to inhibit its phosphorylation at Thr-567 (21). We first demonstrated that the inhibitor of ezrin NSC668394 inhibited, in a dose-dependent manner, the ERM phosphorylation in Jurkat cells, as analyzed by flow cytometry (Fig. 9). Importantly, the decrease of ezrin phosphorylation was associated to induction of apoptosis, as measured by annexin V and PI staining (Fig. 9). This suggests that inhibition of ERM phosphorylation, induced in Jurkat cells by LOK cleavage, could in turn favor the apoptotic process.

Discussion

We demonstrate here that, during apoptosis, LOK is cleaved by caspases at the DAVID332 consensual caspase site, located between the kinase domain and the coiled-coil domain. This cleavage separates the N-terminal kinase domain (p50 N-LOK) from the C-terminal coiled-coil domain (p115 C-LOK). A second cleavage occurs in the C-terminal fragment to generate a smaller C-terminal fragment of 65 kDa (p65 C-LOK). Mutation of potential cleavage sites (LEQD646, QDRD648, LRLD694, and LDRD696) failed to abrogate this second cleavage (data not shown), demonstrating that the cleavage occurs in another site.

The results of time course experiments reveal that this second cleavage occurs after cleavage at the DAVID332 site. Mutation of this site abolishes the generation of p50 N-LOK and p115 C-LOK and partially prevents the second cleavage, confirming sequential cleavage of LOK in the course of apoptosis.

The results of our in vitro cleavage assays performed with purified caspases indicate that caspases 3, 7, 8, and 9 can cleave LOK at the DAVID332 site. The effector caspases 3 and 7 induced stronger cleavages than the initiator caspases 8 and 9, suggesting that LOK is a preferential substrate of the effector caspases. Accordingly, the optimal motif for caspase 3 and 7 cleavage is a DEVD sequence close to the DAVID site identified for LOK. The notion that several caspases may contribute to cleavage at the DAVID332 site is supported by our observation that the pan-caspase inhibitor Z-VAD-fmk, in contrast to Q-VD-Oph, whose inhibition spectrum is broader (22), only reduces this cleavage.

In contrast, only caspase 7 was able to cleave LOK at both the DAVID332 site and at the additional site involved in the generation of the p65 C-LOK fragment from the p115 C-LOK. The observation that p65 C-LOK appears later during apoptosis than p115 C-LOK is consistent with the fact that caspase 7 is an effector caspase. Although caspase 3 is also an effector caspase, its high p115 C-LOK-generating activity suggests that it might intervene fairly early in the process.

Caspase cleavage at the DAVID332 site of LOK generates an N-terminal fragment (p50 N-LOK or LOK332) comprising the entire kinase domain. However, this fragment is devoid of kinase activity because it cannot phosphorylate ERM in transfected cells or either histone H2A or the moesin C-terminal domain in in vitro kinase assays. In contrast, we have shown previously and confirmed in this study that the longer fragment extending from the N terminus to amino acids 422 (LOK422) is an active kinase (5). This suggests that the sequence between amino acids 332 and 422 is a regulatory domain. Interestingly, resolution of the LOK crystal structure has shown that the LOK kinase domain can dimerize, possibly causing trans-phosphorylation of the kinase and activation of its catalytic activity (3). This suggests that LOK might activate itself. Consistently, both full-length LOK and LOK422 display constitutive kinase activity (Fig. 4A). The fact that p50 N-LOK (LOK332) displays no kinase activity suggests that the region following the kinase domain is important in regulating the catalytic activity of the protein by promoting LOK dimerization, for instance.

Interestingly, although LOK becomes inactive when cleaved, several members of the STE 20 family are reported to be activated by caspase cleavages. For instance, caspase cleavage of SLK or MST1 after the kinase domain generates an N-terminal fragment displaying constitutive kinase activity and playing a
direct role in the regulation of apoptosis. This fragment resembles the p50 N-LOK fragment in that it comprises the entire kinase domain but differs from p50 N-LOK by the presence of some extra residues: 109 for SLK and 7 for MST (Fig. 5). The MST1 kinase fragment can phosphorylate histone H2A, an event that, in lymphocytes, participates in chromatin condensation, a hallmark of apoptotic cell death (18, 19). As for the caspase-cleavage-generated SLK kinase fragment, it has been
shown to promote apoptosis, in contrast to that of its kinase-dead version. This suggests that the constitutive kinase activity of this fragment participates actively in apoptosis (16, 17).

The fact that p50 N-LOK displays no kinase activity suggests that the usual targets of LOK are no longer phosphorylated in apoptotic cells. LOK has recently been shown to display high specificity for the ERM phosphorylation sequence and to phosphorylate the ERM proteins in lymphocyte cell lines. Genetic evidence confirms this because hematopoietic cells from LOK-deficient mice display decreased ERM phosphorylation (5), in contrast to mature lymphocytes from SLK-deficient mice. Accordingly, we find here that, in the course of apoptosis, ERM

Figure 7. ERM phosphorylation in Jurkat cells undergoing apoptosis. A, Jurkat cells were treated with anisomycin (50 μM) for the indicated time. Cell lysates were analyzed by Western blotting with an antibody directed against the C-terminal domain of LOK. The membrane was stripped and reprobed sequentially with an anti-ezrin antibody and an anti-PARP antibody. Similar extracts were analyzed by Western blotting (WB) with an antibody directed against moesin. The membrane was stripped and reprobed with an antibody directed against moesin. B, Jurkat cells were treated with anisomycin (50 μM) for the indicated time. ERM phosphorylation was detected by flow cytometry with an antibody directed against phosphorylated ERM (Phospho ERM). C, Jurkat cells were treated for 2 h with 50 μM anisomycin and stained with Annexin V and PI. Cells were separated in three populations (AV+/PI−, AV−/PI−, and AV−/PI+) by cell sorting. Each population was then stained with antibody directed against phosphorylated ERM (Phospho ERM). The percentage of positive cells for ERM phosphorylation was shown for each population (n = 3, mean ± S.D.). D, Jurkat cells were treated with anisomycin (50 μM) for the indicated time. Caspase 3 activation and ERM phosphorylation were detected by flow cytometry using an antibody directed against cleaved caspase 3 and the phosphorylated form of ERM, and T cells were separated by CD4 and CD8 detection. Phosphorylation of ERM is shown in positive and negative population for caspase 3 staining. Presented flow cytometry graphs are representative of obtained results from three mice of each genotype.

Figure 8. ERM phosphorylation in apoptotic lymphocytes from LOK-deficient mice. A, strategy for generating the targeted lok allele is described. Black boxes represent exons and open arrows LoxP sites. The position of the primers is marked by black arrows. B, to confirm deletion of exons 3 and 4, PCRs were performed with primers p1 and p2 amplifying fragment of 571 bp from wild type (lok+/+) allele and primers p3 and p4 amplifying fragment of 646 bp from LOK KO (lok−/−) allele with deletion of exons 3–4 and Neo between two LoxP sites. C, LOK is predominant in lymphocytes. Cell lysates were analyzed by Western blotting (WB) with an antibody directed against mouse LOK in splenocytes. The membrane was stripped and reprobed with an antibody against actin as a loading control.

D, lymphocytes were isolated from spleen of LOK deficient mice (LOK−/−) and heterozygous mice (LOK+/−). Splenic lymphocytes were treated for 3 h with 10 μM etoposide. Caspase 3 activation and ERM phosphorylation were detected by flow cytometry using an antibody directed against cleaved caspase 3 and the phosphorylated form of ERM, and T cells were separated by CD4 and CD8 detection. Phosphorylation of ERM is shown in positive and negative population for caspase 3 staining. Presented flow cytometry graphs are representative of obtained results from three mice of each genotype.
phosphorylation decreases concomitantly with LOK cleavage, whereas the ezrin and moesin levels remain unchanged. Interestingly, although non-apoptotic T-cells from LOK-deficient mice show a lower level of ERM phosphorylation than those of control mice, apoptotic T-cells from control and LOK-deficient mice show a similar low level of ERM phosphorylation. LOK thus no longer seems to participate in ERM phosphorylation during apoptosis. Furthermore, because the ERM phosphorylation level is lower in apoptotic cells than in non-apoptotic LOK-deficient cells, it would seem that one or more other kinases, likely inactivated during cell death, might participate in ERM phosphorylation. Because ERMs are known to be phosphorylated by several other kinases (6), it would be interesting to evaluate their activities in apoptotic lymphocytes.

Phosphorylation of the conserved threonine residues located in the ERM actin-binding domain participates in ERM activation to a phosphorylated form displaying an open conformation. Active ERM can create a link between the plasma membrane and the cytoskeleton through interaction with membrane phospholipids or proteins such as CD44 and ICAM and with cortical actin filaments. In resting lymphocytes, ERM phosphorylation contributes to cell rigidity and to the maintenance of microvilli (23), whereas, in response to chemokines, dephosphorylation of ERM participates in a transition to a flexible, migrating cell form (10). The involvement of ERM phosphorylation in the apoptotic process is poorly understood. Because ERM phosphorylation is drastically reduced upon LOK cleavage by caspases, we speculated that this dephosphorylation could play an active role during cell death. A selective inhibitor of ezrin, able to directly bind its target (21), induced a dose-dependent decrease of ERM phosphorylation. Interestingly, ezrin dephosphorylation is associated with induction of T-cell line apoptosis, suggesting that dephosphorylation of ERM could play a direct role in the regulation of apoptotic cell death. A similar induction of apoptosis with the ezrin inhibitor was recently reported in several diffuse large B cell lymphoma cell lines (24). The drastic dephosphorylation of ERM during lymphocyte apoptosis suggests that membrane rigidity is disrupted during apoptosis. In adherent cells, extensive plasma membrane reorganization plays an active role in the apoptotic process because apoptotic adherent cells display membrane blebbing, which depends upon activation of the myosin light chain via its phosphorylation by Rho-activated protein kinase 1, which is cleaved to a constitutively active form by caspases (25, 26). It has been proposed that membrane blebbing leads ultimately to cell fragmentation and that surface blebs are the progenitors of apoptotic bodies. Loss of membrane rigidity in apoptotic lymphocytes upon LOK inactivation and subsequent ERM dephosphorylation may also be a crucial step in the profound cell morphology changes observed in the course of apoptotic cell death.

Many kinases are cleaved by caspases, and most of them participate in the apoptotic process through the generation of active fragments (27). As mentioned above, cleavages of MST1, SLK, and Rho-associated protein kinase 1 generate active kinase fragments that can phosphorylate specific substrates to promote chromatin condensation or membrane blebbing, for instance. Caspase cleavages can also abolish kinase activity, but the generated fragments acquire novel properties involved in apoptosis. For instance, we have shown recently that the kinase fragment of the receptor tyrosine kinase Met, generated during apoptosis, is devoid of kinase activity but can promote mitochondrial permeabilization involved in cell death amplification (28). Therefore, beside the consequence of LOK caspase cleavage on ERM phosphorylation, LOK fragments could also play an active role during this process.

**Caspase Cleavages of LOK**

**FIGURE 9. Consequences of the ezrin inhibitor on ERM phosphorylation and induction of apoptosis.** Jurkat cells were treated overnight with rising concentrations of the ezrin inhibitor NSC668394 or left untreated. ERM phosphorylation was then detected by flow cytometry with an antibody directed against phosphorylated ERM (Phospho ERM). In parallel, apoptosis was evaluated by staining with Annexin V (FITC, green fluorescence) and PI (red fluorescence).

**Author Contributions**—D. T. conceived and coordinated the study and wrote the paper. C. L. designed, performed, and analyzed the experiments. N. V. B. and S. S. designed, performed, and analyzed the experiments shown in Figs. 6 and 8. T. L. and C. D. constructed tagged and mutated versions of LOK. E. D. performed and analyzed flow cytometry and cell sorting. All authors reviewed the results and approved the final version of the manuscript.
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