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Activation of Hematopoietic Progenitor Kinase 1 Involves Relocation, Autophosphorylation, and Transphosphorylation by Protein Kinase D1

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Received 14 June 2004/Returned for modification 23 July 2004/Accepted 16 December 2004

Adaptive immune signaling can be coupled to stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and NF-κB activation by the hematopoietic progenitor kinase 1 (HPK1), a mammalian hematopoiesis-specific Ste20 kinase. To gain insight into the regulation of leukocyte signal transduction, we investigated the molecular details of HPK1 activation. Here we demonstrate the capacity of the Src family kinase Lck and the SLP-76 family adaptor protein Clnk (cytokine-dependent hematopoietic cell linker) to induce HPK1 tyrosine phosphorylation and relocation to the plasma membrane, which in lymphocytes results in recruitment of HPK1 to the contact site of antigen-presenting cell (APC)–T-cell conjugates. Relocation and clustering of HPK1 cause its enzymatic activation, which is accompanied by phosphorylation of regulatory sites in the HPK1 kinase activation loop. We show that full activation of HPK1 is dependent on autophosphorylation of threonine 165 and phosphorylation of serine 171, which is a target site for protein kinase D (PKD) in vitro. Upon T-cell receptor stimulation, PKD robustly augments HPK1 kinase activity in Jurkat T cells and enhances HPK1-driven SAPK/JNK and NF-κB activation; conversely, antisense down-regulation of PKD results in reduced HPK1 activity. Thus, activation of major lymphocyte signaling pathways via HPK1 involves (i) relocation, (ii) autophosphorylation, and (iii) transphosphorylation of HPK1 by PKD.

Innate and adaptive immune functions are governed by instructive signals from receptors that sense pathogens and toxins. Ligation of lymphocyte antigen receptors triggers a series of characteristic events that is initiated by activation of Src family tyrosine kinases followed by recruitment and activation of Syk and BTK family nonreceptor tyrosine kinases (18). The ensuing tyrosine phosphorylation provides docking sites for SH2 and PTB domains on adaptor proteins and enzymes, which assemble into a spatially highly defined complex that will ultimately cause lymphocyte activation. For instance, in T cells, the adaptor proteins SLP-76 and Gads (Grb2-related adaptor downstream of Shc) are recruited to the transmembranous adaptor LAT, forming a central scaffold for the activation of several effector molecules, such as phospholipase Cγ (PLCγ), Ras, and Rho GTPases (15). These events are followed by the activation of a number of different serine/threonine kinases, which include members of the protein kinase B (PKB), PKC, and PKD families as well as mitogen-activated protein 3 (MAP3) and MAP4 kinases, such as mitogen-activated protein kinase kinase 1 (MEKK1) and hematopoietic progenitor kinase 1 (HPK1). Further downstream, mitogen-activated protein kinases (MAPKs) which contribute to the immune response are efficiently triggered; for instance, stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs) have been shown to regulate critical steps in T-helper-cell differentiation (8). Transcription factors of the NF-κB family constitute an additional major pathway involved in the regulation of lymphocytes and execution of immune functions. NF-κB transcription factors determine life and death decisions in developing lymphocytes and also provide important costimulatory signals in T cells (16).

Lymphocyte receptor signaling can be coupled to SAPK/JNK and NF-κB activation by HPK1 (18). HPK1 is a mammalian hematopoiesis-specific Ste20 homologue belonging to the germinal center (GC) kinase family, which contains at least 20 kinases (6). HPK1 is characterized by an N-terminal kinase domain, followed by an intermediate region and a C-terminal Citron homology domain. Both the N-terminal kinase domain and the C-terminal Citron homology domain are highly conserved among related kinases of the GC family. In the GC family, HPK1 is most closely related to germinal center kinase (GCK), germinal center-like kinase (GLK), and germinal center-related kinase (GCKR), which together form subfamily I of GCKs (19). The biological function of these Ste20 homologues is largely elusive. GCK and GCKR have been implicated in tumor necrosis factor receptor signaling (36, 42). More recently, HPK1 has been shown to be a positive regulator of cell death in T cells (35).

HPK1 has been demonstrated to be a potent and highly specific activator of the SAPKs/JNKs in various cell types, a property shared with the related GCK family members (18, 19). HPK1 likely acts at the level of a MAP4 kinase, resulting in the activation of MAP3 kinases, such as MLK3 or MEKK1, which signal through the well-established MKK4/7 SAPK/JNK
Enhances HPK1 activity upon T-cell receptor engagement, transphosphorylation of serine 171 by PKD1 is prerequisite for autophosphorylation site, serine 171 is efficiently phosphorylated and serine 171. While threonine 165 probably represents an enzymatic activation of HPK1 requires three proline-rich motifs that have been shown to mediate constitutive association with SH3 domain-containing adaptor proteins, such as Grb2 (1), HS1 (30), Crk/Crkl (24, 31), Gads (Grb2-related adaptor downstream of Shc) (22, 26), Bam32/DAPP1 (10), or SH3P7/HPK-55 (9). Recently, it has been shown that HPK1 in association with SH3P7/HPK-55 is recruited to the immunolabile synapse and contributes to the down-regulation of T-cell receptor (TCR) signaling (20).

The serine/threonine kinase PKD1 is the most predominant PKD isoform expressed in mouse T cells (27). In T cells, B cells, and mast cells, PKDs are strongly activated by antigen receptor stimulation but not by costimulatory receptors, chemokines, or cytokines (29). Upon stimulation which depends on the action of protein kinases C (PKCs) and PLCs, PKD can be regulated by its cellular location and context (27).

Because HPK1 is potently activated by immunoreceptors and is a highly selective signal mediator on the road to SAPK and NF-kB activation, PKD1 is an important candidate for the fine tuning and orchestration of immunoreceptor signaling in leukocytes. Despite the increasing evidence for a function of PKD1 in lymphocyte signaling, the molecular mechanism of its activation remains obscure. A more profound understanding of the details of HPK1 activation promises important insights into the regulation of leukocyte function. We show here that the Src family kinase Lck is capable of localizing HPK1 to the plasma membrane. After immunoreceptor stimulation, HPK1 relocates to the contact site of antigen-presenting cell (APC)–T-cell conjugates. Relocation is accompanied by tyrosine phosphorylation and adaptor protein binding and results in enzymatic activation of HPK1. Full activation depends on phosphorylation of key regulatory sites in the HPK1 kinase activation loop; these include threonine 165 and serine 171. While threonine 165 probably represents an autophosphorylation site, serine 171 is efficiently phosphorylated by protein kinase D1 (PKD1). We provide evidence that transphosphorylation of serine 171 by PKD1 is prerequisite for full activation of HPK1. Furthermore, in Jurkat cells, PKD1 enhances HPK1 activity upon T-cell receptor engagement, while antisense down-regulation of PKD1 results in reduced HPK1 inducibility. This identifies PKD1 as a physiological regulator of HPK1 and hence of PKD1-driven SAPK/JNK and NF-kB activation.

MATERIALS AND METHODS

Cell culture and stimulation. Cos1 and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Sigma or Bethesda Research Laboratories), 100 μg of penicillin (Life Technologies, Inc.) per ml, 100 μg of streptomycin (Life Technologies, Inc.) per ml, and 20 mM L-glutamine (Life Technologies, Inc.). Jurkat T cells, mouse T-cell hybridoma DC27.1, DO11.10 T cells, and A20 B cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum ( Gibco/BRL), 100 μg of penicillin (Life Technologies, Inc.) per ml, 100 μg of streptomycin (Life Technologies, Inc.) per ml, 20 mM L-glutamine (Life Technologies, Inc.), and 0.001% monothioglycerol (Sigma). For TCR stimulation, 10^7 T cells were resuspended in phosphate-buffered saline (PBS) together with 2 μg of anti-CD3 antibody (OKT3 for Jurkat T cells and 145-2C11 [BD Bioscience] for DC27.1 T cells) per ml and 20 μg of soluble protein A per ml at 37°C for 3 min.

Transient transfections and NF-κB reporter assays. Luciferase reporter assays were performed as previously described (2). All experiments were repeated at least three times. In the figures, bars depict the averages of duplicate transfection experiments. All values presented in a single diagram are derived from a single transfection experiment. Jurkat or DC27.1 T cells (10^7) were transiently transfected by electroporation (250 V, 950 μF) in 500 μl of cell culture medium using 20 to 30 μg of total DNA. 293T cells were transfected by the CaCl2 transfection method. For in vitro kinase assays, cells were harvested 48 h after transfection.

Immunoblotting, in vitro kinase assays, and SAPK activation. HPK1 and SAPK kinase assays were performed as described previously (17). Polyclonal rabbit sera for anti-HPK1 have been described previously (2). The hemagglutinin (HA) tag was detected using the mouse monoclonal antibody 12CA5 or sc-7392 (Santa Cruz Biotechnology). Phosphoryrosine was detected using mouse monoclonal antibody 4G10 (Upstate Biotechnology). PKD was detected using polyclonal antibody D-20 (Santa Cruz Biotechnology).

Immunofluorescence staining. Cost cells were seeded onto glass coverslips in six-well plates and transfected using the CaCl2 phosphate coprecipitation method with 2 to 5 μg of expression plasmid per well. Raft-like membrane patches were stained by 10-min incubation on ice with Alexa Fluor 594-conjugated cholera toxin subunit B (diluted 1:1,500 in PBS plus 0.1% bovine serum albumin [BSA] [Molecular Probes, Eugene, Oreg.]). After the cells were washed, they were fixed at room temperature for 30 min using PBS containing 4% formaldehyde and washed repeatedly.

For immunofluorescence staining, cells were washed, fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked in PBS containing 5% BSA. Cells were then incubated with either specific antisera for Lck or Chk or a monoclonal antibody against the HA tag (Santa Cruz Biotechnology) for 1 h at 37°C and then washed. Proteins were visualized by incubation with appropriate fluorochrome-conjugated secondary antibodies for 1 h at 37°C. Cell nuclei were counterstained with 2 μg of Hoechst 33258 (Sigma) per ml in PBS for 15 min on ice. Cells were embedded in fluorescence mounting medium (Mowiol) and observed by epifluorescence microscopy.

Cell conjugation analysis. A20 B cells were stained with 1 μM cell tracker green (Molecular Probes) and pulsed with OVA (ovalbumin and ovalbumin) peptide or left alone. A20 cells were then mixed with DO11.10 T cells in a 2:1 ratio and incubated for different times at 37°C. The conjugates were gently resuspended in 4% paraformaldehyde and fixed on poly-l-lysine-coated slides for 30 min. Cells were washed, permeabilized in 0.5% Triton X-100, blocked with PBS containing 2.5% BSA, and stained with anti-HPK1 and anti-CD3 antibodies, followed by anti-rabbit Cy5-labeled and anti-rat fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.). Samples were embedded in Mowiol mounting medium and analyzed by confocal laser scanning microscopy (Leica TCS SP, Heidelberg, Germany).

Phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping. Two-dimensional tryptic phosphopeptide mapping or phosphoamino acid analysis of phosphorylated HPK1 proteins using Hunter thin-layer electrophoresis system HTLE-7000 (C.B.C. Scientific Company, Inc.) was performed closely adhering to the published protocols (4). For two-dimensional peptide maps, peptides were electroblotted in an acidic buffer system (pH 1.9) and subsequently separated orthogonally by ascending thin-layer chromatography (TLC) in phosphor chromatography buffer. Phosphopeptides were visualized by autoradiography. Phosphopeptide spots subjected to phosphoamino acid analysis were marked on TLC plates, and the corresponding cellulose was scraped off using a pipette.
filter tip. The peptides were eluted sequentially with acidic buffer (pH 1.9) and pyridine diluted in acidic buffer (pH 1.9). After the peptides were hydrolyzed in 6 N HCl, the resulting amino acids were separated on TLC plates by two-dimensional electrophoresis using an acidic buffer system (pH 1.9 and 3.5) and exposed to X-ray film. The positions of the different phosphoamino acids were identified by overlay with a reference phosphoamino acid standard stained with ninhydrin (0.25% [wt/vol] in acetone).

**RESULTS**

In Cos1 cells, HPK1 is localized to the cytoplasm and tends to accumulate in intensely fluorescing structures upon overexpression. To better understand the details of HPK1 activation, we investigated the intracellular localization of transiently expressed HA-tagged HPK1 (HPK1-HA) in Cos1 cells by immunofluorescence staining. Cells expressing low, medium, or high levels of HPK1-HA (arrowheads) are identified. Cells expressing low, medium, or high levels of EGFP-HPK1291-827 are shown in panel i, k, or l, respectively.
was excluded from the nucleus (Fig. 1a and b). At intermediate levels of expression of HPK1, the protein appeared to localize in a multitude of small fluorescent spots throughout the cytoplasm, while at high levels of expression, HPK1 was mainly confined to large, intensely fluorescing clusters that were often located perinuclearly (Fig. 1b and c). Interestingly, upon appearance of the intensely fluorescing structures, the remainder of the cytoplasm appeared completely clear of HPK1 (see Fig. 4B). These clusters may have consisted either of vesicles highly loaded with ectopically expressed protein or of protein aggregates forming as a consequence of ectopic overexpression. Antibody specificity was controlled by staining of nontransfected cells; furthermore, in transfected cell cultures, HPK1-expressing cells were unambiguously discerned from nontransfected cells (Fig. 1).

We hypothesized that the proline-rich regions and tyrosine 379 located in the central part of HPK1, which have been shown to act as protein interaction motifs predominantly direct its intracellular localization. To test this assumption, we generated the truncation mutant EGFP-HPK1291-827 in which the kinase domain has been replaced by the enhanced green fluorescent protein (EGFP) (schematically depicted in Fig. 1). Transient expression of EGFP alone in Cos1 cells resulted in a homogeneously lower level of expression (Fig. 1). In contrast, EGFP-driven mislocalization or minor amounts of EGFP generated by proteolysis lead to the generation of EGFP-driven mislocalization or minor amounts of EGFP generated by proteolysis, which has previously been reported to be cleaved by caspases in the central hinge region (2). To assess the extent of EGFP-HPK1291-827 fusion protein cleavage leading to the generation of EGFP or proteolytic degradation, we performed Western blotting of transfected Cos1 cells expressing EGFP-HPK1291-827. Antiserum directed against the C terminus of HPK1 detected mainly the full-length fusion protein, demonstrating that in transfected Cos1 cells, cleavage is of little importance (Fig. 2A). To test for proteolytic degradation, we used antiserum against the EGFP part of the fusion protein and again detected full-length EGFP-HPK1291-827 as the predominant species (Fig. 2A), although deliberate overexposures disclosed the presence of small amounts of degradation products. The excellent coincidence between the cytoplasmic HPK1-HA staining and EGFP-HPK1291-827 fluorescence demonstrated that the intracellular localization of HPK1 is governed by sequence motifs C terminal to the kinase domain and argued against a significant contribution of the EGFP part of the fusion protein to the observed localization. However, the residual nuclear localization of EGFP-HPK1291-827 may be due to EGFP-driven mislocalization or minor amounts of EGFP generated by proteolysis. In summary, upon forced expression in Cos1 cells, the locations of full-length HA-tagged HPK1 and EGFP-HPK1291-827 fusion protein were largely identical.

Src family kinases and SLP-76 family adaptor proteins induce HPK1 tyrosine phosphorylation and translocation to the plasma membrane. In B and T cells, activation of HPK1 relies
on the presence of a SLP-76 adaptor protein (34). Phosphorylation of tyrosine 379 (Y379), which is recognized by SLP-76 adaptor protein SH2 domains, is a prerequisite for HPK1 kinase stimulation in lymphocytes and a mutant with the tyrosine 379 to phenylalanine substitution (Y379F) fails to become enzymatically activated after immunoreceptor ligation (38). Upon forced expression in epithelial cells, HPK1 is constitutively active, irrespective of the presence of SLP-76 family adaptors, which are not expressed in this cell type, and phosphorylation of Y379 is dispensable for HPK1 activity. Upon overexpression in 293T cells, kinase activity of the HPK1 (Y379F) mutant is indistinguishable from wild-type HPK1.

When we probed wild-type HPK1 expressed in Cos1 cells for phosphotyrosine, we detected no evidence of tyrosine phosphorylation (Fig. 2B). However, coexpression of an activated form of the T-cell-specific Src family kinase Lck resulted in low-level tyrosine phosphorylation, which was profoundly enhanced in the presence of Lck and the SLP-76 family adaptor protein Clnk (Fig. 2B). These results indicated that HPK1 can be phosphorylated by Src family kinases and that the SLP-76 family adaptor protein Clnk facilitates HPK1 phosphorylation by Lck. As Src family kinases are constitutively membrane associated, Clnk might contribute to the localization of HPK1 to the plasma membrane. This relocation step appears to be an essential prerequisite for HPK1 activation in leukocytes but is dispensable in epithelial cells, possibly because the protein is clustered in the cytoplasm.

So far, the intracellular localization of HPK1 in resting and activated cells has not been investigated. We decided to address this question and determine in particular the effects of proteins necessary for HPK1 activation in lymphocytes on its subcellular localization. To more precisely assess the effects of Lck and Clnk on the intracellular localization of HPK1, we coexpressed HPK1-HA with either Clnk or Lck or both in Cos1 cells. Using immunofluorescence microscopy, we classified transfectants as expressing low, medium, or high levels of HPK1 (in accordance with Fig. 1); for at least 20 cells of each class, we visually judged the fluorescence intensity in the nucleus, cytoplasm, and cytoplasmic aggregates and at the plasma membrane. Each intracellular location was attributed either a weak, intermediate, or strong fluorescence signal, and the average fluorescence at the different localizations was plotted for the different proteins analyzed. Throughout the analysis, staining specificity was controlled on nontransfected samples.

When Clnk was expressed alone, we found Clnk persistently localized to the plasma membrane and in small spots that were most prominent around the nucleus. Similarly, Lck was found at the plasma membrane but also in an intensely fluorescent area close to the nucleus (Fig. 3, top row, middle panel, and Fig. 4A). Interestingly, at low levels of expression, coexpression of HPK1 and Clnk redirected both proteins into cytoplasmic aggregates and cause a decrease in Clnk membrane localization (Fig. 4C). At intermediate and high levels of expression, HPK1 remained in cytoplasmic aggregates, and again membrane localization of Clnk was reduced (Fig. 3 and 4C). Taken together, Clnk alone was inefficient in localizing HPK1 to the plasma membrane, while HPK1 appeared to divert Clnk away from the plasma membrane. In contrast, coexpression with Lck resulted in HPK1 membrane recruitment even at low levels of expression; at intermediate and high levels of expression, HPK1 and Lck largely colocalized (Fig. 3 and 4D). Simultaneous expression of all three proteins resulted in significant colocalization at all levels of expression analyzed (Fig. 3 and 4E). Membrane localization of HPK1 was again most noticeable at low levels of expression. While cytoplasmic and membrane localization were balanced in cells expressing low levels of HPK1, increasing levels of expression of HPK1 resulted in a concentration of all three proteins in spots in the cytoplasm. Interestingly, in this group, a high proportion of HPK1-expressing cells had already undergone apoptosis 24 h after transfection. From our analysis, it appeared that at low levels of expression of HPK1, which best reflect natural HPK1 levels in lymphocytes, Lck was most efficient in directing HPK1 to the plasma membrane. In Cos1 cells, HPK1 was, at least to some degree, capable of directing the localization of Clnk, arguing in favor of an interaction between both proteins. Surprisingly, in Cos1 cells, localization of the HPK1(Y379F) mutant was similar to that of the wild-type protein (not shown).

The HPK1 kinase domain does not contribute to the subcellular localization. To further substantiate our finding that HPK1 localization is governed by the central and C-terminal portions, we repeated our analysis by coexpressing EGFP-HPK1291-827 with either Clnk or Lck or both in Cos1 cells. As observed for the HA-tagged full-length protein, coexpression of Clnk and EGFP-HPK1291-827 resulted in colocalization of both proteins in spots in the cytoplasm, even at low levels of expression (Fig. 5C), but little plasma membrane association of HPK1. Again, Lck more efficiently recruited EGFP-HPK1291-827 to the plasma membrane (Fig. 5D). The presence of either Clnk or Lck strongly reduced nuclear localization of the fusion protein. Simultaneous expression of all three proteins resulted in higher membrane association than coexpression with Lck alone (Fig. 3 and 5E). At intermediate and high levels of expression of HPK1, the cytoplasmic and membrane-associated proteins disappeared in favor of cytoplasmic aggregates that appeared to include all three proteins. Of note, in this group, there was also a high prevalence of HPK1-expressing cells that had already undergone apoptosis 24 h after transfection.

The virtually identical intracellular distributions of HAtagged full-length HPK1 and EGFP-HPK1291-827 fusion protein suggested that the intracellular localization of HPK1 is dominated by sequences located between amino acids 291 and 827. Apparently, Lck is more efficient in recruiting HPK1 to the plasma membrane than Clnk. Nevertheless, when these proteins are present simultaneously, these proteins colocalize, and through the concerted action of SLP-76 family adaptors and Src and/or Syk family kinases, HPK1 is recruited into a membrane-proximal position where it is phosphorylated on tyrosine.

HPK1 is recruited to the interface of B- and T-cell conjugates. Does immunoreceptor activation in lymphocytes cause translocation of HPK1 to the site of receptor clustering? To directly address this question, we generated conjugates of OVA peptide-pulsed A20 B cells and DO11.10 T-cell hybridoma cells. We then visualized the distribution of endogenous HPK1 30 and 60 min after conjugate formation. HPK1 polarization in pairs of nonpulsed A20 and DO11.10 cells was not discerned. In conjugates of OVA-pulsed A20 and DO11.10 cells, we observed recruitment of HPK1 to the B-cell–T-cell

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FIG. 3. Active Lck and Clnk direct HPK1-HA or the EGFP-HPK1<sub>291-827</sub> fusion protein to the plasma membrane. Cos1 cells grown on coverslips were transfected with expression plasmids encoding either HPK1-HA, Clnk, or Lck alone (top row) or HPK1-HA in combination with Lck or Clnk or Lck and Clnk or EGFP-HPK1<sub>291-827</sub> in combination with Lck and Clnk. Twenty-four hours after transfection, cells were fixed with formaldehyde and stained with the respective antibodies. Hoechst 33258 was used to counterstain nuclei (middle column and top row). Specimens were observed with an epifluorescence microscope using a 63×/1.25 oil immersion objective, and micrographs were acquired using a Spot digital camera. Merging of green (anti-HA or anti-EGFP) and red (anti-Clnk or anti-Lck) fluorescence is depicted as overlay.
interface at 30 and 60 min after conjugate formation (Fig. 6). Somewhat unexpectedly, we detected significantly higher HPK1 levels of expression in nonpulsed A20 B cells than in DO11.10 T cells (Fig. 6, −Ova). Our analysis showed that HPK1 can be recruited in either cell type to the conjugate interface (Fig. 6, +Ova 30 min). Finally, we noted a strong decline in HPK1 immunoreactivity after conjugate initiation (compare staining intensities of the −Ova, +Ova 30 min, and +Ova 60 min groups), suggesting that HPK1 is efficiently cleared from activated lymphocytes.

Our analysis of APC–T-cell conjugates impressively demonstrated that after immunoreceptor stimulation, HPK1 was recruited to a receptor-proximal position. Efficient localization of the EGFP-HPK1291-827 fusion protein demonstrated that the central and C-terminal parts of HPK1 also suffice to direct this translocation event in T cells (not shown).

Three amino acids in the kinase domain activation loop of HPK1 are indispensable for maximal HPK1 kinase activity. GCKs, including HPK1, are constitutively active upon expression in epithelial cells. HPK1 is immunopurified from Cos1 cells in an active conformation that readily undergoes autophosphorylation in vitro. Hence, activation of HPK1 appears to occur preferentially under conditions that are associated with local clustering of the kinase, due to immunoreceptor ligation or forced expression. We hypothesized that the actual activation process may involve auto- or transphosphorylation steps, which rely on local accumulation. In the catalytically active form, all protein kinases adopt a structurally similar conformation. A loop of typically 20 to 30 amino acids localized at the center of the protein kinase domain between subdomains VII and VIII is known to regulate entry of the substrate peptide to the active site and to provide important charge interactions with the active center. For many kinases in which the catalytic aspartate is preceded by an arginine (RD kinases), phosphorylation of the activation loop results in a stable open conformation and enzymatic activation (14). On the basis of a sequence comparison with related serine/threonine kinases, we identified three potentially activating phosphoacceptor sites in the HPK1 activation loop: threonine 165, serine 171, and threonine 175 (Fig. 7A). These sites were mutated into alanine either individually or in combination, and the activity of the resulting HPK1 mutant kinases was assessed in vitro after transient expression in Cos1 cells. We have previously shown that the SLP-76 family adaptor Clnk will bind HPK1 with phosphorylated tyrosines and will also copurify with HPK1 during immunoprecipitation and is efficiently phosphorylated by HPK1 in vitro (41). Therefore, we coexpressed Clnk to provide a substrate for HPK1 kinase which directly binds to HPK1. In addition, a bacterially expressed c-Jun fusion protein (glutathione S-transferase [GST]–c-JunN) was included in the assay as an additional surrogate substrate. Auto-phosphorylation of HPK1 and transphosphorylation of the substrate proteins were assessed.

Single mutation of all three amino acids resulted in a significant reduction of HPK1 kinase activity (Fig. 7B). The highest level of residual activity was maintained by the T165A mutant, while the S171A mutant displayed less than 10% of wild-type activity and a T175A mutant was largely inactive. The activity of double mutant TS165 171AA was slightly reduced from that of the S171A single mutant, while all mutants containing the

**FIG. 4.** Active Lck dominates HPK1 localization upon overexpression in Cos1 cells. Cos1 cells grown on coverslips were transfected with expression plasmids encoding either Clnk or Lck (A) or HPK1-HA (B), HPK1-HA plus Clnk (C), HPK1-HA plus Lck (D), or HPK1-HA plus Clnk and Lck (E) as described in the legend to Fig. 3. Twenty-four hours after transfection, cells were fixed and stained with antibodies plus Clnk and Lck (E) as described in the legend to Fig. 3. Twenty-four.
A triple mutant (TST165,171,175AAA), even upon prolonged autoradiography (not shown), retained no activity and hence was indistinguishable from the published ATP-binding site K46E mutant.

FIG. 5. Intracellular localization of HPK1 is mediated exclusively by its C-terminal part in Cos1 cells. Cos1 cells grown on coverslips were transfected with expression plasmids encoding either EGFP (A) or EGFP-HPK1291-827 (B) alone or EGFP-HPK1291-827 with Clnk (C), Lck (D), or Clnk and Lck (E) as described in the legend to Fig. 3. Twenty-four hours after transfection, cells were fixed and stained with antibodies directed against Clnk or Lck. In each experimental group, successfully transfected cells were then classified as expressing low, middle, or high levels of EGFP or EGFP-HPK1291-827 as judged by their green fluorescence (Fig. 1). For at least 20 cells of each expression level, green fluorescence (GF) in the nucleus (N), cytoplasm (C), and spots in the cytoplasm (S) and at the plasma membrane (M) was graded. The level of fluorescence is indicated on the y axes as follows: weak (●), intermediate (●●), and strong (●●●). The average fluorescence intensity of the different cellular compartments was then

FIG. 6. HPK1 is recruited to the contact site of APC–T-cell conjugates. A20 B cells labeled with cell tracker green were pulsed with OVA peptide and incubated with antigen-specific DO11.10 T hybridoma cells to induce conjugate formation. Conjugates were fixed and stained for HPK1 (red) and CD3 (green). In areas where HPK1 is recruited to the contact sites, the overlay of Cy3-stained HPK1 and FITC-stained CD3 and/or cell tracker appears yellow. The negative control shows A20 cells not pulsed with OVA peptide (+ Ova); conjugate formation with OVA-pulsed A20 cells was allowed to proceed for 30 or 60 min. Representative conjugates analyzed by confocal laser scanning microscopy are shown.

T175A substitution were inactive, as was the T175A single mutant. A triple mutant (TST165,171,175AAA), even upon prolonged autoradiography (not shown), retained no activity and hence was indistinguishable from the published ATP-binding site K46E mutant.
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**HPK1**

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| in vitro kinase activity |

| anti-Clnk Western-blot | |
| HPK1 | Clnk |

| anti-HA Western-blot |
| HPK1 | |

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| in vitro kinase activity |

| 175 kDa |
| HPK1 |

| 83 kDa |
| Clnk |

| anti-HA Western-blot |
| HPK1 |
For all mutants analyzed, the relative ratio of HPK1 auto-
phosphorylation to Clnk and GST–c-Jun₅₆ transfosphorylation
remained constant, indicating that with respect to these
phosphorylation targets, HPK1 was the only active kinase
present in the assay.

**HPK1 activation in response to TCR stimulation is regulat-
ed by the kinase domain activation loop.** At this time, immu-
noreceptor stimulation is the best-characterized physiological
stimulus for HPK1. If immunoreceptor-mediated activation of
HPK1 occurred through phosphorylation of the activation loop,
activation loop mutants should be refractory to stimulation
by TCR cross-linking. To test this hypothesis, we ligated
CD3 in Jurkat T cells and determined the kinase activity of
wild-type or mutant HPK1 in vitro (Fig. 7C). Wild-type HPK1
kinase was markedly activated after CD3 cross-linking. Of the
three single amino acid activation loop mutants, only the
T165A mutant displayed a residual capacity for TCR-mediated
activation. The S171A and T175A single mutants failed to
became stimulated after TCR cross-linking. Concomitantly,
substrate phosphorylation of the coexpressed adaptor protein
Clnk was no longer detected.

We wondered whether activation loop phosphorylation of
HPK1 occurred in vivo. To address this question, we trans-
ferred Jurkat T cells with either wild-type HPK1 or the T165A
or S171A mutant. After metabolic labeling with ³²P, we ligated
the TCR and immunopurified HPK1 proteins (not shown). We
detected no significant difference in phosphate content be-
tween wild-type and mutant forms of HPK1. Even in nonstimu-
lated cells, HPK1 was already phosphorylated, suggesting that
HPK1 is phosphorylated on multiple acceptor sites in vivo.
Some of these sites are likely located outside the regulatory
loop of the kinase domain and are occupied independently of
immunoreceptor stimulation.

The capacity of HPK1 kinase activation loop mutants to
activate downstream effector pathways is strongly impaired.
Next, we tested the capacity of the described HPK1 activation
loop mutants to activate well-established HPK1 effector path-
ways, namely, the SAPK and NF-κB pathways (Fig. 8). The
indicated HPK1 mutant proteins were transiently expressed in
Cos1 cells, and after immunopurification, the activation state
of endogenous SAPK/JNK was determined by an in vitro ki-

FIG. 7. Mutation of potential phosphorylation sites in the HPK1 kinase domain activation loop impairs HPK1 kinase activity and generates
HPK1 mutants refractory to TCR ligation-mediated activation. (A) Alignment of the amino acid sequences of activation loops of different
serine/threonine kinases. Residues reported to be phosphorylated and/or implicated in the regulation of the respective kinase are shown in bold
type. The alignment identified three potential phosphorylation sites in the HPK1 activation loop, which are shown as white letters on a black
background. The signature motifs of the kinase subdomains VII (DFG) and VIII (APE) and a central serine/threonine and an invariant threonine
residue are highlighted by light grey shading. (B) Cos1 cells were transiently transfected with plasmids encoding Clnk and either empty vector
(control) or HPK1 [wild-type (wt)] or the ATP-binding site mutant HPK1(K₄₆E) or various HPK1 constructs containing single or multiple
activation loop mutations at position 165 (threonine to alanine[T165A]), position 171 (serine to alanine[S171A]), or position 175 (threonine to alanine[T175A]).
After immunoprecipitation, the indicated variant proteins were tested for their abilities to autophosphorylate and to transphos-
phorylate a recombinant GST–c-Jun₅₆ fusion protein in an in vitro kinase assay. Reaction products were separated by SDS-PAGE, and phospho-
proteins were visualized by autoradiography (top panel). The migratory positions of HPK1 proteins, communoprecipitated Clnk, and GST–c-Jun₅₆
are indicated to the right of the gels. The levels of expression of HPK1 and Clnk were visualized by Western blotting (bottom panels). A
representative experiment of four experiments is shown. (C) Jurkat T cells were transiently cotransfected with plasmids encoding Clnk and either
empty vector (control), or HPK1 [wild-type (wt)], or HPK1 constructs containing the indicated activation loop mutations. Forty-eight hours after
transfection, cells were stimulated for 3 min using the anti-CD3 antibody OKT3. HPK1 proteins were immunoprecipitated and tested for their
ability to autophosphorylate in an in vitro kinase assay. Reaction products were separated by SDS-PAGE; the migratory positions of HPK1 proteins
and communoprecipitating Clnk are indicated to the right of the gels. Expression of the HPK1 proteins was determined by anti-HA Western
blotting. The positions of the two nonspecific signals are indicated by asterisks. The experiment was repeated twice with similar results.
FIG. 8. HPK1 activation loop mutants are severely impaired in mediating SAPK/JNK and NF-κB activation. (A) Wild-type HPK1 (wt) or HPK1 proteins bearing the indicated activation loop mutations were transiently expressed in Cos1 cells together with pSAPK/JNK. Forty-eight hours after transfection, coexpressed SAPK/JNK protein was immunopurified, and then its kinase activity towards bacterially expressed GST-c-Jun protein was determined. Phosphorylated reaction products were separated by SDS-PAGE, and relative GST-c-Jun phosphorylation levels were determined assessed using a phosphorimager. Expression of HPK1 proteins was demonstrated by anti-HPK1 Western blotting. A representative experiment of two experiments is shown. Bars depict the averages of duplicate samples; the experiment was repeated three times with similar results. (B) Cos1 cells were transfected with increasing amounts of plasmids (1, 2, and 4 μg) encoding either wild-type HPK1 (wt) or HPK1 constructs containing single activation loop mutations as indicated or empty vector (−). Phosphorylated reaction products were separated by SDS-PAGE, and relative GST-c-Jun phosphorylation levels were determined assessed using a phosphorimager. Expression of HPK1 proteins was demonstrated by anti-HPK1 Western blotting. A representative experiment of two experiments is shown. Bars depict the averages of duplicate samples; the experiment was repeated three times with similar results. Matching levels of expression of HPK1 proteins were visualized by anti-HPK1 Western blotting.

even outside the kinase domain. To reduce the difficulties associated with the analysis of such a complex pattern of spots, we generated a truncation mutant of HPK1 containing the first 232 amino acids C terminally flanked by an HA tag. This truncation mutant, designated HPK11-232-HA, encompassed subdomains I to X of the HPK1 kinase domain. In subsequent experiments, HPK11-232-HA served as a phosphorylation target for full-length, kinase-active HPK1. We reasoned that elimination of potential phosphorylation sides in the central and C-terminal domains of HPK1 should result in a significantly simplified spot pattern.

We coexpressed the HPK11-232-HA fragment and full-length, kinase-active HPK1-HA and immunopurified both proteins, making use of their HA tag. Subsequently, the mixture was subjected to an in vitro kinase assay to allow for phosphorylation of the truncation mutant by full-length HPK1. When the reaction products were separated by SDS-PAGE, we found that the 232-amino-acid fragment was efficiently phosphorylated by full-length HPK1, irrespective of whether it was derived from the wild-type sequence or whether it also harbored a mutation (K46E) that precludes ATP binding (Fig. 9B).

Phosphoamino acid analysis of phosphorylated full-length HPK1 expressed in Cos1 cells predominantly revealed phosphorylation on threonine residues and a significantly lower level of serine phosphorylation (Fig. 9C). The HPK11-232-HA fragment contained phosphothreonine at a level comparable to that of wild-type HPK1 and, if at all, minute amounts of phosphoserine. Furthermore, threonine phosphorylation levels were significantly reduced in a triple mutant of the HPK11-232-HA fragment in which the three potential activation loop phosphorylation sites had been changed into alanine (TST165,171,175AAA). These results suggested that HPK1 autophosphorylates on multiple sites in the first 232 amino acids and that autophosphorylation takes place in the activation loop but is restricted to threonine phosphorylation.

Threonine 165 of the HPK1 kinase activation loop is an autophosphorylation target. To test for autophosphorylation of threonine 165, serine 171, and threonine 175, the respective alanine mutations were introduced into HPK11-232(K46E)-HA, and the resulting mutated kinase domain fragments were transiently coexpressed with full-length HPK1-HA in Cos1 cells. After transphosphorylation by full-length HPK1, phosphoproteins were separated by SDS-PAGE and visualized by autoradiography. Compared to the fragment harboring the wild-type activation loop, mutation of the three candidate phosphorylation sites resulted in a relative reduction in phosphorylation levels. However, even mutation of all three candidate sites did not completely abolish fragment phosphorylation, supporting the notion that additional amino acids of the kinase domain outside the activation loop are phosphorylated by full-length HPK1 (Fig. 10A).

The different mutant HPK11-232(K46E)-HA phosphoproteins corresponding to the HPK11-232(K46E)-HA fragments were eluted, oxidized, subjected to tryptic digestion, and separated two dimensionally on cellulose TLC plates to generate phosphotryptic maps. The resulting peptide mixture was separated. As expected, the HPK11-232(K46E)-HA fragment contained significantly fewer autophosphorylation sites than the
FIG. 9. HPK1 proteins autophosphorylate in vitro on multiple amino acids. Autophosphorylation sites in the kinase domain are mostly threonine residues. (A) HPK1 protein was transiently expressed in Cos1 cells and subjected to immunoprecipitation and autophosphorylation in vitro. Reaction products were separated by SDS-PAGE and visualized by autoradiography. Full-length HPK1 phosphoprotein was eluted from the gel (white box in panel B) and subjected to tryptic digestion. Tryptic phosphopeptides were separated by two-dimensional TLC on a Hunter HLTE-7000 machine and visualized by autoradiography. The polarity of the electrophoretic separation and direction of the ascending chromatography are indicated. (B) Full-length HA-tagged HPK1 (wild type) was coexpressed in Cos1 cells with either empty vector (control) or with the HA-tagged HPK11-232 truncation mutant consisting of the first 232 amino acids of HPK1. The truncation mutant was either wild type at amino acid position 46 or harbored a K46E mutation which precludes ATP binding to the kinase domain. Anti-HA immunoprecipitation and a subsequent in vitro kinase reaction demonstrated transphosphorylation of the kinase-dead HPK11-232 fragment (left panel). Expression of all HPK1 proteins was visualized by anti-HA Western blotting (right panel). The white box depicts full-length HPK1 protein eluted for the generation of tryptic phosphopeptide maps as shown in panel A. The black box depicts the HPK11-232 fragment that was eluted for the subsequent phosphoamino acid analysis (C) and tryptic phosphopeptide maps (Fig. 10B). (C) HPK1 (wild type [wt]) or truncated HPK11-232(K46E) or HPK11-232(K46E) containing a triple activation loop mutation (TST165,171,175AAA) were transphosphorylated by full-length HPK1 in vitro as described in panel B. After SDS-PAGE, the depicted HPK1 phosphoproteins were eluted and subjected to phosphoamino acid analysis using a Hunter HTLE-7000 machine. The buffer system, polarity of the electrophoretic separations, and positions of reference phosphoamino acids are indicated. p-Ser, phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine, Pi, free phosphate.
full-length wild-type protein (Fig. 9A), giving rise to a map of only four major spots (Fig. 10B, panel a). This spot pattern is shown schematically in Fig. 10B, panel b. Mutation of threonine 165 to alanine (T165A) resulted in the loss of the two spots marked 1 and 2 in panel b of Fig. 10B (Fig. 10B, panel c).

Due to the tandem arginine residues at amino acid positions 168 and 169, tryptic digestion likely generates two phospho-peptides containing phosphothreonine 165, which are designated peptides 1 and 2 in Fig. 10C. On the basis of work by Boyle et al. (4), we predicted that the 16-amino-acid peptide 1 carries a single positive charge at pH 1.9 and that it is slightly more hydrophobic than the 17-amino-acid doubly charged peptide, peptide 2. Therefore, due to its higher electrical charge, peptide 2 should migrate further towards the cathode during the first dimension of electrophoresis. In contrast, due to its lower hydrophobicity during ascending chromatography, peptide 2 should lag behind peptide 1. On the basis of these predictions, the spots labeled 1 and 2 on the schematic map in Fig. 10B could represent phosphopeptides 1 and 2, which harbor phosphothreonine 165.

Surprisingly, mutation of serine 171 to alanine (S171A) did not lead to the loss of different spots from the phosphotryptic peptide map but yielded a pattern identical to that of the T165A mutation (Fig. 10B, panel d). Possible explanations for this puzzling result are that either the amino acid serine 171 was indispensable for T165 to become phosphorylated or both amino acids were phosphorylated but the phosphorylation events were interdependent. In the latter case, phosphorylation of S171 could be a prerequisite or priming phosphorylation for phosphorylation of T165 or vice versa.
Due to the tandem arginine residues R168 and R169, tryptic digestion would produce two phosphopeptides containing serine 171, which are designated peptides 3 and 4 in Fig. 10C. Peptide 3 is predicted to be 21 amino acids long, carry a net electrical charge of +2 at pH 1.9, and be less hydrophobic than peptide 4, which is predicted to carry a charge of +1 at pH 1.9. Due to the very similar properties of the pairs of peptides (peptides 1 and 2 and peptides 3 and 4), spots 1 and 2 in Fig. 10B could also represent peptides 3 and 4, which contain S171.

As it was impossible to distinguish phosphopeptides containing phosphorlated T165 (pT165) or pS171 on the basis of their migratory behavior in phosphotryptic peptide maps, we eluted the peptides corresponding to spots 1 and 2 from the TLC carrier and performed a subsequent phosphoamino acid analysis. Spots 1 and 2 contained only phosphothreonine, confirming our initial assignment as pT165-containing peptides 1 and 2 (Fig. 10B). Furthermore, this analysis also demonstrated that serine 159 is not an autophosphorylation target.

Mutation of threonine 175 alone (T175A) or in combination with S171 (ST171,175AA) resulted in a general strong reduction of fragment phosphorylation. The observed residual weak phosphotryptic peptide pattern was reminiscent of the pattern generated by the wild-type fragment (Fig. 10B, panels e and f). This result suggested that mutation of T175 to alanine introduced a significant structural perturbation into the activation loop, which affected its binding as a substrate to the active site. Further support for this notion was provided by a mutant in which in addition to the three potential phosphorylation sites, the adjacent tyrosine 177 was mutated into alanine. The phosphopeptide map of this quadruple mutant (TSTY-AAAA) resembled the maps of T165A and S171A, suggesting that the Y177A mutation compensated for the T175A mutation. Possibly, the Y177A mutation released structural constraints introduced by T175A, and substrate binding and phosphorylation were again possible (Fig. 10B, panel g).

Serine 171 in the HPK1 activation loop is a PKD1 phosphorylation site. We were puzzled by the apparent interdependence of T165 and S171 phosphorylation. Intriguingly, mutation of S171 had a significantly stronger effect on overall HPK1 activity than mutation of T165 (Fig. 7B). A possible explanation is that phosphorylation of S171 is a prerequisite or priming phosphorylation for T165 autophosphorylation and that a kinase other than HPK1 is responsible for phosphorylation of S171. This phosphorylation event could take place during transient expression in intact cells and would consequently not be detected in subsequent in vitro kinase reactions. Therefore, we examined whether the sequence context of the mutated amino acid S171 was particularly favorable for any known serine/threonine kinase and found S171 to be a consensus phosphorylation site for PKD family serine/threonine kinases.

To test for phosphorylation of the HPK1 activation loop by PKD1, we made use of mutations in the PKD1 zinc fingers or PH domain which render this kinase constitutively active (13). Coexpression of wild-type PKH1;1,222(K46E)-HA with active PKD1 resulted in efficient phosphorylation of the HPK1 fragment (Fig. 11A). The HPK1 activation loop T165A and T175A mutants were phosphorylated at levels comparable to that of the wild-type fragment. In sharp contrast, a S171A mutant completely failed to become phosphorylated by active PKD1. This result demonstrated that PKD1 was able to phosphorylate the activation loop of HPK1.

A phosphotryptic map of the HPK1 activation loop fragment HPK11-232(K46E)-HA transphosphorylated by PKD1 displayed two predominant spots (Fig. 11B), which were lost in the S171 mutant and are designated spots 3 and 4 in the schematic map in Fig. 11B. In mixing experiments of material that had been autophosphorylated by HPK1 with material transphosphorylated by PKD1 (not shown), spots 3 and 4 (Fig. 11B) did not comigrate with spots 1 and 2 (Fig. 10B). Finally, a phosphoamino acid analysis of spots 3 and 4 revealed exclusively phosphoserine (Fig. 11C). Therefore, spots 3 and 4 (Fig. 11B) indeed represent S171-containing phosphopeptides, i.e., peptides 3 and 4 in Fig. 10C.

In summary, our analysis identified T165 of the HPK1 activation loop as an autophosphorylation target, while S171 is phosphorylated by PKD. Furthermore, we obtained evidence of a priming function of phosphoserine 171 for T165 phosphorylation. Therefore, transphosphorylation by PKD1 and subsequent autophosphorylation appear to be prerequisite for full activation of HPK1.

PKD1 enhances activation of HPK1 after TCR stimulation. To analyze a possible effect of PKD1 on HPK1 activation, PKD1 and HPK1 were coexpressed in Jurkat T cells. The presence of either wild-type PKD1 or the constitutively active PKD1 (PH-Mut) and PKD1 (Zn-Mut) mutants augmented HPK1 kinase activity after TCR stimulation (Fig. 12A). In the presence of wild-type PKD1, HPK1 activity was mildly reduced in the basal state and slightly enhanced after TCR ligation, resulting in a clear increase in inducibility. Constitutively active forms of PKD1 caused a strong stimulation of HPK1 activity, regardless of TCR ligation. A kinase-deficient mutant, PKD1 (KD), had no influence on HPK1 activity, likely due to compensation by other PKD isoforms endogenously expressed in Jurkat cells.

Next, we directly addressed the importance of endogenous PKD for the regulation of HPK1 kinase activity by down-modulation of PKD protein using a PKD1 antisense construct. Transfection of the PKD1 antisense construct into epithelial cells resulted in a clear reduction of PKD1 expression (Fig. 12B). Concomitantly, the kinase activity of cotransfected HPKI-HA as measured by autophosphorylation in vitro was clearly diminished. Protein down-modulation mediated by the PKD1 antisense construct had no effect on the level of HPK1-HA expression. This result demonstrates that in a nonlymphoid cell system, endogenous PKD can also significantly contribute to the full stimulation of HPK1 kinase activity.

We then took advantage of the mouse T-cell hybridoma DC27.1 to analyze the role of PKD1 in the activation of endogenous HPK1 in T cells. TCR ligation mediated by anti-CD3 antibody reproducibly induced endogenous HPK1 activity, as determined by in vitro kinase assays (Fig. 12C, top blot). Again, down-modulation of PKD1 protein after transfection of the PKD1 antisense construct resulted in a clear reduction of HPK1 activation, providing independent confirmation for the importance of PKD1 in TCR-dependent activation of HPK1 (Fig. 12C).

Finally, we wanted to address possible consequences of the functional interaction between PKD1 and HPK1 for the activation of downstream signaling pathways. Upon forced expres-
FIG. 11. Serine 171 in the HPK1 activation loop can be transphosphorylated by PKD1. (A) The indicated HPK1(WT)-HA truncation proteins were transiently coexpressed with activated versions of full-length PKD1-HA. Anti-HA immunopurified proteins were subjected to in vitro kinase reactions and separated by SDS-PAGE. The migratory position of the HPK1(WT) fragment is indicated to the right of the gels. Expression of the different proteins was verified by Western blotting. wt, wild-type protein. (B) (Left) HPK1(WT)-HA protein was transphosphorylated by the PKD1 (Zn-Mut) mutant in vitro. After separation of the reaction mixture by SDS-PAGE, the HPK1 fragments were recovered from the SDS-polyacrylamide gel and subjected to tryptic peptide mapping. Phosphopeptides were separated on a Hunter HTLE-7000 machine and visualized by autoradiography. The maps are oriented as in Fig. 9A. (Right) Schematic depiction of the phosphotryptic map on the left. Major spots which coincide with the presence of S171 in the activation loop peptide are shown in black; these spots are not present after phosphorylation of the HPK1(WT) (K46E,S171T)-HA fragment. Dark grey spots likely represent degradation products of the active kinase PKD1 (Zn-Mut). (C) Phosphoamino acid analysis of phosphopeptides 3 and 4 indicated in the right panel of panel B. The buffer system, polarity of the electrophoretic separations, and positions of reference phosphoamino acids are indicated as described in the legend to Fig. 9C. (D) Activation loop sequence alignment of subfamily I GCKs. Grey boxes highlight the amino acids T165 and S171 that are phosphorylated in and contribute to the activation of HPK1. A hydrophobic residue at position –5 relative to S171 (white letters on black background) is critical for phosphorylation by PKD1 and conserved in all family members.
sion in epithelial cells, we found that the constitutively active PKD1 (PH-Mut) mutant moderately activates an AP-1-dependent reporter gene, while expression of HPK1 alone had no effect on basal AP-1 activity. Coexpression of the PKD1 (PH-Mut) mutant and HPK1 did not result in enhanced PKD-induced AP-1 activation but instead led to mild repression (not shown).

In contrast, wild-type PKD1 profoundly stimulated HPK1-mediated SAPK activation, while the kinase-dead PKD1 (KD) mutant exerted a suppressive effect (Fig. 12D). The stimulatory effect of wild-type PKD1 was most likely mediated by HPK1, as PKD1 by itself failed to elicit any SAPK/JNK activation. Concomitantly, the constitutively active PKD1 (PH-Mut) mutant showed no NF-κB activation, while HPK1-driven NF-κB activation was augmented in the presence of the PKD1 (PH-Mut) mutant (Fig. 12E).

These results demonstrate a nonoverlapping pathway specificity for HPK1 and PKD1 downstream signaling when analyzed independently. However, if coexpressed, activation of HPK1 is significantly augmented by the presence of kinase-competent PKD1, leading to a stronger, more robust signal input into the effector SAPK/JNK and NF-κB pathways.

DISCUSSION

Activation of serine/threonine kinases is readily detected after immunoreceptor stimulation at various developmental stages. However, the biological consequences of serine/threonine kinase action have been less apparent, possibly due to the existence of multiple isoforms and overlapping expression patterns in the relevant kinase families that defy simple genetic strategies, such as the elimination of single genes. The biological function of the subfamily I GCKs, HPK1, GCK, GCKR/KHS, and GLK, has remained particularly enigmatic. Here we describe a study in which we investigated in precise detail the mechanism of HPK1 activation after TCR ligation.

Several SH2 and SH3 domain-containing adaptor proteins have been reported to be HPK1 interaction partners. Some of these molecules are recruited to the plasma membrane upon lymphocyte activation; however, the localization of HPK1 in resting and activated cells has not been described so far. We determined the intracellular localization of HA-tagged full-length HPK1 and an HPK1 fusion protein in which the kinase domain had been replaced by EGFP in Cos1 cells. Both proteins were located predominantly in the cytoplasm but accumulated in multiple clusters in cells expressing intermediate levels of protein that appeared to coalesce into large aggregates in cells expressing high levels of protein. The large overlap in localization of both HPK1 forms suggests that motifs defining HPK1 intracellular distribution reside outside the kinase domain. The strong clustering of HPK1 is likely a consequence of the forced overexpression and without a direct physiological counterpart, yet it is likely to be the basis for the constitutive activation observed under these conditions.

Under more physiological conditions in lymphocytes, HPK1 activation has been reported to depend on Src family kinases and SLP-76 family adaptors (38). Therefore, we investigated the effects of these proteins on HPK1 localization. Surprisingly, coexpression of Clnk had little effect, while Lck efficiently recruited HPK1 to the plasma membrane. When expressed together, all three proteins displayed a large degree of colocalization. Exactly how Lck recruited HPK1 to the membrane remains unclear; a possible mechanism could involve SH3 domain-containing adaptor proteins related to the Grb2 family or HS1 (18, 30). Our results indicate that the membrane-proximal localization is further stabilized by SLP-76 adaptors that are needed for efficient HPK1 tyrosine phosphorylation in epithelial cells and activation in lymphocytes. Of note, the localization of a HPK1(Y379F) mutant that is incapable of binding the Clnk SH2 domain was comparable to that of wild-type HPK1. This finding suggests that neither Lck-mediated localization nor a basal interaction with Clnk depends exclusively on the SH2 domain binding to HPK1.

Finally, we were able to demonstrate recruitment of HPK1 to the clustered immunoreceptor in APC–T-cell conjugates and Jurkat cells (I. M. Patzak, unpublished data). Given that immunoreceptor engagement also results in HPK1 activation, it was tempting to speculate that clustering of HPK1 is an essential prerequisite for its enzymatic activation inasmuch as it makes subsequent auto- and transphosphorylation reactions possible by providing a sufficient local concentration of substrate and enzyme.

Activation of a large number of RD kinases has been demonstrated to depend on phosphorylation of activation loop amino acids (14). Employing alanine mutants, we identified three amino acids in the HPK1 activation segment that are indispensable for full kinase activity and effector pathway stimulation. While mutation of threonine 175 apparently introduced a strong structural perturbation, threonine 165 and serine 171 were characterized as bona fide phosphorylation sites. Surprisingly, phosphorylation of these two sites was interdependent, phosphorylation of serine 171 appeared to be a prerequisite for phosphorylation of threonine 165 and HPK1 appeared not to be the predominant serine 171 kinase. On the basis of its sequence context, we identified serine 171 as a possible PKD phosphorylation site and demonstrated a strong positive influence of PKD1 on HPK1 kinase activity and downstream effector activation.

Therefore, we propose a multistep model for HPK1 activation. Initially, PKD1 is relocalized to the plasma membrane such that activation loop trans- and autophosphorylation steps can take place during the ensuing second phase of activation. Full activity necessitates phosphorylation of threonine 165 and serine 171, with phosphorylation of threonine 165 being dependent on phosphoserine 171. To our knowledge, this is the first identification of a priming phosphorylation in a RD kinase activation segment. We and others have previously reported the puzzling observation that forced expression in epithelial cells renders subfamily I GCKs constitutively active, a property that has significantly hindered the elucidation of upstream activators and biological function. Clustering induced by ectopic overexpression likely alleviates the need for plasma membrane relocalization, thereby allowing activation loop phosphorylation to occur in the absence of upstream input. Could this ectopic activation of HPK1 involve PKD1? A significant proportion of the observed HPK1 fusion protein clusters appeared near or at the Golgi complex (F. Kiefer and B. Neuhau, unpublished data). PKD1 and related family members (PKD2 and PKD3) are ubiquitously expressed and are also present in epithelial cells. In addition, PKD1 has been dem-
Figure A: Bar graph showing relative HPK1 kinase activity with and without CD3 stimulation. The graph includes data points for fold activation with values of 3.1, 8.0, 1.5, 1.6, and 3.0.

Figure B: Western blot analysis of HPK1, PKD, and Tubulin. The panels show in vitro kinase activity with anti-HA, anti-PKD, and anti-Tubulin antibodies.

Figure C: Western blot analysis of HPK1, PKD, and Tubulin. The panels show in vitro kinase activity with anti-HPK1, anti-PKD, and anti-Tubulin antibodies.

Figure D: Western blot analysis of SAPK and HPK1. The panels show in vitro kinase activity with anti-SAPK and anti-HPK1 antibodies.

Figure E: Western blot analysis of HPK1 and PKD. The panels show in vitro kinase activity with anti-HPK1 and anti-PKD antibodies.

onstrated to localize to the Golgi complex where it is part of a vesicle-Golgi network (39). Hence, upon forced expression, PKD1 might encounter, colocalize, and transphosphorylate HPK1 at cellular locations other than the native environment of immunoreceptors.

A mechanism for activation of the MAP3 kinase MEKK1 by the Ste20 homologue GCK has been proposed in which membrane recruitment of GCK/MEKK1 heterodimers by TRAF2 creates a higher-order aggregate that activates MEKK1 (5). This mechanism implies GCK aggregation during MEKK1 activation, although GCK oligomerization per se has so far not been demonstrated. MEKK1 activation clearly involves activation loop phosphorylation; however, activation loop phosphorylation may be entirely self catalyzed or may involve GCK transphosphorylation (5). A rather unusual mode of regulation has recently been reported for GCK itself (43). In this study, GCK was shown to be subject to constant turnover by the proteasome; however, Toll-like receptor agonists caused a TRAF6-dependent stabilization of constitutively active GCK. Therefore, the apparent receptor-mediated kinase activation was the result of protein accumulation rather than modulation of enzymatic activity (43). In contrast to the reported stabilization of GCK, we observed a distinct down-modulation of HPK1 protein after induction of synapse formation, suggesting that there may be very different or possibly overlapping modes of regulation of subfamily I GCKs.

Activating autophosphorylation sites for subfamily I GCKs have not been identified so far, but phosphorylation of the archetypical *Saccharomyces cerevisiae* Ste20p at a threonine residue equivalent to HPK1 threonine 175 has been described (40). Interestingly, a threonine residue is found at a corresponding position in many kinases (Fig. 7A), and alanine mutation results in a strong reduction of kinase activity, but phosphorylation of this most C-terminal activation loop threonine has been seen in only a few kinases, including cdc2, Rsk1, and MEKK1 (37). Indeed, for MEKK1, conflicting data have been reported by Deak and Templeton (7); they failed to detect phosphorylation of this residue and instead proposed a structural function as we do here for HPK1 threonine 175. Phosphorylation of a serine or threonine residue corresponding to HPK1 serine 171 has been reported for numerous RD kinases. Uniquely, we found that this site in HPK1 was not an auto-phosphorylation site but a transphosphorylation site that provides a priming phosphate for threonine 165 autophosphorylation. Somewhat reminiscent, serine 281 of the MAP3K MLK3 has been identified as a site of HPK1 transphosphorylation, while threonine 277 and serine 281 of MLK3 were found to be autophosphorylation sites (21). Furthermore, many kinases of the AGC family (cyclic AMP-dependent protein kinase/protein kinase G/protein kinase C extended family) depend on activation segment phosphorylation by the master kinase PDK1 (32). For instance, PKB/AKT is phosphorylated by PDK1 on threonine 308, which corresponds to HPK1 serine 171, and in this case, phosphorylation also depends on both kinases colocalizing to the cell membrane. Interestingly, monophosphorylated PKB/AKT acquires only 10% of maximal activity, and full activation depends on phosphorylation of a conserved C-terminal motif outside the kinase domain (3, 33). Whether phosphorylation of C-terminal residues contributes to kinase activation in HPK1 remains to be established.

The identified phosphoacceptor sites are conserved in the activation loops of all subfamily I GCKs. Interestingly, the PDK1 target site corresponding to S171 is conserved in all subfamily I GCKs (Fig. 11D); hence, PKDs could act as master regulators for this kinase family. We found that PDK1 enhanced the extent of HPK1 inducibility after CD3-mediated TCR ligation in Jurkat cells, while constitutively active forms of PDK1 generally augmented HPK1 kinase activity. As a result of the more robust signal input, we observed enhanced stimulation of the effector SAPK and NF-kB pathways. Coexpression of the kinase-dead PDK1 (KD) mutant did not inhibit HPK1 activation after TCR cross-linking. In coprecipitation studies, we found no evidence for a stable, direct interaction between HPK1 and PDK1 (I. M. Patzak, unpublished data), which does not contradict the notion that HPK1 is a PKD substrate, if both kinases colocalize with other molecules. Consequently, a kinase-dead version of PDK1 does not necessarily act in a dominant-negative fashion; instead, its function to provide a priming HPK1 phosphorylation could be taken over.
by other PKD family members expressed in Jurkat cells, such as PKD2. We obtained independent support for this assumption when we down-regulated endogenous PKD levels in 293T and DC2.71 cells, which resulted in reduced HPK1 activation levels in both cells.

Our approach throughout this study was inspired by the expectation that mechanistic insight into HPK1 kinase activity regulation will ultimately provide valuable information on HPK1 function. Our results imply a functional interaction between HPK1 and PKD, at least in T cells. The biological activity of PKD1 in T cells has been reported to be exquisitely dependent on cellular localization and the developmental stage of the cell under investigation. Using a transgenic model, active PKD1 initiated a differentiation program similar to β-selection in pre-T cells and was able to trigger the cellular mechanisms that ensure allelic exclusion (27). A possible contribution of HPK1 to these processes deserves experimental investigation.

Rapidly after immunoreceptor stimulation PKD1 is activated at the plasma membrane and is found to remain activated for several hours even after relocation to the cytoplasm (28). Therefore, PKD1 could also cause persistent stimulation of HPK1. This mode of HPK1 activation would no longer be dependent on plasma membrane localization and might elicit HPK1-mediated signaling events other than SAPK and NF-κB activation.

In the subfamily I GCKs, HPK1 displays the most restricted expression pattern, being exclusively expressed in leukocytes. HPK1 appears to be predominantly involved in immunoreceptor signaling, while other family members, such as GCK, may be coupled to different receptors, such as receptors of the tumor necrosis factor receptor family (42) and Toll-like receptors (43). It is tempting to speculate that the subfamily I GCKs act in a similar fashion on different receptor systems in different cell types. Although rapid and transient activation of HPK1 after antigen receptor ligation in Jurkat and splenic T cells has been reported (25), its affinity for ClnK is significantly higher than for SLP-76 (41). Therefore, it appears that HPK1 is most efficiently recruited to the plasma membrane at a time when immunoreceptors have already been engaged for a considerable length of time and may even contribute to the termination of receptor activation. Most recently, supporting evidence for an involvement of HPK1 in the generation of a receptor-off signal was provided by a study that analyzed the interaction of HPK1 and the adaptor protein SH3P7/HIP-55. The HPK1/HIP-55 complex was found to negatively regulate NF-AT signaling and to contribute to basal and ligand-induced TCR down-modulation (20). This function may be correlated with the rapid disappearance of HPK1 we observed in lymphocytes after conjugate induction. Given that PKD1 has also been demonstrated to fulfill important functions in intracellular trafficking (23), PKD1 and HPK1 might regulate antigen receptor transport to and from the plasma membrane and receptor down-modulation in a concerted fashion.

ACKNOWLEDGMENTS

We thank Wolfgang Müller for expert technical assistance and Martin Stehling and Klaus Hessel for help with cell sorting. We are indebted to Ali Hamann, Siegfried Weiß, and Jane McCabe for the gift of murine T- and B-cell lines and Min Li-Weber for providing the AP-1 reporter construct. We thank Kristina Brauburger and Marcel Deckert for valuable discussions. We are grateful to Christian R. Frey and Jörg Kraus for critically reading the manuscript and Peter H. Kramer for stimulating discussions and valuable contributions. We thank Dietmar Vestweber for his continued interest and generous support. J.V.L. thanks Sandy Vandoninck for expert technical assistance.

Research in the J.V.L. lab was supported by the Association for International Cancer Research (AICR grant 02-252), the Belgian Federation against Cancer (SCIE2003-53), the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO-Vl), and the Belgian government (IUAP P5/12). Research in the F.K. lab was supported by the Max-Planck-Society.

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