Caspase-mediated Cleavage of Hematopoietic Progenitor Kinase 1 (HPK1) Converts an Activator of NFκB into an Inhibitor of NFκB*

Received for publication, September 12, 2000, and in revised form, January 12, 2001
Published, JBC Papers in Press, January 29, 2001, DOI 10.1074/jbc.M008343200

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Hematopoietic progenitor kinase 1 (HPK1), a mammalian Ste20-related protein kinase, is a potent stimulator of the stress-activated protein kinases (SAPKs/JNKs). Here we report activation of NFκB transcription factors by HPK1 that was independent of SAPK/JNK activation. Overexpression of a dominant-negative SEK1 significantly inhibited SAPK/JNK activation, whereas NFκB stimulation by HPK1 remained unaffected. Furthermore, activation of NFκB required the presence of full-length, kinase-active HPK1, whereas the isolated kinase domain of HPK1 was sufficient for activation of SAPK/JNK. We also demonstrate that overexpression of a dominant-negative IKKβ blocks HPK1-mediated NFκB activation suggesting that HPK1 acts upstream of the IκB kinase complex. In apoptotic myeloid progenitor cells HPK1 was cleaved at a DDVD motif resulting in the release of the kinase domain and a C-terminal part. Although expression of the isolated HPK1 kinase domain led to SAPK/JNK activation, the C-terminal part inhibited NFκB activation. This dominant-negative effect was not only restricted to HPK1-mediated but also to NIK- and tumor necrosis factor α-mediated NFκB activation, suggesting an impairment of the IκB kinase complex. Thus HPK1 activates both the SAPK/JNK and NFκB pathway in hematopoietic cells but is converted into an inhibitor of NFκB activation in apoptotic cells.

Constant turnover and the capacity to adapt efficiently to a changing environment are hallmarks of the hematopoietic system. Inflammatory cytokines like tumor necrosis factor α (TNFα) and interleukin 1 trigger intracellular pathways resulting in the activation of the stress-activated protein kinases SAPKs/JNKs and p38s as well as of NFκB family transcription factors (reviewed in Refs. 1 and 2). SAPKs are executing enzymes acting at the basal level of a hierarchical three-tiered kinase cascade (3), which upon activation enter the nucleus and phosphorylate nuclear transcription factors (4, 5).

In mammals, two families of serine/threonine kinases have been identified that contain a catalytic domain with extensive homology to Sterile 20 (Ste20) kinase of the yeast Saccharomyces cerevisiae. Kinases prototypically represented by p21-activated kinase (PAK) are characterized by a C-terminal kinase domain and an N-terminal p21-binding domain, flanked by proline-rich sequences that serve as SH3 domain-binding sites (6). PAKs are activated by the GTP-bound forms of the small GTPases Rac/Cdc42 and have been implicated in regulation of cytoskeletal dynamics, cell cycle, and oxidant generation in neutrophils (7).

The second family comprises kinases related to germline center kinase (GCK), which are defined by a N-terminally located kinase domain. Based on homologies within their C-terminal domains GCKs can be grouped into six subfamilies. Four kinases, GCK, GCKR/KHS, GLK, and HPK1, which will be referred to as subfamily I, share a C-terminally located regulatory domain, called citron homology domain (CNH) (8, 9). Of this group GCKR/KHS (10, 11) and GLK (12) are ubiquitously expressed, whereas GCK (13) and HPK1 (14, 15) display tissue specificity, with HPK1 being exclusively expressed in the hematopoietic cells of the adult. Upon overexpression subfamily I kinases are rendered active and activate potently and selectively the SAPK/JNK pathway via MAP3Ks. Kinase activity of endogenous GCK, GCKR/KHS, and GLK is stimulated in response to TNFα (11, 12, 16). In addition GCK and GCKR/KHS have been shown to bind to the TNF receptor-associated factor 2 (TRAF2) (17, 18). In contrast to GCK, GCKR/KHS, and GLK are responsive to UV light. HPK1, which already displays significant kinase activity when immunoprecipitated from nonstimulated tissues or cell lines, has been reported to be activated in response to erythropoietin receptor engagement (19) as well as T and B cell immunoreceptor cross-linking (20, 21).

Four proline-rich stretches located between the kinase domain and CNH domain of HPK1 contain a PXXP motif, the minimal sequence requirement for SH3 domain ligands. Three of these have been shown to interact with small adaptor proteins including Grb2 (22), Nck (22), HSI (19), and Crk (23, 24), providing a possible link to activated transmembrane receptors. SH3 domain-mediated coupling to possible downstream MAP3Ks like mixed lineage kinase 3 (MLK3) has also been described (14). Despite their structural similarity, the SH3 domain ligand motifs are poorly conserved between subfamily I kinases. Therefore, subfamily I kinases appear to be subject to different regulatory mechanisms and most likely serve distinct physiological functions.

NFκB/Rel proteins are dimeric, sequence-specific transcription factors that control many important biological processes, including development, immune responses, cell growth, and apoptosis. NFκB family transcription factors are rendered inactive within the cytoplasm by interaction with IκB inhibitory proteins. In response to extracellular signals, a high molecular
weight IkB kinase (IKK) complex is activated resulting in IkB phosphorylation followed by ubiquitinylation and degradation. De-repressed NFκB proteins translocate to the nucleus, where they bind and transactivate κB sites within the promoter region of NFκB-regulated genes (25).

Tissue-specific signaling molecules such as HPK1 are likely to provide specific inputs in ubiquitous transduction pathways and may function as signaling integrators or branch points. The increasing number of kinases that activate both SAPKs and NFκB family transcription factors prompted us to investigate a possible function of HPK1 in NFκB signaling. Here we report a robust stimulation of NFκB activity by HPK1 in hematopoietic cells. In apoptotic cells HPK1 was cleaved by a caspase 3-like activity, resulting in the generation of a dominant-negative C-terminal fragment that inhibited NFκB stimulation.

**EXPERIMENTAL PROCEDURES**

**Generation of NFκB Reporter and Expression Plasmids**—The pGL8xNFκB-fos reporter plasmid contains 8 repeats of the mouse major histocompatibility class complex I h2kκ gene κB site fused to the mouse c-fos minimal promoter driving a luciferase reporter gene. For normalization we generated pfos-LacZ that contains the identical mouse c-fos minimal promoter lacking the κB-binding sites. pSP64T-HPK1(D383E):HA and pSP64T-HPK1(D383N):HA were generated by site-directed mutagenesis using the U.S.E. Mutagenesis Kit (Amerham Pharmacia Biotech) according to the manufacturer’s protocol. pMT2-based HPK1 expression plasmids were described previously (14). For detection by immunoblotting T7-tagged versions of the GC family kinases GCK, GRK, and GLK were generated in pCATT.

For pCATT: FL-GLK an Ncol/Baf120I fragment from pCRI-1-FL-GLK was inserted into the Smal site of pCATT-neo. For pCATT:GCK an EcoRI/Smal fragment from pFLAG-GLK was inserted into pCATT-neo, and pCATT:GCK was created by blunt insertion of an EcoRV/XbaI fragment from pCCMV-GCK into the EcoRI site of pCATT-neo.

**Generation of Deletion Mutants in the Proline-rich Motifs P1, P2, and P4 of mHPK1—** A Kan fragment comprising 1233 base pairs of mHPK1 cDNA containing the proline-rich motifs P1 to P4 was subcloned into P4 of mHPK1 from pRC/CMV-GCK into the EcoRI site of pCAT7:GCK was created by blunt insertion of an XhoI fragment comprising 1233 base pairs of mHPK1 and reinserted into pB-mHPK1: D KpnI site of pCAT7-neo. For pCAT7:GCKR an NcoI/StyI fragment comprising 1233 base pairs of mHPK1 was obtained from Cell Signaling Technology, the IκBα antibody 2/PC142 antibody from Oncogene Research Products. The T7 tag was detected by the monoclonal antibody 12CA5. A phospho-specific antibody against serine 32 of IkBα was obtained from Cell Signaling Technology, the IkBa (antibody 29C2) antibody from Oncogene Research Products. The T7 tag was detected by the monoclonal antibody T7Tag (Novagen).

**NFκB Reporter Assays**—Soluble proteins were prepared and used for electrophoretic mobility shift assays as described (31, 32). For each shift reaction 10–20 fmol of 32P-labeled κB-binding oligonucleotide (5'-TCGAGATGGGATATCCAGCCTAGC-3') were employed.

**RESULTS**

HPK1 Activates the NFκB Pathway in Hematopoietic Cell Lines—We tested the capacity of HPK1 to activate NFκB in Jurkat T cells, which endogenously express HPK1. Cotransfection of increasing amounts of HPK1 expression plasmid and a NFκB-dependent reporter resulted in HPK1-dependent NFκB activation (Fig. 1A), whereas expression of the kinase-deficient mutant HPK1(K46E) failed to activate NFκB. These results suggest that HPK1 is an activator of NFκB in Jurkat T cells.

To investigate HPK1-dependent NFκB activation in a myeloid progenitor cell line, FDC-P1 cells (26) were used, which also endogenously express HPK1. We demonstrated HPK1-dependent NFκB activation taking advantage of two retrovirally transduced FDC-P1 clones FDC-P1/C9 and FDC-P1/D4 that stably express an exogenous, HA-tagged variant of HPK1. FDC-P1/D4 cells harbor approximately double the amount of HPK1 kinase activity present in FDC-P1/C9 cells (Fig. 1B). FDC-P1/C9 cells contain about double the amount of HPK1 kinase activity of FDC-P1 wild-type cells. Comparing nuclear extracts derived from FDC-P1 wild-type cells and the cell clones C9 and D4, we observed an increase in NFκB bandshift activity that paralleled HPK1 kinase expression levels. These results provide an independent demonstration of HPK1-mediated NFκB transcription factor activation.

**Activation of NFκB Depends on Full-length Kinase-active HPK1—** To analyze the HPK1-driven NFκB activation in more detail and to determine the presence of endogenous HPK1, we used COS1 cells for further analysis. For subsequent transfection experiments we generated a double reporter gene system, in which NFκB-dependent transcription of a luciferase reporter...
Fig. 1. Activation of NFκB transcription factors by HPK1 and the related kinases GLK, GCKR/KHS, and GCK. A, upper panel, Jurkat T cells were transiently cotransfected with expression plasmids encoding full-length HPK1, the kinase-deficient mutant HPK1(K46E), or empty vector DNA (control) and an NFκB-dependent reporter system. Values are averages of triplicates ± S.D. Lower panel, expression of HPK1 was assessed by anti-HA Western blotting. B, upper panel, NFκB mobility shift activity was determined in nuclear extracts of wild-type (WT) FDC-P1 myeloid progenitor cells (FDC-P1/WT) or retrovirally transduced FDC-P1 clones stably overexpressing HA-tagged HPK1 (FDC-P1/C9 and FDC-P1/D4). Equal amounts of nuclear extracts prepared from 4 × 10⁶ cells were incubated with a 32P-labeled DNA fragment comprising an NFκB-binding site. Nucleoprotein complexes were separated by native PAGE, visualized by autoradiography, and quantified using image analysis software. Middle panel, expression of endogenous and HA-tagged HPK1 in FDC-P1/WT, FDC-P1/C9, and FDC-P1/D4 cells was assessed by anti-HPK1 Western blotting using rabbit serum 7. Lower panel, endogenous and HA-tagged HPK1 was immunopurified from FDC-P1/WT, FDC-P1/C9, and FDC-P1/D4 cells using anti-HPK1 antisera 5/6 and tested for the ability to autophosphorylate in vitro. Electrophoretic mobilities of endogenous and HA-tagged HPK1 are indicated by arrows. C, dual reporter plasmid system used to assess activation of NFκB in transiently transfected COS1 cells. Transcription of a luciferase reporter gene driven by 8 NFκB-binding sites, fused to a c-fos minimal promoter, was normalized on the basis of β-galactosidase expression from an identical c-fos promoter lacking NFκB-binding sites. D, upper panel, Western blotting using rabbit serum 7. Middle panel, in parallel identical amounts of the same plasmids were assayed for p54-SAPKβ activation. After 36 h control cells were stimulated with 2 nM recombinant hTNFα and incubated for additional 12 h followed by cell lysis. After 48 h NFκB-driven luciferase activity was determined and normalized against β-galactosidase activity using a chemiluminescence assay system. Relative activation of NFκB-driven luciferase activity normalized for transfection efficacy is shown. Depicted are averages of a representative experiment, in which all transfections were performed in duplicate. Middle panel, in parallel identical amounts of the same plasmids were assayed for p54-SAPKβ activation. After 48 h cells were lysed, and p54-SAPKβ was immunopurified. SAPK/JNK activation was determined by in vitro phosphorylation of a bacterially expressed c-Jun N-terminal fragment fused to GST. Phospho-proteins were separated on SDS-PAGE and visualized by autoradiography. Lower two panels, expression of the different proteins was visualized by Western blotting using the polyclonal anti-HPK1 rabbit serum 3 (directed against kinase domain subdomain XI) or 7 (directed against the HPK1 C terminus). E, upper panel, increasing amounts of expression plasmids for the indicated GCK-related kinases or empty vector DNA were cotransfected into COS1 cells. The total amount of transfected DNA was kept constant. Lower panel, expression of the different proteins was visualized by Western blotting using the polyclonal anti-HPK1 rabbit serum 7 or anti-T7 tag antibody. F, identical amounts of expression plasmids for the GCK-related kinases were cotransfected in COS1 cells and assayed for their ability to undergo autophosphorylation (upper panel) or activate an HA-tagged p54-SAPKβ (middle panel). p54-SAPKβ expression levels were visualized by anti-HA Western blotting (lower panel).
gene was normalized against basal transcription of a galactosidase reporter gene (Fig. 1C). To delineate the requirements for NFκB activation, we transiently expressed wild-type HPK1, the kinase-deficient variant HPK1(K46E), or the isolated kinase domain HPK1-Ko in COS1 cells. NFκB activity levels were compared with the activity level observed after treatment with the inflammatory cytokine TNFα, a well-established inducer of NFκB (Fig. 1D). HPK1 caused a robust activation of NFκB, comparable to TNFα stimulation, whereas HPK1(K46E) was not able to activate NFκB. Interestingly, HPK1-Ko failed to stimulate NFκB, although it still activated the SAPK/JNK pathway.

These results confirm our observation in Jurkat T cells demonstrating again that HPK1 kinase activity is essential for NFκB activation. Furthermore, NFκB activation required the presence of full-length HPK1, whereas the C-terminal regions of HPK1 are dispensable for SAPK/JNK activation.

The Ste20-related Kinases of the GC Family Show Variable Capacities to Activate NFκB—We next addressed the question whether the potential to activate NFκB is shared by other members of subfamily I GCK-related kinases, besides HPK1. After transient expression of increasing amounts of HPK1, GLK, GCKR/KHS, or GCK in COS1 cells, we found a dose-dependent and profound activation of NFκB by HPK1 closely followed by GCKR/KHS (Fig. 1E). Moderate NFκB activation by GCK was only seen at the highest expression level, whereas
FIG. 3. **NFκB activation is dependent on the proline-rich SH3-binding sites in HPK1.**  
**A,** upper panel, COS1 cells were transiently cotransfected with expression plasmids for HPK1 or HA-tagged MLK3Δ alone or in combinations. 48 h after transfection NFκB activation was determined. Lower two panels, expression of the proteins was visualized by Western blotting using the polyclonal anti-HPK1 rabbit serum 7 or anti-HA antibody.  
**B,** the indicated expression plasmids were transiently transfected into COS1 cells, and after 48 h NFκB activation was determined. Lower panel, expression of the HA-tagged forms of HPK1 and Grb2 was visualized by anti-HA Western blotting.  
**C,** HPK1 or HA-tagged deletion mutants lacking the indicated SH3 domain-binding motifs and the NFκB reporter system were cotransfected into COS1 cells. After 48 h NFκB activation was determined. Expression of HPK1 proteins was demonstrated by anti-HPK1 Western blotting using rabbit serum 7. Localization of the proline-rich sites (P1, P2, and P4) and the amino acid sequence DDVD on the HPK1 protein is depicted (inset).  
**D,** HPK1 polyproline stretch deletion mutants are not impaired in their ability to activate p54-SAPKβ. Top panel, the HPK1 mutants assayed in **C** were transiently coexpressed in COS1 cells with p54-SAPKβ, immunopurified, and tested for their ability to autophosphorylate in vitro. Middle panel, equal expression was demonstrated by anti-HA Western blotting. **Bottom panel,** p54-SAPKβ activation was determined as described in Fig. 1D.
NFκB activation by GLK was largely blunted. At expression levels that resulted in comparable levels of autophosphorylation activity (Fig. 1F, upper panel), the capacity of the GCK-related kinases to activate NFκB correlated with their ability to activate the SAPK/JNK p54b (Fig. 1F, middle panel). These experiments clearly demonstrate that within the GCK subfamily I of Ste20 kinases HPK1 and the most closely related GCKR/KHS are potent activators of NFκB.

HPK1-mediated NFκB Activation Is Independent of HPK1-mediated SAPK/JNK Activation—We wondered whether NFκB activation by HPK1 was secondary to HPK1-mediated SAPK/JNK activation or caused by a component of the SAPK/JNK pathway. Three MAP3Ks, MEKK1 (34, 35), displayed comparable potency in activating NFκB, whereas MLK3 failed to activate NFκB (Fig. 2A, upper panel). By using identical conditions HPK1, MEKK1, and MLK3 all activate the SAPK/JNK p54b to a comparable extent (Fig. 2A, lower panel). Furthermore, we detected no synergism between HPK1 and MEKK1 in NFκB nor in SAPK/JNK p54b activation. Surprisingly, we found that coexpression of MLK3 potently inhibits HPK1-mediated NFκB activation, whereas it had no influence on SAPK/JNK p54b activation, suggesting that NFκB activation by HPK1 does not involve MLK3.

To demonstrate formally that HPK1-mediated NFκB and SAPK/JNK activation utilize distinct effector pathways, we took advantage of a mutant form of SEK1/MKK4, HPK1 is cleaved in vitro and in apoptotic cells by a caspase 3-like activity at a DDVD motif immediately preceding the P2 region. A, FDC-P1/D4 (left panel) or 32D-C13 (right panel) cells were rendered apoptotic by IL-3 withdrawal and lysed, and proteins were separated by SDS-PAGE. HPK1 was visualized by Western blotting with the polyclonal anti-HPK1 rabbit serum 7 directed against the C terminus of HPK1. Electrophoretic mobilities of full-length HPK1 and its cleaved forms are indicated. B, sequence comparison of the hinge region between the N-terminal kinase domain and the C-terminal Citron homology (CNH) domain of subfamily I GCK-related kinases. Kinase and CNH domains are indicated by gray boxes. The proline-rich motifs P1, P2, and P4 as well as the DDVD cleavage site are indicated by bars. Black boxed amino acids are conserved in all four kinases, and gray boxed amino acids identify conservative exchanges. C, top panel and middle panel, [35S]methionine-labeled HPK1 was generated by in vitro translation using cell-free reticulocyte lysates. Aliquots of the in vitro translation reaction were incubated at 37 °C for the indicated intervals with apoptotic cell extracts derived from HL60 cells. Where indicated apoptotic extract plus Me2SO or heat-pretreated apoptotic extract was added. Addition of an Ac-YVAD-CHO caspase inhibitor had no influence on the cleavage of in vitro translated [35S]methionine-labeled HPK1. In contrast, an Ac-DEVD-CHO caspase inhibitor blocked HPK1 cleavage completely. Bottom panel, the [35S]methionine-labeled HPK1 mutants HPK1(D383E) and HPK1(D383N) were incubated at 37 °C for 60 min in the absence (−) or presence (+) of apoptotic extracts. Reaction products were separated by SDS-PAGE and visualized by autoradiography. Electrophoretic mobilities of HPK1 and two predominant cleavage products are indicated by asterisks.
SEK1(S220A,T224L), which we will refer to as SEK1(AL). Mutations of the critical activation loop residues Ser-220 and Thr-224 render SEK1 refractory to upstream activating kinases and turn it into a potent dominant-negative inhibitor of SAPK/JNK activation at the MAP2K level. SEK1(AL) potently inhibits SAPK/JNK activation in a dominant-negative fashion at the MAP2K level (36). Whereas SEK1(AL) caused no change in HPK1 or MEKK1-induced NFκB activation (Fig. 2B, upper panel), SAPK/JNK activation was profoundly inhibited (Fig. 2B, lower panel), demonstrating that HPK1-mediated NFκB activation is independent of HPK1-mediated SAPK/JNK activation.

Kinase-deficient IkB Kinase β (IKKβ) Abrogates HPK1-mediated Activation of NFκB—To define further the level of HPK1 action and to test whether IKK functions downstream of HPK1, we coexpressed HPK1 and dominant-negative forms of NIK and IKKβ, NIK(KK429,430AA), and IKKβ(K444A). The dominant-negative variants of NIK and IKKβ both inhibited TNFα-stimulated as well as HPK1-mediated NFκB activation (Fig. 2C). Kinase-active NIK displayed an NFκB activation potential comparable to that of HPK1, whereas no synergy between both kinases was detectable. Blockage of HPK1 signaling to NFκB by overexpression of dominant-negative NIK or IKKβ protein suggested that HPK1 might act upstream of the IKK complex.

HPK1 kinase activity and phosphorylation status were not responsive to TNFα, neither did overexpression of HPK1 augment TNFα-mediated NFκB activation (not shown). Taken together these findings argue against a direct involvement of HPK1 in TNFα signaling.

HPK1-mediated NFκB Activation Is Blocked by Overexpression of SH3 Domain-containing Molecules—The surprising finding that MLK3 potently inhibited HPK1-mediated NFκB activation (Fig. 2A) led us to speculate that the SH3 domain-driven interaction between MLK3 and HPK1 (14) could result in sequestration of HPK1 from an NFκB-activating complex. To test this hypothesis we coexpressed MLK3Δ, a truncation mutant of MLK3 consisting of the N terminus, which includes the SH3 domain and 21 amino acids of the adjacent kinase domain (14) with HPK1 (Fig. 3A). According to our hypothesis we detected a potent suppression of HPK1-driven NFκB activation. Therefore we reasoned that other HPK1-binding SH3 domain-bearing molecules should also interfere with NFκB activation. The small adaptor Grb2, which consists of a central SH2 domain flanked by SH3 domains and HPK1, we decided to generate HPK1 mutants, in which the three proline-rich SH3 domain-containing molecule that does not bind to HPK1. Because of the possibility of residual interactions between overexpressed SH3 domains and HPK1, we decided to generate HPK1 mutants, in which the three proline-rich SH3 domain-binding sites (P1, P2, and P4) were deleted either singularly or in combination. When we tested the HPK1 proline deletions for activity comparable to wild-type HPK1 (Fig. 3B). These results lend further support to our notion that SH3 domain interactions are likely critically involved in HPK1-mediated NFκB activation.

NFκB Activation Is Dependent on the Proline-rich SH3-binding Sites in HPK1—Non-specific inhibition of HPK1-mediated NFκB activation could be excluded by cotransfection of an SH3 domain-containing molecule that does not bind to HPK1. Because of the possibility of residual interactions between overexpressed SH3 domains and HPK1, we decided to generate HPK1 mutants, in which the three proline-rich SH3 domain-binding sites (P1, P2, and P4) were deleted either singularly or in combination. When we tested the HPK1 proline deletions for their ability to activate NFκB, none of them displayed an activity comparable to wild-type HPK1 (Fig. 3C). The mutations did not impair protein stability, as they did not decrease HPK1-associated kinase activity or the capacity of HPK1 to activate SAPK/JNK (Fig. 3D). These results demonstrate a strong dependence of NFκB activation on the proline-rich SH3-binding sites in HPK1, whereas those sites were dispensable.

**Fig. 5.** The isolated C-terminal portion of HPK1 inhibits NFκB activation. A, top panel, HPK1, the isolated kinase domain (HPK1-Ko) or the C-terminal portion (HPK1-ΔN) were transiently expressed alone or in combination and tested for their ability to activate NFκB in COS1 cells. 2nd panel from top, the HPK1 proteins assayed in A were transiently coexpressed in COS1 cells with p54-SAPKβ, immunopurified, and tested for their ability to phosphorylate a c-Jun N-terminal fragment fused to GST in vitro. Lower two panels, expression of the different proteins was visualized by anti-HPK1 Western blotting using rabbit serum 3 or 7. B, increasing amounts of HPK1-ΔN cDNA were expressed either alone or in combination with NIK and the NFκB reporter system in COS1 cells. Luciferase activity was measured as described above. Inset, expression of HPK-ΔN was visualized by anti-HPK1 Western blotting using rabbit serum 7. C, FDC-P1 myeloid progenitor cell pools infected with either the empty retroviral vector MSCV (FDC-P1/vector only) or a virus transducing HPK1-ΔN (FDC-P1/HPK1-ΔN) were stimulated with 2 ng recombinant hTNFα and incubated for 2, 5, or 10 min followed by cell lysis. Endogenous phospho-IκBα was visualized by Western blotting using a phospho-IκBα (Ser-32)-specific antibody (upper panel). Total levels of IκBα and HPK1 proteins were visualized by anti-IκBα and by anti-HPK1 (rabbit serum 7) Western blotting.
for SAPK/JNK activation. Furthermore, we found a C-terminally HA-tagged version of HPK1 to be less efficient in activating NFκB as compared with the native protein (Fig. 3C), indicating a critical role of the HPK1 C terminus in NFκB activation.

Taken together our findings demonstrate that SH3 domain-mediated interactions are a prerequisite for NFκB activation and that HPK1-mediated SAPK/JNK and NFκB activation differ significantly in their molecular requirements.

**HPK1 Is Proteolytically Degraded in FDC-P1 Cells Rendered Apoptotic by IL-3 Withdrawal—**NFκB target genes have been implicated in a plethora of pro- and anti-apoptotic processes, and a number of NFκB regulators have been shown to be subject to caspase cleavage in apoptotic cells. Growth and survival of the hematopoietic progenitor cell line FDC-P1 is strictly IL-3-dependent (37). We tested HPK1 stability in apoptotic FDC-P1/D4 hematopoietic progenitor cells after induction of apoptosis by IL-3 withdrawal for 18 h. At this time point DNA fragmentation, a hallmark of the apoptotic cell death program, was maximal (not shown). By using Western blot analysis, we detected reduced HPK1 levels after IL-3 deprivation and observed the appearance of HPK1 cleavage products (Fig. 4A). These results were reproduced under identical conditions using the IL-3-dependent myeloid progenitor cell line 32D-C13 indicating that HPK1 is proteolytically degraded in hematopoietic cells rendered apoptotic after growth factor withdrawal.

**The Hinge Region between the HPK1 Kinase and the Citron Homology Domain Contains a Caspase Recognition Motif—**Caspases, the effector proteases during apoptosis (38), display overlapping substrate specificity, with the four N-terminal amino acids preceding their cleavage site being most important for substrate recognition and turnover. An Asp residue at the position N-terminally flanking the cleavage site is indispensable for all caspases. Caspase 3/7 cleavage sites are defined by the DXx(D motif, where X denotes a wide variety of amino acids and ψ a hydrophobic amino acid (39, 40). Inspection of the primary sequence of the HPK1 hinge region between kinase and CNH domain, which also contains the proline-rich motifs, revealed several potential caspase recognition sites with a DDVD motif, which also contains the proline-rich motifs, revealed a number of potential caspase recognition sites with a DDVD motif domain, which also contains the proline-rich motifs, revealed a number of potential caspase recognition sites with a DDVD motif domain.

**HPK1 Is Efficiently Cleaved by Apoptotic Cell Extracts in Vitro—**To address the question, if the HPK1 DDVD motif is recognized by caspases, we first employed an *in vitro* test system, in which [35S]methionine-labeled HPK1 generated by *in vitro* translation was exposed to cytoplasmic extracts of apoptotic HL60 cells. Such extracts contain abundant activated caspases. During a 60-min incubation HPK1 was efficiently cleaved into two fragments, the size of which correlated well with the calculated fragment sizes of 43 and 48 kDa. Heat pretreatment of the apoptotic extracts completely abolished proteolytic degradation (Fig. 4C, top panel).

**HPK1 Cleavage Is Blocked by the Caspase 3/7 Inhibitor Ac-DEVD-CHO and Depends on a DDVD Motif—**Caspases 3 and 7 can be blocked by incubation with the inhibitory peptide Ac-DEVD-CHO, whereas the Ac-YVAD-CHO inhibitor blocks caspases 1 and 4 preferentially. When HPK1 was incubated with apoptotic HL60 extracts in the presence of Ac-DEVD-CHO, proteolytic cleavage was efficiently blocked, whereas the Ac-YVAD-CHO inhibitor showed no effect (Fig. 4C, middle panel). Addition of the vehicle Me₃SO alone had no inhibiting effect on HPK1 cleavage (Fig. 4C, top panel).

Two point mutants HPK1(D383N) and HPK1(D383E), in which the DDVD motif was either changed to DDVN or DDVE, were found to be completely cleavage-resistant (Fig. 4C, bottom panel). These data identify the DDVD motif in the hinge region as a relevant target site for HPK1 cleavage by apoptotic extracts *in vitro*.

Our data indicated that in apoptotic myeloid progenitor cells proteolytic cleavage of HPK1 occurs after growth factor withdrawal.

**The Isolated HPK1 C Terminus Suppresses NFκB Activation by HPK1—**To address possible implications of HPK1 proteolytic cleavage during apoptosis, we tested the signaling capacity of the isolated HPK1 kinase domain (HPK1-Ko) or the HPK1 C-terminal fragment (HPK1-ΔN). HPK1-ΔN comprises the proline-rich motifs P2 and P4 as well as the CNH domain. Whereas expression of full-length HPK1 caused robust stimulation of NFκB-mediated transcription, none of the two fragments caused a detectable activity (Fig. 5A, upper panel). In agreement with our previous results the intact kinase domain HPK1-Ko was necessary and sufficient for SAPK/JNK activation (14), whereas HPK1-ΔN failed to activate SAPK/JNK (Fig. 5A, 2nd panel from top). We did not detect an augmented kinase activity of HPK1-ΔN as a result of the removal of the C-terminal part (not shown). When we coexpressed HPK1 fragments in combination with full-length HPK1, HPK1-ΔN inhibited HPK1-induced NFκB stimulation, whereas HPK1-Ko failed to exert an effect. Under identical conditions activation of SAPK/JNK by full-length HPK1 was not altered.

These data indicate that the C-terminal part of HPK1 was capable of inhibiting HPK1-driven NFκB activation, although it did not alter HPK1-mediated SAPK/JNK activation.

**HPK1-ΔN Inhibits NFκB Activation by NIK—**To investigate whether the C-terminal part of HPK1 was able to inhibit NFκB activation by stimuli other than HPK1 itself, we tested a potential effect on NIK that displayed an NFκB activation capacity comparable to HPK1 (Fig. 2C). Coexpression of increasing amounts of NIK and HPK1-ΔN resulted in a dramatic dose-dependent decrease in NFκB activation (Fig. 5B). This observation clearly showed that a C-terminal fragment of HPK1, like HPK1-ΔN, is a potent inhibitor of NFκB activation.

**HPK1-ΔN Reduces Phosphorylation of IκBα in FDC-P1 Cells—**To assess the capacity of HPK1 cleavage products to act as inhibitors of NFκB activation at physiological levels, we derived retrovirally infected FDC-P1 cells that stably expressed the C-terminal fragment HPK1-ΔN. Selected clones were pooled to avoid effects due to clonal variation. These clones expressed endogenous full-length HPK1 and HPK1-ΔN at a similar ratio to that observed in FDC-P1/D4 cells rendered apoptotic by growth factor withdrawal (see Fig. 4A and Fig. 5C, bottom panel). To test for a possible impact of HPK1-ΔN on NFκB activation, we compared the appearance of IκBα phosphorylated on serine 32 in FDC-P1/HPK1ΔN cells and FDC-P1 cells infected with the parental retroviral vector pMSCV (FDC-P1/vector only) after application of TNFα.

In FDC-P1/vector only cells IκBα phosphorylation sharply peaked at 5 min after stimulation and was barely detectable after 10 min (Fig. 5C, top panel), at the same time a decrease in total IκB levels became apparent (Fig. 5C, middle panel). In FDC-P1/HPK1-ΔN cells the accumulation of phospho-IκBα was found to be significantly reduced indicating a suppression of NFκB activation (Fig. 5C, top panel). Therefore, it appears that cleavage of HPK1 in apoptotic hematopoietic cells may be utilized as an effective tool to block NFκB activation.

**DISCUSSION**

Hematopoietic progenitor kinase (HPK1), a GC kinase-related mammalian Ste20 homologue, has been implicated as an upstream regulator of SAPK activity. We show here that HPK1 also potently activates NFκB transcription factors in hemato-
NFκB activation by HPK1

NFκB activation by HPK1 is not mediated by the TNF family of cytoplasmic serine/threonine kinases. Here we have presented data that show activation of NFκB by HPK1 and proteolytic cleavage of HPK1 during apoptosis in growth factor–deprived myeloid progenitor cells. Proteolytic cleavage converts HPK1 into an inhibitor of NFκB. These findings significantly enhance our knowledge on HPK1 activity and open novel avenues to test the biological roles of HPK1 which is likely to fulfill yet undefined functions that rely on NFκB activation.

Acknowledgments—We thank M. Grell for an NFκB-binding site containing plasmid; J. Kehrl for a GCKR expression plasmid; D. Kitamura for the pCATH-neo plasmid; D. Goeddel (Tularik) for IKKα and IKKβ expression plasmids; J. Kehrl for a GCKR expression plasmid; D. Kita-mura for the pCAT7-neo plasmid; D. Goeddel (Tularik) for IKKα and IKKβ expression plasmids. We are grateful to W. Vogel, V. Jassal, and B. Bock for critically reading the manuscript. We thank B. Engelhardt for stimulating discussions and valuable contributions.

References

1. Kyriakis, J. M. (1999) Gene Expr. 7, 217–231
2. Mercurio, F., and Manning, A. M. (1999) Curr. Opin. Cell Biol. 11, 226–232
3. Dhanasekaran, N., and Premkumar, R. R. (1998) Oncogene 17, 1447–1455
4. Tibbles, L. A., and Woodgett, J. R. (1999) Cell. Mol. Life Sci. 55, 1230–1245
5. Karin, M. (1996) Philos. Trans. R. Soc. Lond.-Biol. Sci. 351, 127–134
6. Kanaus, U. G., and Bokoch, G. M. (1998) Int. J. Biochem. Cell. Biol. 30, 857–862
7. Bagrodia, S., and Cerione, R. A. (1999) Trends Cell. Biol. 9, 350–355
8. Kyriakis, J. M. (1999) J. Biol. Chem. 274, 5259–5262
9. Schultz, J., Copley, R. R., Doerk, T., Ponting, C. P., and Bork, P. (2000) Nucleic Acids Res. 28, 231–234
10. Tung, R. M., and Blenis, J. (1997) Oncogene 16, 653–659
11. Shi, C. S., and Kehrl, J. H. (1997) J. Biol. Chem. 272, 32102–32107
12. Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Keesler, G., Zukowski, M., Tan, T. H., and Yao, Z. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9687–9692
13. Katz, P., Whalen, G., and Kehrl, J. H. (1994) J. Biol. Chem. 269, 16802–16809
14. Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996) EMBO J. 15, 7013–7025
15. Hu, M. C., Gu, W. R., Wang, X. M., Meyer, C. F., and Tan, T. H. (1996) Genes Dev. 10, 2251–2264
16. Pombio, C. M., Kehrl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T., and Kehrl, J. H. (1995) Nature 377, 750–754
17. Shi, C. S., Leonard, A., Kyriakis, J., Siebenlist, U., and Kehrl, J. H. (1999) J. Immunol. 163, 3279–3285
18. Yuasa, T., Ohno, S., Kehrl, J. H., and Kyriakis, J. M. (1998) J. Biol. Chem. 273, 22681–22692
19. Nagata, Y., Kiefer, F., Watanabe, T., and Todokoro, K. (1999) Blood 93, 14683

Poietic and COS1 cells. These findings corroborate a previous report showing stimulation of IKKα/β-mediated IκBα phosphorylation after forced expression of HPK1 (41). Among the GCK subfamily I kinases, we also observed NFκB activation by the closest HPK1 homologue GCKR/KHS, whereas the more distantly related GCK caused moderate NFκB activation, and GLK failed to elicit any activity in COS1 cells. NFκB activation by GCK-related kinases may be highly cell type-dependent as GCK and GCKR/KHS failed to affect NFκB in HEK293 cells (11), whereas in melanoma cells GCK activated NFκB moderately (42).

The molecular requirements for NFκB activation differed substantially from those for activation of SAPK, which appear to necessitate the HPK1 kinase domain mainly (14). Our data suggest the existence of distinct HPK1-containing complexes responsible for SAPK/JNK and NFκB activation. The scaffolding protein JIP1 has been described to coordinate a SAPK/JNK-activating complex that contains SAPK/JNK, MKK7/SEK2, MLK3, and HPK1 and likely facilitates the interaction of the pathway compounds (43). NFκB activation has been shown to be dependent on the activity of a high molecular mass complex of 700–900 kDa containing the IκB-kinases (IKKs) α and β (44). Whether HPK1 is physically associated with this complex remains to be established. Kinase-deficient mutants of NFκB-inducing kinase (NIK) and IKKβ efficiently inhibited HPK1-mediated NFκB activation, suggesting that HPK1 acts upstream of the IKK complex. Suppression of NIK-induced NFκB activation by an N-terminal HPK1 deletion mutant implicated HPK1 downstream of NIK. The apparent paradox of HPK1 being able to act upstream and downstream of NIK might result from the action of overexpressed mutant forms of both proteins on the same NFκB-inducing complex. Furthermore, SH3 domain interactions are likely to contribute crucially to the formation of the HPK1-containing NFκB-activating complex, as coexpression of SH3 domain-containing molecules strongly interfered with this process, and NFκB activation was dependent on the presence of intact polyproline sites in HPK1.

MLK3, which has been shown to interact with HPK1 via its SH3 domain (14), was recently reported to phosphorylate directly IKKα and IKKβ (45) raising the interesting possibility that MLK3 might mediate NFκB stimulation by HPK1. Although we and others (46) failed to detect NFκB activation by MLK3, this could be a consequence of the different cell types used in the assay systems (HeLa versus COS1) or different amounts of DNA transfected (45).

In the adult, HPK1, which is exclusively hematopoietic, displays the most restricted expression pattern of the GCK subfamily I kinases. In contrast to GCK, GCKR/KHS and GLK, which appear to be elements of TNF signaling pathways, HPK1 is not activated by TNFα in vivo. GCK and GCKR/KHS were reported to bind TRAF2 (11, 18) and therefore are likely to act at a receptor proximal position in TNF signaling. However, GCK binding is dispensable for TRAF2-mediated p38, SAPK/JNK, and NFκB activation (47). The TRAF2-GCK complex was recently described to protect melanoma cells against UV-induced apoptosis (48). Increasing expression of TRAF2 and GCK during melanoma progression was positively linked to JNK and NFκB activity. In T cells HPK1 has been shown to be constitutively associated with the adaptor protein Grb2 and its homologue Grap (20), whereas association with the Grb2 family member Gads was only observed after T cell receptor ligation (21). Both B and T cell receptor engagement cause stimulation of HPK1 kinase which is dependent on Src and Syk/ZAP-70 tyrosine kinases and the adaptor proteins LAT, SLP76/BLNK (20). Taken together these studies suggest that HPK1, which is dependent on inducible tyrosine phosphorylation of immunospecific adaptors like LAT and SLP76/BLNK, mediates immunoreceptor signals in a receptor proximal position resulting in the stimulation of SAPK/JNK and NFκB effector pathways. The subfamily I GCK kinase HPK1 therefore appears to have adopted a specific function in hematopoietic cells coupling cell type-specific receptor systems to ubiquitous effector pathways.

The precise role of HPK1 during apoptosis of murine hematopoietic cells is not well understood. We found HPK1 to be cleaved by a caspase 3-like activity in vitro, and we detected corresponding proteolytic products in apoptotic cells in vivo. The caspase C-terminal fragment inhibited NFκB activation by HPK1 and NIK in a dominant-negative manner and strongly reduced phosphorylation of IκBα in FDC-P1 cells. Although HPK1 is not likely to be involved in the induction of apoptotic processes in hematopoietic cells, its conversion from an activator of NFκB to an inhibitor of NFκB may contribute significantly to the efficient execution of the apoptotic program.

While our manuscript was in preparation, caspase-mediated cleavage of human HPK1 was demonstrated in Jurkat T cells following Fas ligation (48).

Caspase cleavage has been reported for several mammalian Ste20 kinases. Proteolysis of SPAK/PASK has been implicated in the regulation of subcellular localization (49), whereas caspase 3 cleavage of MST/Krs (50, 51), SLK (52) and PAK2 (53, 54) results in the generation of an activated apoptosis-inducing kinase domain fragment. Interestingly, we did not detect an enhanced kinase activity of the isolated HPK1 kinase domain, suggesting that the HPK1 regulatory C terminus fulfills no autorepressive function as has been described for PAK2.

Mammalian GCK-related kinases are a rapidly growing family of cytoplasmic serine/threonine kinases. Here we have presented data that show activation of NFκB by HPK1 and proteolytic cleavage of HPK1 during apoptosis in growth factor-deprived myeloid progenitor cells. Proteolytic cleavage converts HPK1 into an inhibitor of NFκB. These findings significantly enhance our knowledge on HPK1 activity and open novel avenues to test the biological roles of HPK1 which is likely to fulfill yet undefined functions that rely on NFκB activation.
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3347–3354
20. Liou, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M. H., Kurosaki, T., and Weiss, A. (2000) Immunity 12, 399–408
21. Anafi, M., Kiefer, F., Gish, G. D., Mhamalu, G., Iscove, N. N., and Pawson, T. (1997) J. Biol. Chem. 272, 27804–27811
22. Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998) Oncogene 17, 1893–1901
23. Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N., and Pawson, T. (1997) J. Biol. Chem. 272, 27804–27811
24. Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrl, W., Feller, S. M., and Tan, T. H. (1999) Mol. Cell. Biol. 19, 1036–1047
25. Sakakeeny, M. A., and Greenberger, J. S. (1982) J. Natl. Cancer Inst. 68, 305–317
26. Dexter, T. M., Garland, J., Scott, D., Scolnick, E., and Metcalf, D. (1980) J. Exp. Med. 152, 1036–1047
27. Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998) Oncogene 17, 1893–1901
28. Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N., and Pawson, T. (1997) J. Biol. Chem. 272, 27804–27811
29. Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998) Oncogene 17, 1893–1901
30. Fearnhead, H. O., McCurrach, M. E., O'Neill, J., Zhang, K., Lowe, S. W., and Lazebnik, Y. A. (1997) Genes Dev. 11, 1266–1276
31. Schlaeger, T. M., Qin, Y., Fujisawa, Y., Magram, J., and Sato, T. N. (1995) Development 121, 1089–1098
32. Arnold, R., Burcin, M., Kaiser, B., Muller, M., and Renkawitz, R. (1996) Nucleic Acids Res. 24, 2640–2647
33. Zhou, G., Lee, S. C., Yao, Z., and Tan, T. H. (1999) J. Biol. Chem. 274, 13133–13138
34. Lee, S. C., Hagler, J., Chen, Z. J., and Maniatis, T. (1997) Cell 88, 213–222
35. Lee, S. C., Hagler, J., Chen, Z. J., and Maniatis, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 95, 9319–9324
36. Tan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. F. (1994) Nature 372, 798–800
37. Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998) Oncogene 17, 1893–1901
38. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
39. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghatry, T., Brady, R. D., and Wong, W. W. (1997) J. Biol. Chem. 272, 9677–9682
40. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordström, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholsen, D. W. (1997) J. Biol. Chem. 272, 17907–17911
41. Hu, M. C., Wang, Y., Qiu, W. R., Mikhail, A., Meyer, C. F., and Tan, T. H. (1999) Oncogene 18, 5514–5524
42. Ivanov, V. N., Kehrl, J. H., and Ronai, Z. (2000) Oncogene 19, 933–942
43. Whitemarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) Science 281, 1671–1674
44. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) Science 278, 866–869
45. Hehner, S. P., Hofmann, T. G., Ushmorov, A., Dientz, O., Leung, I. W., Lassam, N., Scheidererit, C., Droge, W., and Schmitz, M. L. (2000) Mol. Cell. Biol. 20, 2556–2565
46. Zhao, Q., and Lee, F. S. (1999) J. Biol. Chem. 274, 8355–8358
47. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Genes Dev. 13, 1297–1308
48. Chen, Y. R., Meyer, C. F., Ahmed, B., Yao, Z., and Tan, T. H. (1999) Oncogene 18, 7370–7377
49. Johnston, A. M., Naselli, G., Genez, L. J., Martin, R. M., Harrison, L. C., and DeAizpurua, H. (2000) Oncogene 19, 4290–4297
50. Graves, J. D., Gotuh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) EMBO J. 17, 2224–2234
Caspase-mediated Cleavage of Hematopoietic Progenitor Kinase 1 (HPK1) Converts an Activator of NFκB into an Inhibitor of NFκB

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J. Biol. Chem. 2001, 276:14675-14684.
doi: 10.1074/jbc.M008343200 originally published online January 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M008343200

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