Hematopoietic Progenitor Kinase 1 Associates Physically and Functionally with the Adaptor Proteins B Cell Linker Protein and SLP-76 in Lymphocytes

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B cell linker protein (BLNK) is a SLP-76-related adaptor protein essential for signal transduction from the BCR. To identify components of BLNK-associated signaling pathways, we performed a phosphorylation-dependent yeast two-hybrid analysis using BLNK probes. Here we report that the serine/threonine kinase hematopoietic progenitor kinase 1 (HPK1), which is activated upon antigen-receptor stimulation and which has been implicated in the regulation of MAP kinase pathways, interacts physically and functionally with BLNK in B cells and with SLP-76 in T cells. This interaction requires Tyr<sup>452</sup> of HPK1 and the Src homology 2 (SH2) domain of BLNK/SLP-76. Via homology modeling, we defined a consensus binding site within ligands for SLP family SH2 domains. We further demonstrate that the SH2 domain of SLP-76 participates in the regulation of AP-1 and NFAT activation in response to T cell receptor (TCR) stimulation and that HPK1 inhibits AP-1 activation in a manner partially dependent on its interaction with SLP-76. Our data are consistent with a model in which full activation of HPK1 requires its own phosphorylation on tyrosine and subsequent interaction with adaptors of the SLP family, providing a mechanistic basis for the integration of this kinase into antigen receptor signaling cascades.

The most proximal events following the stimulation of lymphocyte antigen receptors are the activation of Src family (Lck, Lyn, and Fyn) and Syk family (Syk and ZAP-70) protein-tyrosine kinases (PTKs). Many of the substrates of these PTKs are adaptor proteins composed of modular domains, which mediate various types of protein-protein interactions (1–6). For example, the reversible phosphorylation of tyrosine residues creates transient binding surfaces for SH2 or PTB domains (7). Thus, phosphotyrosine interactions allow the dynamic modulation of signaling complexes.

In T cells, tyrosine phosphorylation of the palmitoylated transmembrane protein LAT is critical for signaling from the TCR, because it allows the SH2/PTB domain-mediated recruitment of signal transducers like PLC<sub>γ</sub>1, Shb, and Grb2 adaptors (Gads, Grb2 and Grap) (3) into proximity with the TCR within glycosphingolipid-enriched membrane microdomains (3–6, 8). Through independent interactions with their SH3 domains, Grb2 adaptors recruit several other signal transducers, including Cbl, p85<sup>PTK</sup>, HPK1, Sos, and SLP-76 (3, 4, 9–12). These interactions are required for activation of the Ras pathway and the elevation of intracellular calcium, ultimately leading to the activation of transcription factors like AP-1 and NFAT. In particular, gene disruption studies demonstrate that SLP-76 is essential for T cell development and assists in linking TCR signaling to activation of PLC<sub>γ</sub>1, Ras, and Erk (13–15). These functions rely on dynamic associations of tyrosine-phosphorylated SLP-76 with the SH2 domains of Itk, Vav, and Nck (3, 6). SLP-76 itself also possesses an SH2 domain for which only one ligand has been identified so far, SLAP-130/FYB (16, 17). Upon TCR stimulation, SLAP-130 is phosphorylated on tyrosine and subsequently binds to SLP-76, thereby modulating the TCR-induced signaling cascade (16–21).

While most cytoplasmic components implicated in TCR signaling have direct counterparts in B cells, no functional analog of LAT has been identified in B cells, and the mechanism mediating recruitment of Grb2-family members to the B cell receptor (BCR) complex remains enigmatic. Although LAT is not expressed in B cells (6), B lymphocytes do contain a relative of SLP-76, BLNK/SLP-65/BASH (hereafter referred to as BLNK; Fig. 1A) (22–24). BLNK undergoes Syk-dependent tyrosine phosphorylation and is recruited into glycosphingolipid-enriched membrane microdomains upon BCR stimulation (1, 25–27). Prior studies demonstrated that BLNK is essential for BCR-induced Erk, JNK, and p38 activation, PLC<sub>γ</sub>2 activation, IP<sub>3</sub> production, Ca<sup>2+</sup> mobilization, up-regulation of CD69 and N-terminal kinase; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; TCR, T cell receptor; CLNK, cytokine-dependent hemopoietic cell linker; MIST, mast cell immunoreceptor signal transducer.
CD86/B7–2, proliferation, and B cell development (28–33). Importantly, BLNK undergoes molecular interactions characteristic of those documented for both LAT and SLP-76 (25). Following its tyrosine phosphorylation, the N-terminal domain of BLNK binds to the SH2 domains of Vav, Nck, Btk (the B cell Itk homolog), PLCγ2, and Cbl(22, 26, 27, 34). BLNK also contains proline-rich regions that recruit Grb2 adapters. While these interactions explain some of the functions of BLNK, it is still unknown how BLNK connects the BCR to activation of JNK and p38.

BLNK harbors an SH2 domain at its C terminus, for which no functional ligands have been identified (3). To explore the functions of the BLNK SH2 domain, we employed a Syk-driven yeast two-hybrid screen, which could identify novel phosphotyrosine-dependent binding partners for BLNK in B cells. Such molecules could provide links between BLNK and the JNK or p38 pathways. We isolated multiple interactors for BLNK baits, including Gads, Vav, and the serine/threonine kinase HPK1 (35, 36). Interestingly, HPK1 has been shown to be activated upon antigen receptor stimulation, whereupon it acts as a regulator of MAP kinase pathways (9, 10). We show that HPK1 and SLP-76 can be efficiently co-immunoprecipitated from lysates of TCR-stimulated, but not unstimulated, Jurkat cells. This interaction requires Tyr379 of HPK1 and an intact SLP-76 SH2 domain. In cotransfection experiments with SLP-76-deficient J14 cells (13), we demonstrate that the SLP-76 SH2 domain participates in the regulation of AP-1 and NFAT activation in response to TCR stimulation. Finally, HPK1 acts as a dose-dependent inhibitor of HPK1 (9, 10) and an intact SLP-76 SH2 domain. In cotransfection experiments with SLP-76-deficient J14 cells (13), we demonstrate that the SLP-76 SH2-domain participates in the regulation of AP-1 and NFAT activation in response to TCR stimulation. Full activation of HPK1 in response to antigen receptor stimulation requires both tyrosine phosphorylation of HPK1 and its subsequent interaction with the SH2 domains of SLP-76 (in T cells) or BLNK (in B cells). Thus, HPK1 acts in part to connect SLP family adaptors to mitogen-activated protein kinase signaling and the negative regulation of AP-1 activation in lymphocytes.

EXPERIMENTAL PROCEDURES
DNA Constructs, Reverse Transcription PCRs, and Site-directed Mutageneses—We based our Syk-driven yeast two-hybrid system on the MATCHMAKER LexA Two-hybrid System (CLONTECH Laboratories, Inc., Palo Alto, CA). To generate a bait vector that allows coexpression of a bait and Syk as a driver kinase, we inserted a cDNA fragment encoding murine Syk into the Gal-inducible expression cassette of pYES2 (NT 1–876; Invitrogen, Inc., San Diego, CA) and subcloned the Syk expression cassette into the unique Nru I site of pLexA, generating pLexASyk. Grb2 SH2 (R85K) was generated via PCR mutagenesis. Full-length murine BLNK was obtained via reverse transcriptase-PCR from mouse spleen poly(A) RNA. The R327K mutation was introduced via PCR mutagenesis. Identity and sequence of all constructs were confirmed via automated DNA sequence analysis. Target cDNA libraries in pB42AD were constructed using the Two-hybrid cDNA Library Construction Kit (CLONTECH) following the user manual. We used double oligo(dT)-purified mouse spleen or human bone marrow poly(A) RNA (CLONTECH) or prepared our own triple oligo(dT)-purified poly(A) RNA from thymocytes of 6–9-week-old C57BL/6 mice using FastTrack 2.0 kits (Invitrogen). 50% of each cDNA were random- or oligo(dT)-primed, pooled, and size-selected to exclude cDNAs of less than 500 base pairs. Each library represents >1.4 × 106 independent clones and contains at least 76% recombinant clones with average insert sizes of 0.7–0.8 kb. pEF-BOS, pEFFLAG SLP-76 WT, pEFFLAG SLP-76 R45K, and pCDEF-HPK1-HA have been described (9, 37). cDEF-HPK1(YF):HA was generated via standard PCR mutagenesis methods.

Yeast Two-hybrid Screens and Analyses—Yeast handling, analyses, and plasmid DNA isolation were carried out as described in the MATCHMAKER LexA Two-hybrid System user manual or the CLONTECH yeast protocols handbook. Yeast transformations were performed with the Yeastmaker yeast transformation system (CLONTECH). Yeast cell protein extracts were analyzed via immunoblotting with mAb-LexA, mAb-BLNK, or 4G10 antibodies for the presence and abundance of bait proteins or Syk or Syk activity. Two-hybrid screens were performed on inducing selection agar containing X-gal (H-U-T-F-JR/GX) for up to 10 days, and single candidate positive colonies were restreaked onto the same medium for further purification and propagation. For restriction endonuclease mapping, inserts of isolated target cDNA clones were amplified via PCR. Representatives for each class, defined by a unique restriction pattern, were sequenced and subjected to further analysis.

Homology Modeling and Data Base Searches—Homology models for the three major domains of HPK1 were generated based on the sequence alignment shown in Fig. 7A. Various SLP adapter sequences were modeled into the SH2 domain taken from the crystal structure of full-length Src, with ligand peptides modeled into the Src C-terminal peptide bound to the Src SH2 domain (38). A cartesian average of 10 intermediate homology models was subjected to steepest descent minimization to obtain the nearest approximation to the starting model. Modeling was performed using MODE version 2000.02 (Chemical Computing Group, Montreal, Canada). Backbone structures of human Src and hBLNK were rendered with RIBBONS (M. Carson, University of Alabama at Birmingham).

Antibodies and Immunoblot Analyses—Immunoprecipitations and immunoblots were performed using the following antibodies: sheep anti-(human SLP-76 (gift from G. Koretzky, University of Pennsylvania, Philadelphia, PA); α-HPK1 (9) or α-HPK1 (N-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); α-HPA, clone 3F10 (Roche Molecular Biochemicals); α-FLAG, clone M2 (Sigma); mAb-phosphotyrosin, clone 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY); and mAb-LexA (CLONTECH). For co-immunoprecipitation studies, antibodies were prebound to protein A + G-agarose beads (Santa Cruz Biotechnology). Immunoblot analyses were performed using ECL or ECF (Amersham Pharmacia Biotech) with quantification on a STORM system (Molecular Dynamics, Inc., Sunnyvale, CA).

T and B Cell Stimulation, Lysate Preparation, and Co-immunoprecipitations—Jurkat or J14 cells were kept in phosphate-buffered saline (154 mM NaCl, 10 mM sodium phosphate, pH 7.4) or stimulated either with C305 (anti-Jurkat Ti β-chain monoclonal antibody) as described in the MATCHMAKER user manual or with human UCD body; BD Pharmingen/OKT4 in phosphate-buffered saline/× 10 5 cells/ml followed by goat anti-mouse IgG cross-linking for 3 min at 37 °C, lysed by the addition of 1/4 volume of 5× YNP/PI (250 mM Tris-HCl, pH 8.0, 134 mM NaCl, 5% Nonidet P-40, 100 mM NaF, 1.5 mM Na3VO4, 0.1% NaN3, 2.9 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mg/ml TPC or, 50 μg/ml soybean trypsin inhibitor; all from Sigma) and rocking at 4 °C. Extracts were prepared and precleared with 10 μg of normal sera. Input extracts were denatured by the addition of 1/4 volume of 3× sample buffer and boiling. For co-immunoprecipitations, extracts corresponding to 2–9 × 10 5 cells were rotated at 4 °C with 4–20 μg of the respective antibody. After washing in Y-NPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 0.3 mM NaN3, 0.2% NaOAc, precipitated proteins were eluted by boiling in 3× sample buffer.

Cell Transfections, HPK1 Kinase Assays, and Reporter Gene Assays—Cell transfections, HPK1 kinase assays, and AP-1 or NFAT-luciferase reporter gene assays were performed as described in Ref. 9, except that pNFAT-TaLuc (CLONTECH) or pGL-2E-Luc (39) was used for NFAT reporter assays, and luciferase assays were performed with luciferase Plus kits (Packard). J14 lines stably reconstituted with various SLP-76 mutants (described in detail in Ref. 40) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin, streptomycin, and gentamicin. Cells were stimulated with C305 or stimulated with UCHT1 alone or in combination with OKT4 ascites fluid and CD28.2 (BD Pharmingen) followed by cross-linking with goat anti-mouse IgG (Jackson).

RESULTS
Isolation of Novel BLNK Interactors in a Syk-dependent Yeast Two-hybrid Screen—We employed a modified yeast two-hybrid screen strategy to search for novel phosphotyrosine-dependent and independent interactors that bind full-length BLNK (Fig. 1A). BLNK was co-expressed with murine Syk and used to screen various cDNA libraries. We isolated 166 reproducibly positive clones from 1.6 × 10 8 colony-forming units of an amplified murine spleen cDNA library representing ~1.9 × 10 6 independent clones. The isolates represented three classes of cDNAs. Class 1 (111 clones) encodes the C-terminal SH3...
Moreover, no interaction occurred when a mutant HPK1 construct was used, in which Tyr^{277} was replaced by phenylalalanine. Likewise, the interaction was abrogated in the absence of Syk. In control analyses, BLNK baits did not interact with LAT or Grb2 SH2 domain targets, and Lck SH2 domain or Lamin baits did not interact with any target (data not shown). These results suggest that HPK1 and BLNK can interact when coexpressed in the presence of Syk and that this interaction requires an intact BLNK SH2 domain and Tyr^{277} of HPK1.

Therefore, further study focused on HPK1 as a potential mediator of SLP adaptor signaling. In particular, although the Vav isolates from our screen interacted with wild type BLNK in the presence of Syk, interactions of varying intensities were also observed in the absence of Syk (data not shown). The variability of this interaction in yeast most likely reflects its overall low avidity and in any case rendered further studies impossible.

Association of HPK1 and SLP-76 in T Cells—HPK1 has recently been implicated in signal transduction from antigen receptors (9, 10). Engagement of BCR or TCR results in activation of HPK1. This activation was strongly reduced in B cells lacking BLNK or in T cells lacking SLP-76 (9). A possible explanation would be that molecular interactions with SLP adaptors are required for full activation of HPK1 in both B cells and T cells. To test if HPK1 associates with SLP-76 in T cells, we immunoprecipitated SLP-76 from lysates of unstimulated or anti-TCR-stimulated Jurkat cells, or SLP-76-deficient J14 cells as specificity controls, and visualized co-precipitating proteins on immunoblots (Fig. 2A). HPK1 could readily be detected in SLP-76 immunoprecipitates from anti-TCR-stimulated Jurkat cells but not in precipitates from unstimulated cells containing equal amounts of SLP-76 or in precipitates from J14 cells. Prolonged exposure of the blot permitted visualization of a weak HPK1 signal in anti-SLP-76 precipitates from unstimulated Jurkat cells, probably reflecting a weak basal association between HPK1 and SLP-76, which could be mediated by Grb2/Gads (data not shown). Stripping and reprobing of the completely stripped blot with an anti-phosphotyrosine antibody revealed that the SLP-76-associated HPK1 was possibly tyrosine-phosphorylated. In addition, a protein most likely representing tyrosine-phosphorylated LAT (pp36−38) co-precipitated with SLP-76. No co-precipitation of LAT was found in lysates from SLP-76-deficient cells. The presence of a weak band in precipitates from unstimulated cells (lane 3) could reflect a participation of LAT in basal interactions with SLP-76. The presence of tyrosine-phosphorylated proteins with similar mobility as SLP-76 and LAT could be demonstrated, albeit at low intensity, in HPK1 precipitates from lysates of ConA-stimulated Jurkat cells and normal murine thymocytes (data not shown). Taken together, these results demonstrate that TCR stimulation induces the molecular interaction of SLP-76 and HPK1 in a complex that may also include tyrosine-phosphorylated LAT.

We next investigated which regions of HPK1 and SLP-76 are required for their interaction. J14 cells were cotransfected with cDNAs encoding HA-tagged wild type or Y379F mutant HPK1 in combination with FLAG-tagged wild type or SH2 R448K mutant SLP-76, which is incapable of binding to tyrosine-phosphorylated ligands (37). As shown in Fig. 2B, immunoprecipitation of similar amounts of SLP-76 permitted significant co-precipitation of HA-HPK1 only in cells that express wild type HA-HPK1 and wild type FLAG-SLP-76 (lanes 3 and 4). In all other cases, no significant interaction was observed. The only slightly weaker interaction in unstimulated cells (lane 3) may result from the high expression levels of exogenous SLP-76 and HPK1 in combination with significant levels of...
basal tyrosine kinase activity in these cells as revealed by anti-phosphotyrosine probing of the lysate blots (data not shown). Moreover, basal interactions between SLP-76 and HPK1 could be mediated by Grb2/Gads or other adaptors. This notwithstanding, our findings suggest that both Tyr379 of HPK1 and the SH2 domain are required for the HPK1/SLP-76 interaction in T cells, which is augmented by TCR stimulation, particularly in the case of endogenous proteins.

**Fig. 2.** TCR stimulation induces the association of SLP-76 and HPK1 in a manner dependent on Tyr379 of HPK1 and the SLP-76 SH2 domain. A, Jurkat cells or SLP-76-deficient J14 cells were stimulated with anti-TCR antibody C305 for 2 min, lysed, and subjected to immunoprecipitation (IP) with polyclonal antibodies against human SLP-76. Lysates representing 1 × 10^5 cells or IP eluates representing 9 × 10^3 cells with (+) or without (−) stimulation were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with antibodies against HPK1 or human SLP-76 followed by ECL detection. Subsequently, the blots were stripped and reprobed with antibody 4G10 against phosphotyrosine (anti-p-tyr). Stripping was complete, because control bands containing HPK1 did not reappear on the 4G10-probed blot (data not shown). Positions of important proteins are indicated on the right. B, J14 cells were cotransfected with 10 μg each of plasmids encoding HA-tagged wild type (wt) or Y379F mutant (YF) HPK1 and FLAG-tagged wild type (wt) or SH2 mutant (RK) SLP-76. Following stimulation with medium (−) or anti-CD3 and anti-CD4 antibodies for 3 min (+), cells were lysed and subjected to IP with polyclonal anti-human SLP-76 antibodies. Input lysates representing 6.5 × 10^5 cells or precipitates representing 7 × 10^4 cells were analyzed via SDS-polyacrylamide gel electrophoresis and immunoblotting. Shown are blots probed with monoclonal antibodies against the HA (α-HA) or FLAG (α-FLAG) tags, along with a Ponceau staining of a portion of a lysate blot as a loading control.

**Fig. 3.** Full HPK1 activation by the TCR requires Tyr379 of HPK1 as well as the Gads-binding and SH2 domains of SLP-76. A, Jurkat cells were transiently transfected with HA-tagged wild type or Y379F mutant HPK1 to equal expression levels (data not shown) and stimulated for 1 min with buffer or the anti-TCR antibody C305. Cells were then lysed, and anti-HA immunoprecipitates were assayed for HPK1 kinase activity. B, SLP-76-deficient J14 cells stably reconstituted with vector, wild type SLP-76, or one of the SLP-76 mutants AP-1, Y3F, ΔGads, or SH2mut, which harbors a phosphotyrosine binding-deficient SH2 domain (37), were transfected with HA-tagged HPK1 and treated with the anti-TCR antibody C305 or a buffer control for 1 min. HPK1 catalytic activity was measured as described above. In all experiments, immunoprecipitates contained similar amounts of HPK1 protein as determined by immunoblotting with anti-HA antibodies. A blot from one representative experiment is shown in the bottom panel. Cell surface expression of the TCR on all J14 lines was comparable. In A and B, HPK1-activities are shown as the mean ± fold induction compared with unstimulated cells from three independent experiments. Error bars represent S.D.
tyrosine residues required for effector interactions with the SLP-76 N terminus were changed to phenylalanine (Y3F) (42) both rescued HPK1 activation. These results suggest that binding of HPK1 via phosphorylated Tyr775 to the SH2 domain of SLP-76 is required for its activation. In addition, our results demonstrate that binding of Grb2 type adaptors to SLP-76 is required for full HPK1 activation.

The SH2 Domain of SLP-76 Participates in the Regulation of AP-1 and NFAT Activation in Response to TCR Stimulation—Previous studies involving overexpression of SLP-76 in Jurkat cells (37) or expression of LAT/SLP-76 chimeric proteins in LAT-deficient J.CaM2 cells (43) have suggested a positive but nonessential role for the SLP-76 SH2 domain in TCR-induced NFAT and IL-2 promoter activation. To further investigate the role of the SLP-76 SH2 domain in the regulation of IL-2 promoter elements, we examined TCR-induced activation of AP-1 and NFAT in SLP-76-deficient J4.14 cells transiently transfected with either wild type or R448K mutant SLP-76 to similar expression levels as judged on immunoblots (Fig. 4). A weak background band that migrates at the position of SLP-76 on blots of untransfected (data not shown) or vector-transfected cells could represent a nonspecific cross-reactivity of the anti-SLP-76 antibody or a very weak residual expression of SLP-76 in J14 cells. However, anti-SLP-76 immunoprecipitation experiments argue against a significant expression of SLP-76 in J14 cells (Fig. 2A and Ref. 13), and compared with Jurkat cells, J14 cells are clearly impaired in TCR-induced pIL-2, NFAT, and AP-1 activation, arguing against significant residual SLP-76 function (13). As expected, J14 cells showed no significant AP-1 or NFAT activation in response to anti-TCR stimulation with two different antibodies (C305 and UCHT1) in the absence or presence of CD4/CD28 co-cross-linking (Fig. 4). Transfection of wild type SLP-76 allowed efficient activation of both reporter genes, which was maximal in the presence of costimulatory antibodies. However, transfection of R448K mutant SLP-76 rescued AP-1 and NFAT activation less efficiently, particularly in the absence of costimulation. Experiments with two different NFAT reporter constructs (either pGL-IL-2E, which contains three copies of the IL-2E element, which binds NFAT and Ets-factors (Fig. 4) (39), or pNFAT-TA-Luc, which contains three tandem copies of the NFAT consensus sequence upstream of the minimal TA promoter (data not shown)) yielded similar results. SLP-76 R448K consistently yielded lower activation levels of AP-1 than wild type SLP-76 over a range of SLP-76 protein expression levels in titration experiments (data not shown). Moreover, analyses of J14 cells stably reconstituted with various mutants of SLP-76 yielded similar results (40). However, the defect in NFAT activation in cells expressing the SH2 mutant is less severe than the defect seen when other domains of SLP-76 were mutated. Reconstitution of LAT-deficient cells with various LAT/SLP-76 chimeras provided similar results (43). Taken together, these results demonstrate that the SLP-76 SH2 domain contributes to activation of both AP-1 and NFAT in response to TCR stimulation but does not play an essential role.

Stimulation downstream of SLP-76 via PMA/ionomycin elicited strong responses even in untransfected (data not shown) and vector-transfected J14 cells (Fig. 4), indicating that the cells have no inherent signaling defect. Notably, we consistently observed a reproducible increase in PMA/ionomycin-induced AP-1 or NFAT activation with decreasing SLP-76 function; reporter gene activation was minimal (albeit high) in wild type SLP-76-transfected cells, intermediate in cells expressing R448K mutant SLP-76, and maximal in vector-transfected J14 cells. Thus, in addition to being required for TCR-induced activation of AP-1 and NFAT, SLP-76 may also be involved in the transmission of inhibitory signals that limit activation of AP-1 and NFAT via pathways downstream or independent of PLC-γ, RasGRP-, or Ca2+/-mediated events. The SLP-76 SH2 domain ligand SLAP-130 could possibly participate in such signals, since its overexpression had negative effects on NFAT/AP-1/pIL-2 activation under certain circumstances (16, 18). These possibilities require further investigation.

HPK1 Inhibits TCR-stimulated AP-1 Activation in a Manner Partially Dependent on Tyr779 of HPK1 and an Intact SLP-76 SH2 Domain—We recently demonstrated an inhibitory role for HPK1 in TCR-induced AP-1 activation in Jurkat cells. This inhibition requires the kinase activity of HPK1 (9). Full activation of HPK1 in response to TCR stimulation appears to require the physical interaction of HPK1 with the SLP-76 SH2 domain (Figs. 2 and 3). Therefore, we next investigated the effects of mutations that abrogate or reduce this interaction on TCR-induced AP-1 activation (Fig. 5). J14 cells were transiently transfected with either wild type or R448K mutant SLP-76 to similar expression levels (compare immunoblots in Fig. 5B) and cotransfected with increasing amounts of wild type or Y379F mutant HPK1. As shown in Fig. 5A, wild type

![Figure 4](http://www.jbc.org/content/jbc/article-pdf/121/10/45211/953574/45211.pdf)
HPK1 acts as a strong, dose-dependent inhibitor of AP-1 activation in response to CD3 stimulation in the absence and presence of costimulatory signals, in cells expressing wild type or R448K mutant SLP-76. However, Y379F mutant HPK1, expressed at levels similar to those of wild type HPK1, inhibits AP-1 activation much less potently in cells expressing wild type SLP-76. The difference in inhibition between wild type and Y379F mutant HPK1 is maximal at low HPK1 expression levels but still significant over the entire dose range except for maximal expression levels, where almost complete inhibition of AP-1 activation is observed and both curves converge. In striking contrast, no significant difference in inhibitory potency between wild type and Y379F mutant HPK1 is observed in cells that coexpress R448K mutant SLP-76. Thus, Tyr$^{379}$ of HPK1 is required for full inhibitory activity of HPK1 on TCR-induced AP-1 activation, but only in the context of a functional SLP-76 SH2 domain. This result suggests that Tyr$^{379}$ of HPK1 and the SLP-76 SH2 domain interact functionally, and in view of our other results (Figs. 2 and 3), this functional interaction is likely to reflect the molecular interaction of HPK1 and SLP-76 via its SH2 domain. This result suggests that Tyr$^{379}$ of HPK1 and the SLP-76 SH2 domain interact functionally, and in view of our other results (Figs. 2 and 3), this functional interaction is likely to reflect the molecular interaction of HPK1 and SLP-76 via its SH2 domain and phosphorylated Tyr$^{379}$ of HPK1.

**Definition of a Structural Motif Mediating HPK1 Binding to SLP Family SH2 Domains**—The importance of Tyr$^{379}$ in HPK1/BLNK and HPK1/SLP-76 interactions prompted us to evaluate HPK1 sequence motifs in more detail. SLAP-130/FYB has been identified as a phosphotyrosine-dependent ligand for the SLP-76 SH2 domain (16, 17, 20, 21). We therefore compared the amino acid sequences of human (h) and murine (m) HPK1 and SLAP-130 in the region of HPK1 surrounding Tyr$^{379}$ and in four related regions (labeled a–d) in SLAP-130 (Fig. 6A). Regions b (Tyr$^{595}$) and c (Tyr$^{651}$) of human HPK1 and SLP-76 SH2 domain target sites (Y) shown in A within the respective full-length proteins. SKAP56 and Vav represent the corresponding gene families. Domains are labeled as in Fig. 1. PPP, proline-rich domain; NLS, nuclear localization sequence. In Vav, the candidate target site is located within the acidic region.
(Tyr⁵) flanked by acidic residues at positions -6 (corresponding to -5 in mHPK1, which carries a one-amino acid deletion N-terminal of -3), -2, +1, and +2. At position +3, a hydrophobic residue is strictly conserved. Some features are only shared by HPK1 and subsets of the SLAP-130 fragments. These include acidic residues at positions -3, +4, +27, and +28. Based on these sequence similarities, we defined a candidate consensus motif for BLNK/SLP-76 SH2 domain ligands (Fig. 6A, bottom).

Although we screened yeast clones at ~86-fold redundancy of the library, our analysis did not identify all known BLNK interactors. Both Vav and HPK1 clones were highly underrepresented relative to Gads. No SLAP-130 homolog was found. We therefore searched protein data bases and GenBank™ for genes that are related to our consensus motif or to SLAP-130 and that might therefore represent novel BLNK or SLP-76 SH2 domain interactors. PATTERNFIND, PHI-BLAST, and manual searches identified both SLAP-130 and HPK1, but the BLNK/SLP-76 consensus motif was also found in additional proteins, including the SKAP55 and Vav families (Fig. 6, A and B).

Sequence comparisons by themselves do not suggest identical ligand specificities for SLP-76, BLNK, and CLNK/MIST, a recently identified third SLP family member (Data not shown; see Refs. 44 and 45). We therefore analyzed the structural features of their SH2 domains. Compared with published SH2 domain alignments (46), SLP family SH2 domains are characterized by extended BC, FB, and BG loops (Fig. 7, A and B). Loops AB and CD carry deletions. Most of the residues frequently involved in ligand interactions are conserved between all SLP family members, with the exceptions of β6, β4, α9, BG3, and BG4 (Fig. 7A) (46, 47). A three-dimensional representation, in which the aligned hBLNK SH2 domain sequence was modeled based on the crystal structure of the SH2 domain of full-length Src complexed to the Src C terminus (38), illustrates that the ligand binding groove accommodates a tyrosine-phosphorylated HPK1 undecapeptide including residues -4 to +6 flanking the phosphotyrosine (Fig. 7B). Similar results were obtained for all known SLP family SH2 domains binding to tyrosine-phosphorylated HPK1 or SLAP-130c ligand peptides. An enlarged view in which SH2 domain amino acids within a 4-Å distance from the ligand peptide are superimposed for hBLNK, mCLNK, and hSLP-76 reveals that most residues that can interact with the phosphopeptide ligand are homologous (Fig. 7, A and C). The phosphotyrosine appears embedded in a pocket in which basic side chains (α2A, βB5) participate in...
phosphate binding. Asp\(^{+1}\) extends inward, likely interacting with the basic side chains of BG4 and the backbone carbonyl of B\(\delta\)D4, in addition to a nonpolar interaction with B\(\delta\)D5 and a possible \(\pi\)-interaction with the aromatic ring of the conserved tyrosine B\(\delta\)3. Asp\(^{-2}\) extends away from the SH2 domain surface toward the solvent. Val\(^{+3}\) is embedded in a pocket and might be involved in hydrophobic interactions with the conserved L\(_{E,F1}\) and stabilizing backbone interactions with BG4. In addition, Asp\(^{+4}\) could undergo polar interactions with EF3, and Asp\(^{-3}\) could interact weakly with a N\(_{a3}\) in hBLNK and mCLNKL.

Other positions including +2 do not appear to participate in specificity determination. These observations are consistent with the definition of our consensus motif and suggest that HPK1 and SLAP-130 a and c (Fig. 6A), along with SKAP55 and Vav family proteins, could be optimal ligands. SLAP-130 c (Tyr\(^{651}\)) has indeed recently been implicated in Fyn-T-mediated binding to the SLP-76 SH2 domain (20, 21). A region of SLAP-130 (RTARGSY\(^{659}\)GYIKTTAV) that does not match our consensus has recently been identified as another SLP-76 SH2 domain ligand (18). Modeling analyses suggest that this peptide can be accommodated, but the interaction is predicted to be much weaker than with peptides that harbor the consensus motif (data not shown).

**Discussion**

Identification of Novel BLNK Interaction Partners—We isolated fragments of Vav, Gads, and HPK1 as BLNK interactors in a Syk-driven yeast two-hybrid screen. The isolation of Vav as a partially Syk-dependent interactor in yeast corroborates previous reports that Vav shows a basal interaction with SLP-76 and BLNK, which is enhanced by antigen receptor stimulation and involves the Vav SH2 domain (22, 23, 48, 49). The identification of Gads as a phosphotyrosine-independent interaction partner for BLNK is reminiscent of the SH3-dependent interactions of Grb2 with BLNK (22, 23) or Grb2 and Gads with SLP-76 (3, 4, 6). Gads has recently been found to be expressed in certain B cell subsets including naive tonsillar B cells and associates with BLNK in MP-1 cells (50).

**HPK1 Is a Phosphotyrosine-dependent Interactor for SLP Adaptor SH2 Domains**—We identified HPK1 (35, 36) as a strictly phosphotyrosine-dependent BLNK interactor. Two-hybrid analyses demonstrate that this interaction is mediated by phosphorylation of HPK1 on Tyr\(^{379}\) and its binding to the BLNK SH2 domain (Fig. 1C). These results are confirmed and complemented by findings of Tsuji et al. (81), of which we learned while our studies were in progress. This group isolated HPK1 as a protein that associates with the BLNK SH2 domain in lyses of BCR-stimulated B cells. The interaction depends on Tyr\(^{379}\) of HPK1 and an intact BLNK SH2 domain (Fig. 1C). Full activation of HPK1 requires both Tyr\(^{379}\) and an intact BLNK SH2 domain (81).

We demonstrate the occurrence and functional significance of a similar interaction of HPK1 with SLP-76 in T cells. HPK1 associates with SLP-76 and a tyrosine-phosphorylated protein most likely representing LAT in anti-TCR stimulated Jurkat cells (Fig. 2A). In cotransfected J14-cells (Fig. 2B), the HPK1/SLP-76 interaction requires Tyr\(^{379}\) of HPK1 and the SLP-76 SH2 domain, both of which are also required for full activation of HPK1 in response to TCR stimulation (Fig. 3). Finally, we show that the SLP-76 SH2 domain contributes to full AP-1 and NFAT activation in response to TCR stimulation (Fig. 4) and that HPK1 inhibits AP-1 activation in a manner partially dependent on Tyr\(^{379}\) of HPK1 and the SLP-76 SH2 domain (Fig. 5). Importantly, the dependence on Tyr\(^{379}\) of HPK1 is relieved by concomitant mutation of the SLP-76 SH2 domain. This provides genetic evidence for a functional interaction between HPK1 and SLP-76 that involves these complementary interaction domains.

Associations of the SLP-76 SH2 domain with a tyrosine-phosphorylated protein and a putative serine/threonine kinase, both of ~100 kDa, in anti-TCR-stimulated Jurkat cells have been reported previously (37). Moreover, an association of Gads with BLNK and an unknown serine/threonine kinase activity, which was increased after BCR stimulation, has recently been found in B cells (50). Our results suggest that these activities represent HPK1, a 97-kDa protein. Complementing our detection of possibly tyrosine-phosphorylated HPK1 in SLP-76 immunoprecipitates from anti-TCR-stimulated Jurkat cells (Fig. 2A), TCR stimulation has recently been shown to induce tyrosine phosphorylation of a small fraction of HPK1 in murine DO11.10 T hybridoma cells and Jurkat cells (10, 11).

Based on homology models, we defined a structural motif that mediates ligand binding to SLP adaptor SH2 domains (Fig. 6A). Both Src- and Syk-type PTKs show strong preferences for substrate tyrosine residues (Tyr\(^{0}\)) in an acidic sequence context. However, while Syk family PTKs strongly prefer acidic residues as immediate neighbors of Tyr\(^{0}\) (51, 52), Src-type PTKs favor hydrophobic amino acids at position −1 (52–55). Thus, HPK1 Tyr\(^{378,381}\) should be a perfect substrate for SYK kinases, while the tyrosines in SLAP-130 b–d should be preferred substrates for Src-PTKs. This could explain why we isolated HPK1 as a Syk-dependent BLNK interactor, whereas SLAP-130 was in part isolated as a Fyn-dependent SLP-76 SH2 domain binder (16, 17, 20, 21). The affinities between SH2 domains and their ligands are mainly determined by interactions with the phosphorylated Tyr\(^{0}\) and the 3–6 residues C-terminal of it. Positions +1, +2, and +3 generally appear most critical (7, 46, 47). Our structural modeling analyses suggest that all SLP adaptor SH2 domains show strong preferences for acidic amino acids at +1 and hydrophobic residues at +3. Acidic side chains at −3 and +4 may increase ligand affinity, particularly for BLNK (Fig. 7C). Precedents for participation of positions +4 or +5 or of residues N-terminal of Tyr\(^{0}\) do exist (46, 56–59). The similarity between the SLP family SH2 domain target motif and that of Src-type SH2 domains (YXXL in an acidic context, where X is preferably acidic) (46, 56), suggests that HPK1 and SLAP-130 can bind to both. Indeed, SLAP-130 has been shown to interact with the SH2 domain of Fyn (14). Finally, our data base searches revealed that the Vav and SKAP55 families have a structural propensity for SLP adaptor SH2 domain interactions (Fig. 6A). While SKAP55 adaptors associate with SLAP-130 via SH3 domain interactions (60, 61), Vav can bind to tyrosine-phosphorylated SLP adaptors via its SH2 domain (3, 6, 22, 26, 27). Thus, HPK1, SLAP-130, SKAP55 adaptors, and Vav have all been described in SLP adaptor assemblies and almost certainly participate in complex, possibly competitive, interactions with SLP adaptor SH2 domains.

**A Model for Antigen Receptor-stimulated Activation of HPK1**—HPK1 has only recently been implicated in signal transduction from antigen receptors (9–12). Engagement of TCR or BCR results in activation of HPK1. This activation was abrogated in T cells lacking Lck, Zap-70, or LAT or in B cells lacking Lyn and Syk or lacking Grb2 and Grap (9). In T cells, HPK1 associates via its proline-rich domains P2 and P4 (Fig. 1B) with the SH3 domains of Grb2 adaptors (10–12, 62). These interactions and the Gads SH2 domain are required for maximal tyrosine phosphorylation of HPK1 (10, 62). These results are consistent with a model in which activation of antigen receptors results in the recruitment of HPK1 to membrane-bound, tyrosine-phosphorylated linker proteins (LAT in T cells).
HPK1 Interacts with SLP-76 and Inhibits AP-1 Activation

FIG. 8. A model for TCR-induced membrane recruitment and activation of HPK1. TCR stimulation leads to activation of TCR-proximal Src family (Lck, Fyn) and ZAP-70 family (ZAP-70, Syk) PTKs. PTK-mediated phosphorylation of the transmembrane adaptor protein LAT permits Grb2/Gads-dependent membrane recruitment of SLP-76 and HPK1. While this may lead to partial activation of HPK1, full activation of HPK1 requires its own tyrosine phosphorylation, most likely by ZAP-70/Syk, and its subsequent binding to the SLP-76 SH2 domain. This may occur in parallel or antagonistically to interactions of tyrosine-phosphorylated SLAP-130 with the SLP-76 SH2 domain. HPK1 negatively regulates Erk and AP-1 activation (Ref. 9 and this study). In addition, HPK1 may connect antigen receptors to activation of the JNK and IKK cascades (9, 12, 75, 79–81). For further explanations, see “Discussion.”

via its constitutive interaction with Grb2 family members (Fig. 8). In T cells, TCR stimulation induces the parallel recruitment of SLP-76 to LAT via Gads, with which it associates constitutively via SH3 domain interactions (3, 4, 6). Our co-precipitation data support the recruitment of LAT, SLP-76, and HPK1 into a molecular complex (Fig. 2). Upon relocation into antigen receptor complexes, HPK1 is phosphorylated on Tyr379, presumably by Syk family PTKs. This allows HPK1 to interact with the SH2 domains of SLP adaptors. These interactions appear essential for full activation of HPK1, because HPK1 activation is strongly reduced in SLP-76-deficient T cells or BLNK-deficient B cells (Fig. 3B and Ref. 9). In addition, full activation of HPK1 in response to TCR stimulation requires Tyr379 plus the Gads-binding and SH2 domains of SLP-76 (Fig. 3). Since Tyr379 is immediately adjacent to proline-rich region P2 of HPK1 (Fig. 1B), SLP adaptor binding could abrogate or modify P2-dependent Grb2 adaptor interactions (10, 12). This might modulate HPK1 activity or substrate access, with concomitant effects on downstream signaling events.

A Role for HPK1 in Signal Transduction via SLP-76 in T Cells and BLNK in B Cells—Previous studies suggested a positive, but nonessential, role for the SLP-76 SH2 domain in the TCR-induced activation of NFAT and pIL-2 (37, 43). The results of our cotransfection experiments in SLP-76-deficient J14 cells extend this observation and suggest that the SLP-76 SH2 domain contributes to full activation of both AP-1 and NFAT in response to TCR stimulation (Figs. 4 and 5). Studies with stably reconstituted J14 cells yielded similar results but demonstrate that the SH2 domain is less important than other regions of SLP-76 (40). We recently demonstrated that HPK1 acts as a negative regulator of TCR-induced Erk and AP-1 activation and that the inhibitory function of HPK1 requires its kinase activity (9). Here we confirm this finding and show that the interaction of HPK1 with the SLP-76 SH2 domain is required for activation of HPK1 (Fig. 3) and facilitates inhibition of AP-1 activation by HPK1 (Fig. 5). Thus, the SLP-76 SH2 domain is involved in both positive (AP-1/NFAT activation via as yet unknown pathways) and negative (inhibition of AP-1 activation via HPK1) branches of TCR signaling. This may explain why SLP-76 SH2 domain mutants so far showed only modest phenotypes (this study and Refs. 37, 40, and 43) and why the function of SLAP-130 is still unclear (16–21, 63).

HPK1 could in principle inhibit AP-1 activation by antagonizing the recruitment of positive effectors to the SLP-76 SH2 domain. A positive role for SLAP-130 in TCR-stimulated pIL-2 and NFAT activation has been suggested (17, 19–21, 63). Thus, it will be interesting to address experimentally a potential antagonism of HPK1 on SLP-130 signaling. However, HPK1 still inhibits activation of AP-1 (Fig. 5) and NFAT (data not shown) in the presence of mutations that abrogate the SLP-76 interaction, albeit less efficiently. One possible explanation could be competition of HPK1 with positive effectors for binding to other components of the TCR complex (e.g. Grb2 family adaptors). Experiments with a kinase-inactive mutant of HPK1 suggest that the kinase activity is required for its inhibitory effect on Erk, AP-1, and NFAT activation (Ref. 9 and data not shown). Thus, the mechanism by which HPK1 inhibits TCR signaling appears to include the HPK1-dependent phosphorylation of signaling proteins. This could provide an alternative explanation for the requirement for the HPK1/SLP-76 SH2 domain interaction for full inhibitory activity of HPK1, because this interaction is required for full activation of HPK1. The residual, weaker inhibitory activity of HPK1 in the absence of SLP-76 binding (Fig. 5) could be explained by additional, SLP-76-independent mechanisms of HPK1 activation in T cells, possibly mediated by interactions with other adaptors, including Nck or the Grb2 or Crk families (10–12, 62). Since HPK1 fails to inhibit AP-1 activation in cells treated with PMA alone (9) or in combination with ionomycin (Fig. 5 and data not shown), it is likely to act upstream of PKC and Ras activation. Thus, it will be interesting to investigate if HPK1 regulates the activity of proximal components of the TCR complex via their phosphorylation. Possible candidates are components of the Erk pathway, particularly PLCγ, RasGRP, and Sos. A negative regulation of Sos via phosphorylation by serine/threonine kinases has been reported (64–68), although the situation may be more complex in lymphocytes (69, 70).

Multiple studies have implicated HPK1 as a potent activator of the JNK cascade (see Refs. 35, 36, and 71 and references within Refs. 72 and 73). Therefore, HPK1 may provide a novel link between SLP adaptors and the JNK pathway. However, the role of HPK1 in JNK regulation in T cells is still unclear (9, 12). A likely explanation is the involvement of costimulatory signals in JNK activation (74). In one study, HPK1 has recently been implicated in a signaling cascade that activates JNK in response to TCR/CD28-costimulation in Vav-transfected Jurkat cells (75). Gene disruption studies have shown that BLNK is required for BCR-induced activation of the JNK cascade via as yet incompletely understood mechanisms (28). A participation of HPK1 in JNK regulation in B cells has not been demonstrated, but it represents a testable hypothesis that can be addressed by interfering with HPK1 function in lymphocytes. Apart from gene disruptions, transgenic overexpression of dominant negative HPK1 mutants, which disrupt functional interactions of endogenous proteins, in developing lymphocytes
in mice provides a powerful approach to analyze functions of HPK1 in lymphocyte signaling in a physiological context, even in a situation of potential functional redundancies with other STE-20-related protein kinases.

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Note Added in Proof—An interaction of HPK1 with CLN4 has recently been reported in Yu et al. (Yu, J., Ricu, C., Davidson, D., Minhas, R., Robson, J. D., Julius, M., Arnold, R., Kiefer, F., and Veillette, A. (2001) Mol. Cell. Biol. 21, 6102–6112).
Hematopoietic Progenitor Kinase 1 Associates Physically and Functionally with the Adaptor Proteins B Cell Linker Protein and SLP-76 in Lymphocytes
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