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Functional Interactions of HPK1 With Adaptor Proteins

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Abstract Hematopoietic progenitor kinase 1 (HPK1 or MAP4K1) is a hematopoietic-specific mammalian STE20-like protein serine/threonine kinase, comprised of a STE20-like kinase domain in its N-terminus, four proline-rich motifs, a caspase cleavage site, and a distal C-terminal Citron homology domain. HPK1 is involved in many cellular signaling cascades that include MAPK signaling, antigen receptor signaling, apoptosis, growth factor signaling, and cytokine signaling. HPK1 binds many adaptor proteins including members of the Grb2 family, Nck family, Crk family, SLP-76 family, and actin-binding adaptors like HIP-55. HPK1 tyrosine phosphorylation and kinase activation depend on the presence of adaptor proteins. Adaptor proteins are required not only for linking HPK1 to cell surface receptors like the EGFR, but also for downstream gene transcription like NFAT, AP-1 and IL-2. The HPK1 association with Crk, CrkL, and HIP-55 mediate HPK1-dependent c-Jun N-terminal kinase (JNK) activation, while the association of HPK1 with SLP-76, Gads, CrkL, Grb2, and Grap affect T- and B-cell dependent gene transcription. Interestingly, HPK1 has been implicated in both increasing and decreasing NFAT, AP-1, and IL-2 gene transcription in T-cells where adaptor proteins play a key role. Lastly, HPK1 will phosphorylate Crk and CrkL, in vitro, which presents a novel possibility for the regulation of adaptor proteins and downstream signaling events. J. Cell. Biochem. 95: 34–44, 2005.

Key words: HPK1; adaptor proteins; T-cell receptor signaling; MAPK; JNK; NF-κB; apoptosis

The transmission of extracellular stimuli to downstream effector molecules requires a variety of cellular proteins including cell surface receptors, kinases, phosphatases, adaptor molecules, and transcription factors. The signal, as well as which of these cellular protein signaling cascades is turned on, elicits a multitude of cellular processes including proliferation, transformation, differentiation, activation, and apoptosis. An important component of receptor signaling is the activation of the mitogen-activated protein kinase (MAPK) signaling cascade [Chen and Tan, 1999, 2000]. The basic MAPK signaling module is a MAP3K kinase (MAP4K) phosphorylating a MAP2K kinase (MAP3K) that phosphorylates a MAPK kinase (MAP2K) that finally phosphorylates a MAPK itself. The STE20-related protein kinases are classified into two major subgroups: the p21 Cdc42/Rac1-activated protein kinase (PAK) and germinal center kinase (GCK)/hematopoietic progenitor kinase 1 (HPK1) subgroups. The GCK/HPK1 subgroup of STE20-related protein kinases is defined by a STE20-like kinase domain in the N-terminus and proline-rich motifs flanked by a citron homology domain in the distal C-terminus [Ling and Tan, 2002]. Some of the GCK/HPK1 family of kinases act as MAP4Ks in the MAPK signaling module where the function of the citron homology domain may mediate signaling via Rap2 to activate c-Jun N-terminal kinase (JNK).

Intracellular adaptor proteins are defined by a lack of intrinsic enzymatic activity (kinase and/or phosphatase) and transcriptional activity, but contain the presence of noncovalent protein–protein interaction domains [Buday, 1999; Wilkinson et al., 2004]. Adaptor-mediated protein complexes potentiate an extracellular signal from a receptor to downstream cytoplasmic and nuclear effector molecules through a variety of protein interaction domains including tyrosine-based signaling motifs (TBSMs) or modular protein–protein domains such as Src

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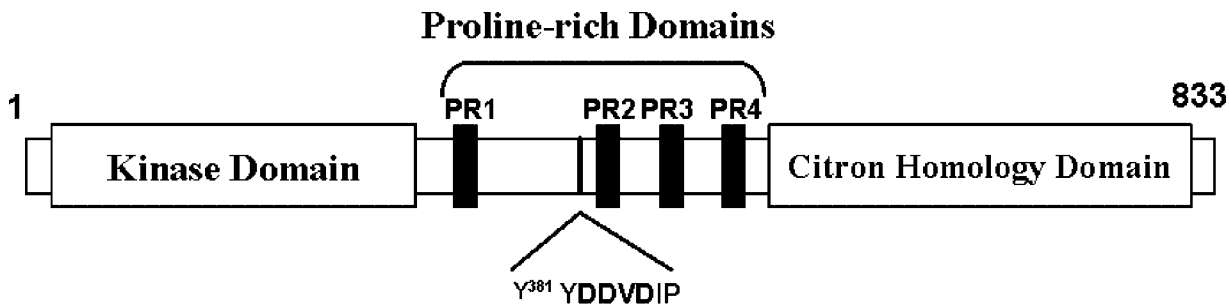
homology 2 (SH2), SH3, PDZ, WW, PH and PTB. SH2 domains comprise ~100 amino acid residues that contain a conserved phosphate-binding sequence FLVRES. Src homology 3 (SH3) domains comprise ~60 amino acid residues that contain a characteristic protein fold present within the secondary structure. SH2 domains bind a phosphorylated tyrosine (pTyr) residue while SH3 domains bind the consensus sequence (-**P**-x-x-**P**-). This review will describe the HPK1 interaction with various adaptor proteins, mainly ones that contain the SH2 and SH3 protein modules, and the effect of these interactions on cellular functions.

HPK1-MEDIATED CELLULAR SIGNALING

HPK1 or MAP4K1 is a hematopoietic-specific mammalian STE20-like protein serine/threonine kinase, which was cloned from mouse hematopoietic progenitor cells using a PCR-based subtraction strategy [Hu et al., 1996; Kiefer et al., 1996]. HPK1 comprises a STE20-like kinase domain in its N-terminus, four proline-rich motifs (-**P**-x-x-**P**-) (PR), a caspase cleavage site, and a distal C-terminal Citron homology domain also present in NIK, GLK and GCK [Ling et al., 1999] (Fig. 1). These proline-rich motifs are capable of binding proteins that contain SH3 domains [Oehrl et al., 1998; Ling et al., 1999]. HPK1 contains 13 potential tyrosine phosphorylation residues, some of which may be phosphorylated by ZAP-70, which provide potential docking sites for SH2 domains containing proteins [Ling et al., 2001]. Tyrosine residue 379 (mouse) or 381 (human) of HPK1 mediates the ability of HPK1 to bind SLP-76, when phosphorylated [Sauer et al., 2001].

HPK1 activates the JNK/stress-activated protein kinase (SAPK) signaling pathway in HEK293T cells and T-cells [Hu et al., 1996; Kiefer et al., 1996; Ensenat et al., 1999; Hehner et al., 2000; Liou et al., 2000; Ling et al., 2001; Ma et al., 2001; Han et al., 2003b]. HPK1 activates JNK through the signaling pathway MAP3K (an example is MEKK1, TAK1 or MLK3) to a MAP2K (an example is MKK4 or MKK7) to JNK [Hu et al., 1996; Kiefer et al., 1996; Zhou et al., 1999]. A dominant-negative HPK1 construct that has a Met substituted for Lys-46 (HPK1-M46), which abrogates ATP binding, fails to activate JNK in these systems [Hu et al., 1996; Ling et al., 2001]. The kinetics of HPK1 kinase activation occurs optimally at 1 min post T-cell receptor (TCR) engagement followed by a rapid decrease in Jurkat T-cells [Liou et al., 2000; Ling et al., 2001]. TCR



Proline-rich Domains

- PR1 (308-407): PELP**P**AI**P**RR
- PR2 (394-402): P**P**PL**P**P**K**PK
- PR3 (432-443): PPPNSPRPGPPP
- PR4 (468-477): KP**P**LL**P**P**K**KE

Fig. 1. HPK1 structure. HPK1 contains an N-terminal serine/threonine kinase domain, a C-terminal citron homology domain (CHD), four proline-rich motifs (PR1, PR2, PR3, and PR4), a caspase cleavage site (DDVD), and tyrosine residue 381 when phosphorylated is responsible for HPK1 binding to SLP-76. The four proline-rich domain sequences are also listed.

signaling or TCR plus CD28 co-stimulation induces HPK1 kinase activity; however, CD28 co-stimulation alone does not appear to induce HPK1 kinase activity or HPK1 tyrosine phosphorylation in Jurkat T-cells [Liou et al., 2000; Ling et al., 2001]. These results suggest that HPK1 function is more closely associated to events related to signaling of the TCR rather than signaling in co-stimulation. JNK activation plays a role in integrating the TCR and CD28 signals involved in gene transcription (NFAT) and IL-2 production. The JNK family of MAPKs are implicated in T-cell responses that include proliferation, Th1/Th2 differentiation, and activation-induced cell death (AICD) [Chen and Tan, 1999, 2000; Ling and Tan, 2002]. The ability of HPK1 to activate JNK provides a means by which HPK1 is involved in cellular activation and apoptosis, but what signals upstream of HPK1 control these differential processes remains unclear.

HPK1 mediates JNK activation after treatment with TGF-β [Wang et al., 1997; Zhou et al., 1999] and HPK1 is involved in cell growth and differentiation as determined by studies using antisense oligonucleotides in erythropoietin signaling [Nagata et al., 1999]. HPK1 is activated by both EGF and PDGF stimulation where adaptor proteins are involved in mediating the localization of effector molecules to cell surface receptors [Anafi et al., 1997; Ling et al., 1999]. HPK1 also binds the cytoplasmic tail of IgE [Geisberger et al., 2002], but the functional significance of this interaction is unknown.

HPK1 does not appear to interact with or affect other members of the mammalian MAPKs including the extracellular signal-regulated kinases (ERKs) and the p38-MAPKs [Kiefer et al., 1996], although HPK1 negatively affects ERK2 in an overexpression system using T-cells [Liou et al., 2000]. Reports indicate that HPK1 negatively affects AP-1 [Liou et al., 2000; Sauer et al., 2001] transcriptional activity, while other reports show that HPK1 enhances both AP-1 and IL-2 trasnscriptional activity [Hu et al., 1996; Ling et al., 1999; Ma et al., 2001]. HPK1 activates IKK-α and -β of the IKK complex that leads to IκB degradation and NF-κB activation [Hu et al., 1999]. The activation of HPK1 by prostaglandin E2 (PGE2) results in the inhibition of *fos* gene transcription [Sawasdikosol et al., 2003], which presents an example of how HPK1 may negatively regulate gene transcription. Is it possible that HPK1 is both a positive and negative regulator of T-cell activation that could be determined by cellular factors like adaptor proteins?

HPK1 AND APOPTOSIS

HPK1 also increases spontaneous, TCR/CD3 or reactive oxygen species (ROS)-mediated apoptosis in $CD4^+$ T-cells [Schulze-Luehrmann et al., 2002]. In murine $CD4^+$ T-cells, the overexpression of HPK1 increases FasL expression and JNK activity [Schulze-Luehrmann et al., 2002]. Although the role of HPK1 in T-cell apoptosis is not yet clear, JNK has been well implicated not only in inflammatory responses and cellular proliferation, but also in apoptotic processes [Chen and Tan, 2000]. HPK1 is cleaved during apoptosis at DDVD (amino acids 382–385) into two fragments where the N-terminal region contains the kinase domain and the first proline-rich motif while the C-terminal region contains the Citron homology domain and the last three proline-rich motifs [Chen et al., 1999; Arnold et al., 2001] (Fig. 1). The N-terminal domain of HPK1 retains the ability to activate JNK, but not to bind adaptor proteins, while the C-terminal domain of HPK1 has an inhibitory effect on NF-κB when compared with full-length HPK1 [Chen et al., 1999; Arnold et al., 2001] (Fig. 2, right). It has been confirmed in mouse primary T-cells that after apoptosis induction the C-terminal domain of HPK1 inhibits NF-κB activation, while the N-terminal domain retains the ability to activate JNK [Schulze-Luehrmann et al., 2002] (Fig. 2, right). HPK1 also induces the surface expression of FasL, a known inducer of apoptosis in T-cells [Schulze-Luehrmann et al., 2002]. These results provide a potential mechanism where HPK1 promotes apoptosis by activating JNK (independently of Crk and Grb2), increasing surface expression of FasL, and inhibiting NF-κB transcriptional activation (Fig. 2, right). These results suggest that HPK1 plays a role in a variety of signaling systems including cellular survival and apoptosis, but what determines when HPK1 is involved in activation versus apoptosis remains to be elucidated.

HPK1 is capable of interacting with a multitude of adaptor proteins (Table I); the function of each adaptor protein is very different in terms of kinetics and signaling. HPK1 interacts with members of the Grb2, Nck, Crk, SLP-76

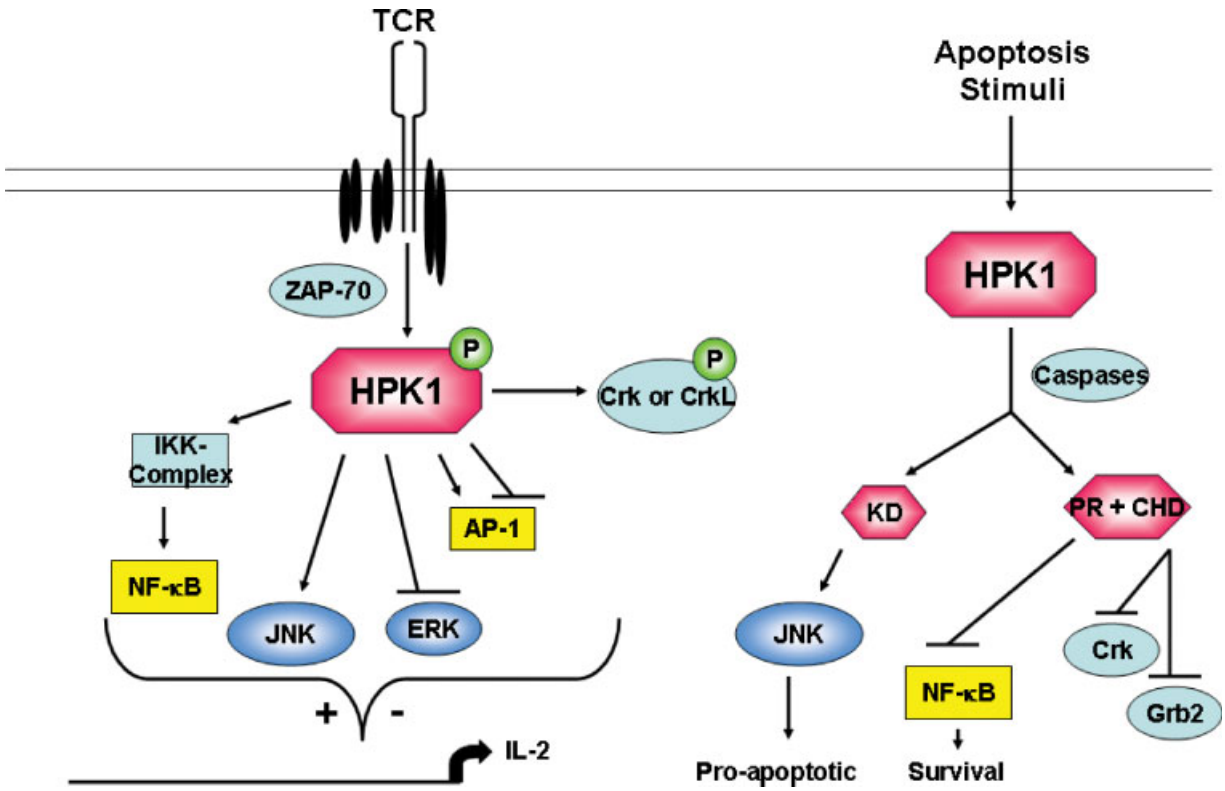


Fig. 2. HPK1 function in TCR signaling. After TCR engagement, HPK1 is tyrosine phosphorylated and becomes activated. HPK1 inhibits ERK2, AP-1, and IL-2 and activates JNK, NF-κB, AP-1, and IL-2 depending on the cell type and stimuli used. Interestingly, HPK1 may also serine/threonine phosphorylate Crk and CrkL, in vitro, which has an unknown physiological function (**left side**). HPK1 is believed to serve a pro-apoptotic role by activating JNK and inhibiting NF-κB activation after caspase cleavage in T-cells (**right side**). KD, kinase domain; PR, proline-rich motifs; CHD, citron homology domain. Arrows indicate activation while bars indicate inhibition. HPK1 is labeled in (pink), transcription factors are colored (yellow), signaling molecules are shown as (light blue), P = phosphate group (green) and MAPK kinases are colored (dark blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

adaptor families, and actin-binding domain containing adaptor proteins [Buday, 1999; Ling and Tan, 2002; Jordan et al., 2003].

CONSTITUTIVE BINDING WITH CRKL

The Crk adaptors consist of Crk I, Crk II, and Crk-like protein (CrkL), which shares 60% overall homology of the full-length Crk (Table I) [Buday, 1999]. The CrkL protein is similar to the Crk II protein, which is widely expressed and contains two SH3 domains. The Crk family of adaptor proteins is not only involved in cellular transformation, but in many signaling pathways including the EGF receptor, the TCR, the B-cell receptor (BCR), integrin stimulation, apoptosis, phagocytosis, cell migration, and focal adhesions.

In the Jurkat T-cell line, CrkL forms a constitutive complex with HPK1 up to 60 min post TCR stimulation [Ling et al., 2001]. Interestingly, the second and fourth proline-rich motifs in HPK1 contain the (-**P**-x-**L**-**P**-x-**K**) motif, which the Crk family of adaptors preferentially use for binding to their first SH3 domain in a very selective and high affinity interaction [Oehrl et al., 1998; Ling et al., 1999]. Studies performed in vitro have shown that HPK1 second and fourth proline-rich motifs bind the SH3 domain of CrkL at the N-terminus [Oehrl et al., 1998; Ling et al., 1999, 2001]. In HEK293T cells, CrkL activates both HPK1 and JNK1 kinase activity using immunocomplex kinase assays [Ling et al., 1999]. This study also determined that the proline region of HPK1 acts as a dominant-negative inhibitor of CrkL-induced JNK1 activation, while the addition of dominant-negative downstream HPK1 effector molecules (such as TAK1, MEKK1, and SEK1/MKK4) also blocks JNK1 activation [Ling et al., 1999]. In contrast to the results from using HEK293T cells, the overexpression of both CrkL and HPK1 does not further activate JNK1 in COS-7 cells [Oehrl et al., 1998]. These

TABLE I. HPK1 Binding Adaptor Proteins

Adaptor protein	Size (kDa)	Structure	Binding	Domain in adaptor	Domain in HPK1	Reference
Grb 2	25	SH3 | SH2 | SH3	Constitutive	SH3 (N)	**PR1**, **PR2**, PR4	Anafi et al. [1997]; Oehrl et al. [1998]; Ling et al. [1999, 2001]; Liou et al. [2000]
Grap	27	SH3 | SH2 | SH3	Constitutive	N.D.	N.D.	Liou et al. [2000]
Gads	40	SH3 | SH2 | Pro | SH3	Inducible	SH3(C)	PR2, **PR4**	Liou et al. [2000]; Ma et al. [2001]; Lewitzky et al. [2004]
Nckα	47	SH3 | SH3 | SH3 | SH2	Inducible	All SH3	All PR motifs	Anafi et al. [1997]; Ling et al. [2001]
Crk l	28	SH2 | SH3	Inducible	SH3	PR2, PR4	Oehrl et al. [1998]; Ling et al. [1999, 2001]
Crk ll	42	SH2 | SH3 | SH3	Inducible	SH3(N)	PR2, PR4	Oehrl et al. [1998]; Ling et al. [1999, 2001]
CrkL	38	SH2 | SH3 | SH3	Constitutive	SH3(N)	PR2, PR4	Oehrl et al. [1998]; Ling et al. [1999, 2001]
SLP-76	76	Y-site | Proline-rich | SH2	Inducible	SH2	$pTyr^{379}$(m), $pTyr^{381}$(h)	Sauer et al. [2001]
Bink	65	Y-site | Proline-rich | SH2	Inducible	SH2	$pTyr^{379}$(m), $pTyr^{381}$(h)	Liou et al. [2000]; Sauer et al. [2001]; Tsuji et al. [2001]
Clnk	54	Y-site | Proline-rich | SH2	Inducible	N.D.	N.D.	Yu et al. [2001]
Bam32	32	SH3 | PH	Constitutive	SH3?	N.D.	Han et al. [2003b]
HIP-55	55	ADF | Y-site | SH3	Constitutive	SH3	PR2	Ensenat et al. [1999]; Han et al. [2003b]; Le Bras et al. [2004]
LAT	36	EX | TM | Y-sites	Inducible	Indirect	Indirect	Ling et al. [2001]

The current list of adaptor proteins, which are capable of binding to HPK1, is presented. Adaptor proteins are listed by family, size in kDa, structure, whether they bind HPK1 inducibly or constitutively, and the domain of the adaptor and HPK1 required for the interaction and the associated references. SH3, Src 3 homology domain; SH2, Src 2 homology domain; (N), N-terminal; (C), C-terminal; PR, proline-rich motif; **PR**, proline-rich motif that binds with the highest affinity; 'Y,' tyrosine residue; ADF, actin-binding domain; TM, transmembrane domain; PH, pleckstrin homology domain; EX, extracellular domain; (m), mouse; (h), human; pTyr, phosphorylated tyrosine residue; N.D., not determined.

results strongly suggest that CrkL acts upstream of HPK1 to modulate HPK1 activation and JNK activity, which depends on the cell type and accessory proteins present in each cell type [Ling et al., 1999]. The CrkL-HPK1 interaction may also serve to localize HPK1 in the cytoplasm where upon stimulation HPK1 translocates to areas of signal transduction, such as lipid rafts.

CONSTITUTIVE BINDING WITH GRB2 FAMILY MEMBERS GRB2 AND GRAP

The Grb2 family consists of growth factor receptor bound 2 (Grb2), Grap (Grb2-related adaptor protein), and Gads (Grb2-related adaptor downstream of Shc; also called Grap2, GrpL, Grf40, GRID, and Mona) [Buday, 1999; Liu et al., 2000; Ling and Tan, 2002]. The structure of the Grb2 family of adaptor proteins is SH3-SH2-SH3, where Grap has a similar SH2 and SH3 binding profile to Grb2 [Liu et al., 2000] (Table I). Grb2 is recruited to the plasma membrane to interact with receptors by Shc, SHP-2, and LAT where activation of Ras occurs through guanine nucleotide exchange factors. Grb2 interacts with C3G and Vav, multiple receptors (EGFR, CD28, and RPTPα), other adaptor proteins (Cbl, LAT, SLP-76, phosphotyrosine phosphatases SHP-2 and PEST), serine/threonine kinases (e.g., MEKK1), and cytoskeletal proteins (dynamin, dystroglycan, and WASP). Grb2 may be a positive regulator of Ras through its interaction with Sos and Vav while the Grb2-Cbl interaction is thought to be a negative signal in some systems including T-cells.

Grb2 and Grap constitutively associate with HPK1 [Liou et al., 2000; Ling et al., 2001; Ma et al., 2001]. Grb2 binds well to the consensus motif (-**P**-x-x-**P**-**R**/**K**-) with its N-terminal SH3 domain [Oehrl et al., 1998; Ling et al., 1999]. An in vitro study showed that the Grb2 N-terminal SH3 domain binds HPK1 [Oehrl et al., 1998], which was later determined to be the first, second, and fourth (weakest of the three) proline-rich motifs of HPK1 that binds to the N-terminal SH3 domain of Grb2 [Anafi et al., 1997; Ling et al., 1999] (Table I). The Grb2-HPK1 and Grap-HPK1 interactions are consti-

tutive in the Jurkat T-cell line, while the absence of both Grb2 and Grap in B-cells severely impairs HPK1 kinase activity after BCR stimulation [Liou et al., 2000; Ling et al., 2001]. In B-cells, the absence of Grb2 or Grap mildly affects HPK1 kinase activity after BCR stimulation, which suggests that Grb2 and Grap share overlapping functions in the activation of HPK1 [Liou et al., 2000]. The exact binding domain of HPK1 to Grap has not been identified. Grap inhibits the Ras/ERK pathway while not affecting JNK activation [Shen et al., 2002], which may provide a potential mechanism for how HPK1 negatively affects the ERK pathway in T-cells.

CONSTITUTIVE BINDING WITH HIP-55

HPK1-interacting protein of 55 kDa (HIP-55 also called SH3P7 or mAbp1), like HS1, Cortactin and Drebrin contains an N-terminal actin-binding domain [Ensenat et al., 1999; Nagata et al., 1999; Han et al., 2003b]. HIP-55 is a substrate for Src and Syk/ZAP-70 family tyrosine kinases and it is tyrosine phosphorylated after TCR or BCR stimulation [Larbolette et al., 1999; Nagata et al., 1999; Han et al., 2003b]. HIP-55 constitutively binds HPK1 in Jurkat T-cells [Le Bras et al., 2004]. HIP-55 binds the second proline-rich motif of HPK1 through its C-terminal SH3 domain [Ensenat et al., 1999] (Table I). The co-expression of HIP-55 with HPK1 in HEK293T cells markedly increases both HPK1 and JNK kinase activity [Ensenat et al., 1999]. The activation of JNK by HIP-55 is mediated by HPK1 as the kinase-dead HPK1 mutant (HPK1-M46) abolishes JNK activation by HIP-55 co-expression [Ensenat et al., 1999]. In Jurkat T-cells, the knockdown of HIP-55 by RNAi results in a significant decrease in HPK1 and JNK, but not ERK kinase activity after TCR stimulation [Han et al., 2003b]. Both HIP-55 and HPK1 translocate to the T-cell/APC contact site and GEMs (glycolipid enriched microdomains, also called lipid rafts) after TCR stimulation [Ling et al., 2001; Han et al., 2003b; Le Bras et al., 2004]. The HIP-55-HPK1 complex inhibits NFAT activity in Jurkat T-cells and is partially reversed by the kinase-dead HPK1 mutant using antigen-pulsed Raji B-cells [Le Bras et al., 2004]. After RNAi knockdown of either HIP-55 or HPK1, NFAT activity increases in this system [Le Bras et al., 2004]. Taken together, these results suggest that HIP-55 may bind to HPK1 and mediate the movement of HPK1 into the immunological synapse, where HPK1 could interact with its substrates and/or downregulate the TCR.

CONSTITUTIVE BINDING WITH BAM32

The B-cell associated molecule (Bam32, also known as Dapp1 or PHISH) is a recently identified adaptor protein that contains an N-terminal SH2 domain and a C-terminal pleckstrin homology domain (PH) involved in recruitment to the membrane [Han et al., 2003a]. Bam32 is important in ERK and JNK activation after BCR stimulation in B-cells [Han et al., 2003a]. Bam32 knockout mice show a defect in ERK, JNK, and HPK1 activity as well as the Bam32-HPK1 interaction [Han et al., 2003a]. The Bam32-HPK1 association occurs in mouse and human B-cells independently of BCR stimulation [Han et al., 2003a] (Table I). The exact relationship between Bam32 and HPK1 as it relates to B-cell activation is only beginning to be determined.

INDUCIBLE BINDING WITH CRK

The Crk I protein is expressed in embryonic cells, contains an N-terminal SH2 domain followed by one SH3 domain, while the Crk II protein is widely expressed and contains an N-terminal SH2 domain followed by two SH3 domains [Buday, 1999]. Crk I and Crk II are alternative splice products of the human *crk* gene. Crk interacts with two, guanine nucleotide exchange factors for Ras family members, Sos and C3G. Sos is a known activator of Ras and C3G is an exchange factor of Rap1A and Rap1B, negative regulators of Ras signaling [Buday, 1999]. Crk is involved in JNK activation possibly through C3G [Buday, 1999].

Although the in vitro binding of HPK1 to the Crk family of proteins has been well described, less is known about what physiological stimuli can induce these complexes in vivo [Oehrl et al., 1998; Ling et al., 1999, 2001]. Unlike CrkL, Crk binds HPK1 10 min after TCR stimulation in Jurkat T-cells [Ling et al., 2001]. Like CrkL, the N-terminal SH3 domain of Crk binds the second and fourth proline-rich motifs of HPK1 in vitro [Oehrl et al., 1998; Ling et al., 1999] (Table I). The Crk family of proteins also increases HPK1 kinase activity and subsequent JNK activity in overexpression systems [Ling et al., 1999]; however, this remains to be observed under

physiological conditions. The major difference between CrkL and Crk binding to HPK1 is in their kinetics. CrkL, a constitutive binder of HPK1 up to 60 min post TCR stimulation [Ling et al., 2001], may be a negative regulator of T-cell activation through its interactions with Cbl or Rap1 proteins, while Crk (an inducible binder of HPK1 [Ling et al., 2001]) may mediate Ras and subsequent JNK activation.

INDUCIBLE INTERACTION WITH GADS OR LAT

Gads has a similar SH2 binding profile to Grb2, but the SH3 domain of Gads is not able to interact with some proline-rich proteins [Buday, 1999; Liu et al., 2000]. After TCR stimulation, the Gads-HPK1 complex increases in both humans and mice [Liu et al., 2000; Ma et al., 2001]. The TCR-induced phosphorylation of HPK1 increases its ability to bind Gads in Jurkat T-cells [Liu et al., 2000; Ma et al., 2001]. The SH3 domain at the C-terminus of Gads mediates the binding with the second [Ma et al., 2001] or fourth [Liu et al., 2000] proline-rich motif of HPK1 in vitro (Table I). Recently, X-ray crystallography studies show that the HPK1 sequence P-**P**-L-L-**P**-P-**K**-K-E-**K** (corresponds to the fourth proline-rich region of HPK1 [Ma et al., 2001]) mediates the interaction between HPK1 and Gads [Lewitzky et al., 2004]. Within this HPK1 sequence, the last lysine is essential for the HPK1-Gads interaction. The first lysine in this HPK1 sequence replaces an otherwise conserved arginine residue present in the SLP-76 sequence, which is responsible for the SLP-76-Gads interaction. Using COS-7 cells, the overexpression of both HPK1 and Gads results in increased HPK1 and JNK kinase activity and activation of the transcription factor c-Jun [Ma et al., 2001]. The deletion of the fourth proline-rich motif of HPK1 or the presence of an SH2 mutant of Gads inhibits TCR-stimulated HPK1 tyrosine phosphorylation [Liu et al., 2000]. In Jurkat T-cells, the co-transfection of HPK1 and Gads increases IL-2 transcriptional activation after TCR plus PMA stimulation [Ma et al., 2001]. These results indicate that Gads plays a role in HPK1 tyrosine phosphorylation and function during TCR stimulation probably by bringing HPK1 in close proximity to ZAP-70 or another tyrosine kinase that is responsible for HPK1 tyrosine phosphorylation and its activation.

The linker for activated T-cells (LAT) is a palmitoylated transmembrane-associated adaptor protein that becomes tyrosine phosphorylated by Syk/ZAP-70 family tyrosine kinases [Wilkinson et al., 2004]. LAT is essential for TCR signaling, which includes Ca^{2+} mobilization and Ras-MAPK activation. After TCR signaling, the proline-rich region of HPK1 is essential for the HPK1-LAT interaction; however, HPK1 and LAT lack appropriate protein–protein binding domains for a direct interaction [Ling et al., 2001] (Table I). The HPK1-LAT interaction is probably mediated by Grb2 or Gads, which bind LAT through their SH2 domain leaving their SH3 domain available for their interaction with HPK1. Gads or Grb2 may bring HPK1 to the LAT signaling complex where HPK1 would be in close proximity to its effector molecules and substrates during TCR stimulation.

INDUCIBLE BINDING WITH NCK

The *nck* gene encodes Nckα while a novel gene encodes Nckβ (Grb4) that shows 68% amino acid identity to Nckα [Buday, 1999]. The Nck protein contains one SH2 domain and three SH3 domains, and undergoes phosphorylation on serine, threonine, and tyrosine residues. Nck regulates intracellular signaling cascades that include Ras signaling, the JNK pathway, actin polymerization, and cytoskeletal reorganization. Nckα interacts directly with HPK1 [Anafi et al., 1997; Ling et al., 2001], while the interaction of Nckβ with HPK1 has not been studied. The HPK1 proline-rich motifs bind in vitro to any of the three SH3 domains of Nck [Anafi et al., 1997] (Table I). In Jurkat T-cells while Crk was shown to bind HPK1 later (10 min) after TCR stimulation, the Nck interaction with HPK1 occurs early (1–5 min) after TCR stimulation [Ling et al., 2001]. The interaction between HPK1 and Nck may also be induced by EGF stimulation [Anafi et al., 1997]. Interestingly, both Nck and HPK1 will translocate to lipid rafts during TCR stimulation [Ling et al., 2001; Le Bras et al., 2004], where Nck may interact with the CD3ε chain or mediate downstream signaling complexes [Wilkinson et al., 2004]. This poses the possibility that Nck may aid in the recruitment of HPK1 to the activated TCR complex and lipid rafts to mediate actin cytoskeletal rearrangement or in the transmission of downstream signals.

INDUCIBLE BINDING WITH SLP-76 FAMILY ADAPTORS (SLP-76, BLNK, AND CLNK)

SLP-76, its B-cell homolog BLNK (also called SLP-65 or BASH), and Clnk all have a similar structure with a N-terminal acidic region, tyrosine residues, a central proline-rich region, and a C-terminal SH2 domain [Yu et al., 2001]. SLP-76 and BLNK are tyrosine phosphorylated by Syk/ZAP-70 family members and interact with many components during TCR and BCR signaling that include Vav, Nck, Itk, PLC-γ1, and PLC-γ2 [Yu et al., 2001; Wilkinson et al., 2004]. SLP-76 and BLNK are adaptor proteins specific to T- and B-cells, respectively, which bind HPK1 after TCR or BCR stimulation [Sauer et al., 2001; Tsuji et al., 2001]. Most of the HPK1-adaptor protein interactions described have been mediated by the proline-rich regions of HPK1 and the SH3 domains of the adaptor protein; however, Tyr-379 (mouse) or Tyr-381 (human) when phosphorylated in HPK1 mediates its binding to the SH2 domain of both SLP-76 and BLNK [Sauer et al., 2001; Tsuji et al., 2001] (Table I). The HPK1-SLP-76 interaction in T-cells or the HPK1-BLNK interaction in B-cells is required for the induction of kinase activity of HPK1 after TCR or BCR stimulation, respectively [Liou et al., 2000; Sauer et al., 2001; Tsuji et al., 2001]. The HPK1 inhibition of AP-1 [Liou et al., 2000] depends on this HPK1-SLP-76 interaction [Sauer et al., 2001]. In B-cells, HPK1 enhances IKKβ activation by BCR stimulation showing that the HPK1-BLNK interaction is a necessary component of B-cell activation [Tsuji et al., 2001].

A recently identified third member of the SLP-76 family of adaptor proteins called Clnk is a cytokine inducible (IL-2 and IL-3) adaptor protein expressed in hematopoietic cells that also binds HPK1 [Yu et al., 2001]. Interestingly, unlike SLP-76 and BLNK, Clnk does not interact with Vav, Nck, and/or PLC-γ isoforms [Yu et al., 2001], nor is Clnk required for immune system functions [Utting et al., 2004]. Clnk will restore the TCR signaling defect in SLP-76-deficient cells even though Clnk and SLP-76 have different binding partners, showing that Clnk is capable of replacing the SLP-76 function [Yu et al., 2001] possibly through HPK1. The HPK1-Clnk interaction is enhanced by immunoreceptor and cytokine stimulation [Yu et al., 2001]. Clnk induces HPK1 kinase activity and IL-2 transcriptional activity in Jurkat T-cells after TCR stimulation [Yu et al., 2001]. The domains required for HPK1 binding to Clnk have not been described (Table I) nor has the Clnk effect on HPK1-dependent JNK activation been determined. The Clnk-HPK1 interaction presents a novel possibility for HPK1 to be involved in T-cells that have been activated by cytokines such as memory or suppressor T-cells.

FUTURE DIRECTIONS

There are several controversies in the literature regarding HPK1, with one of the biggest being the role of HPK1 in T-cells. In regards to JNK activation during T-cell co-stimulation, the kinase-dead mutant and proline-rich regions of HPK1 fail to block JNK activation by TCR plus CD28 stimulation [Liou et al., 2000; Ling et al., 2001]. Another study shows that the kinase-dead mutant of HPK1 inhibits Vav-mediated JNK activation in TCR co-stimulation [Hehner et al., 2000]. Furthermore, it has been shown that HPK1 inhibits AP-1 and ERK2 activation by TCR stimulation [Liou et al., 2000; Sauer et al., 2001]; in contrast, other studies show that the overexpression of HPK1 with Gads or Clnk activates the IL-2 promoter [Ma et al., 2001; Yu et al., 2001]. It has also been shown that the HPK1 proline-rich region or a Crk mutant partially inhibits IL-2 transcription in T-cells [Ling et al., 1999]. These results highly suggest that HPK1 is involved in both positive and negative TCR signaling pathways, but how HPK1 mediates such signals remains unknown. The role of adaptor proteins may be to localize HPK1 to various effector molecules thereby controlling whether HPK1 functions as a positive or negative regulator of TCR signaling, but this remains to be shown (Fig. 3). HPK1 is also an important player in the NF-κB pathway in T- and B-cells [Hu et al., 1999; Tsuji et al., 2001]. The N-terminal domain of HPK1 retains the ability to activate JNK, but does not bind adaptor proteins, while the C-terminal domain of HPK1 has a decreased ability to bind Crk and Grb2 and an inhibitory effect on NF-κB when compared with full-length HPK1 [Chen et al., 1999; Arnold et al., 2001] (Fig. 2, right). The effect from the loss of HPK1 interaction with intracellular adaptor molecules (Crk and Grb2) after apoptosis induction remains to be determined. The exact molecular mechanism of

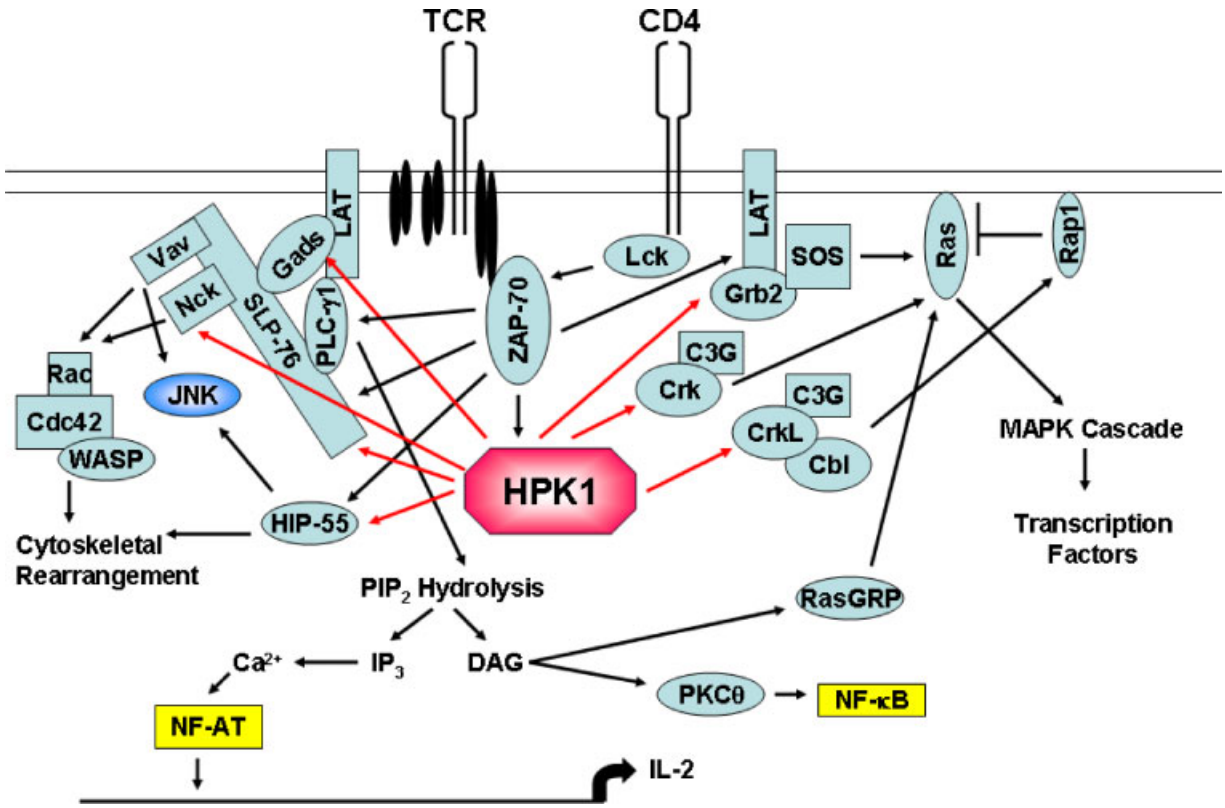


Fig. 3. HPK1 binds adaptor proteins during TCR signaling. The current understanding of TCR signaling is shown, where the phosphorylation of the ITAMS in the TCR/CD3 complex induces ZAP-70 to bind, which in turn is phosphorylated by Lck. ZAP-70 phosphorylates numerous proteins including LAT, SLP-76, Vav, PLCγ1, HIP-55, and HPK1. These phosphorylated proteins mediate protein–protein interations through SH2 and SH3 domains that lead to intracellular scaffolding complexes that mediate Ras-MAPK activation, cytoskeletal rearrangement, Ca^{2+} release, and finally transcriptional factor activation (NFAT) and subsequent IL-2 production. HPK1 (pink) is centrally located in this model due to its interaction with many adaptor proteins that are capable of modulating many intracellular signaling pathways. The diversity of HPK1 function (both positive and negative) may be explained by the ability of HPK1 to bind or phosphorylate various adaptor proteins during T-cell activation. Arrows indicate activation while bars indicate inhibition. The arrows (→) indicate HPK1 directly binds to the protein. HPK1 is labeled in (pink), transcription factors are colored (yellow), signaling molecules are shown as (light blue), and MAPK kinases are colored (dark blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the HPK1 interaction with the NF-κB pathway and how this interaction affects cellular survival especially in T-cells remains to be described in detail (Fig. 2, right).

HPK1 is activated by both EGF and PDGF stimulation where adaptor proteins like Grb2, Nck, Crk, and CrkL are involved in mediating the localization of effector molecules to cell surface receptors [Anafi et al., 1997; Ling et al., 1999]. Both Grb2 and Crk will enhance the HPK1 association with the tyrosine-phosphorylated EGF receptor suggesting that the adaptor proteins Grb2 or Crk provide a mechanism linking HPK1 to cell surface receptors and JNK activation [Anafi et al., 1997; Ling et al., 1999]. Although the CD28 receptor alone does not significantly alter HPK1 tyrosine phosphorylation or kinase activity, the HPK1-associated adaptor proteins Grb2 and Gads interact with the CD28 tail [Ellis et al., 2000]. Adaptor proteins may mediate the movement of HPK1 from the cytoplasm to receptors localized at the cell surface or in lipid rafts to mediate downstream signaling complexes and events.

Finally, HPK1 in vitro phosphorylates the adaptor proteins Crk and CrkL [Ling et al., 1999] (Fig. 2, left), while Clnk was shown to have increased phosphorylation in the HPK1 immunocomplex kinase assay with cells overexpressing HPK1 [Yu et al., 2001]. Although these results need to be analyzed in vivo, they present an interesting mechanism by which HPK1 may serve to directly alter adaptor protein function through serine/threonine

phosphorylation events. The phosphorylation of these adaptors may affect their localization, stability or half-life, and ability to form protein–protein complexes and bind their effector molecules presenting a novel way for HPK1 to be both a positive and negative regulator of intracellular signaling pathways.

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