Title

The regulation and function of hematopoietic progenitor kinase-1 in antigen receptor signal transduction

Author

Liou, Jen,

Publication Date

2001

Peer reviewed|Thesis/dissertation

The Regulation and Function of Hematopoietic Progenitor Kinase-1 in Antigen Receptor Signal Transduction

by

Jen Liou

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

Dedicated to my parents,

and

my husband Lun Tsuei

PREFACE

There are many people I would like to acknowledge. First, I would like to express my sincere gratitude to my graduate advisor, Dr. Art Weiss, for his guidance and support. He has invested time and effort into making me a better scientist and ensuring that I made good progress. His enthusiasm for science has always been an inspiration for me. I was fortunate to follow him for my graduate training, and conduct my thesis work in the great research environment that he has provided.

I also would like to thank the members of the Weiss lab. They have created a collaborative atmosphere in the lab, and made it a stimulating and fun place to be. A special acknowledgment goes to Tomek Sosinowski for sharing his bench with me and helping me get started in the lab. I enjoyed many discussions with him and have learned a lot from him. I also would like to thank Debbie Yablonski, Jeanne Baker, Mike Tomlinson, Joseph Lin, Greg Ku, Ginny Shapiro, and Jun Wu for valuable suggestions on my work. Acknowledgments are also due to Marianne Mollenauer and Terri Kadlecek for patiently listening to me at my frustrating times.

I am very grateful to Dr. Tony DeFranco and Dr. Jason Cyster for serving on my thesis committee. They have helped me make several important decisions on my research projects, and thoroughly reviewed this thesis. I truly appreciate their inputs on my work.

One person who requires a special acknowledgment is Dr. Richard Locksley. He was the chairman of the admissions committee, and interviewed me via an international call the year I applied to the UCSF Graduate Program in Immunology from Taiwan. It is

safe to say that the decision of selecting me for admission to UCSF made by him and other committee members has changed my life. I am very grateful to him and other faculties for providing me the great opportunity to study and work at this excellent research community at UCSF.

The data presented in Chapter 2 and most of the text was presented in the following publication:

Liou, J., Kiefer, F., Dang A., Hashimoto A., Cobb M., Kurosaki T., and Weiss A. (2000). HPK1 is activated by antigen receptors and negatively regulates AP-1. Immunity: 12 (4): 399-408.

The co-author Dr. Friedemann Kiefer generously provided me with HPK1 reagents and knockout mice. Dr. Tomohiro Kurosaki and Ari Hashimoto supplied me with DT40 mutant cell lines. Dr. Melanie Cobb and Alphons Dang provided a constitutively-active JNK construct. The work performed in Chapter 3 was in communication with Dr. Karsten Sauer. I appreciate all the inputs from the collaborators on my project.

I would like to acknowledge my parents, parents-in-law, and my sisters. This degree would not be possible without their support and encouragement. I also thank my friends and fellow graduate students who made my life in San Francisco very enjoyable. Finally, I would like to address my deepest gratitude to my husband, Lun (Tristan) Tsuei, for his endless love and understanding. Building a family with him has been the greatest achievement I have ever made.

ABSTRACT

THE REGULATION AND FUNCTION OF HEMATOPOIETIC PROGENITOR KINASE-1 IN ANTIGEN RECEPTOR SIGNAL TRANSDUCTION

Jen Liou

HPK1 is a Ste20-like serine/threonine kinase expressed in hemapoietic cells. It contains proline-rich motifs that have been shown to bind to several SH3 domain-containing adaptor proteins, and has been implicated as an upstream regulator of MAP kinase cascades. However, its regulation and function in physiological pathways are poorly understood. Here, I demonstrate that HPK1 plays a novel role in antigen receptor signaling. The catalytic activity of HPK1 was rapidly and greatly induced following antigen receptor stimulation in both T and B lymphocytes. Studies with mutant lines of Jurkat T leukemic cells and gene-targeted DT40 B cells revealed that HPK1 activation was dependent on the presence of Src and Syk families of protein tyrosine kinases, as well as the adaptor proteins LAT, SLP-76, BLNK, Grb2, and Grap. Biochemical approaches further demonstrated that HPK1 was complexed with Grb2 and Grap in T cells. Following stimulation, it became associated with SLP-76 and LAT. The inducible association of tyrosine phosphorylated HPK1 with the SH2 domain of SLP-76 was required for the full activation of HPK1. These observations suggest a model for HPK1 activation by the TCR, in which HPK1 is recruited to tyrosine phosphorylated LAT via

Grb2, where it can be tyrosine phosphorylated and thereby bind to the SH2 domain of SLP-76, which leads to the full induction of HPK1 catalytic activity. Surprisingly, overexpression of HPK1 suppressed TCR activation of ERK2 and AP-1, whereas a kinase-inactive HPK1 mutant potentiated these responses. Neither form of HPK1 affected PMA- or v-Ras-mediated activation of AP-1 and ERK2. Thus, HPK1 negatively regulates AP-1 at a step proximal to Ras activation in the T cell receptor signaling pathway.

TABLE OF CONTENTS

CHAPTER 2: HPK1 IS ACTIVATED BY ANTIGEN RECEPTORS AND NEGATIVELY REGULATES AP-1

CHAPTER 3: HPK1 IS REGULATED BY ITS INDUCIBLE ASSOCIATION WITH THE ADAPTOR PROTEIN SLP-76 IN T LYMPHOCYTES

LIST OF FIGURES

CHAPTER 1: HPK1 IS AN UPSTREAM REGULATOR OF MAP KINASES AND MAY PARTICIPATE IN T-CELL ANTIGEN RECEPTOR SIGNALING

CHAPTER 2: HPK1 IS ACTIVATED BY ANTIGEN RECEPTORS AND NEGATIVELY REGULATES AP-1

CHAPTER 1

HPK1 IS AN UPSTREAM REGULATOR OF MAP KINASES AND MAY PARTICIPATE IN T-CELL ANTIGEN RECEPTOR SIGNALING

Summary

Evolutionarily conserved mitogen-activated protein (MAP) kinase cascades respond to signals from cell-surface receptors to induce transcriptional responses within cells. Following T cell activation, multiple MAP kinase cascades are activated, and participate in thymocyte selection, T cell differentiation, and cytokine production. The activity of MAP kinase cascades can be regulated by the Ste20 group of kinases or small GTPases. Many novel mammalian Ste20 group kinases have been identified in the past few years. Among them, hematopoietic progenitor kinase-1 (HPK1) is of particular interest because its expression is restricted in hematopoietic tissues. In addition, it contains multiple proline-rich motifs, and therefore may be recruited to the T cell antigen receptor (TCR) signaling complex by SH3 domain-containing proteins. In this introductory chapter, I briefly review the TCR signaling pathway, focusing on the assembly of multiple signaling components by the adaptor protein LAT, and the activation of MAP kinase cascades. In addition, I discuss Ste20 group kinases as upstream regulators for MAP kinases with emphasis on HPK1, and the closely-related GCK-I subfamily of kinases. I conclude the chapter by describing HPK1 as a Grb2-interacting protein, which may participate in antigen receptor signal transduction.

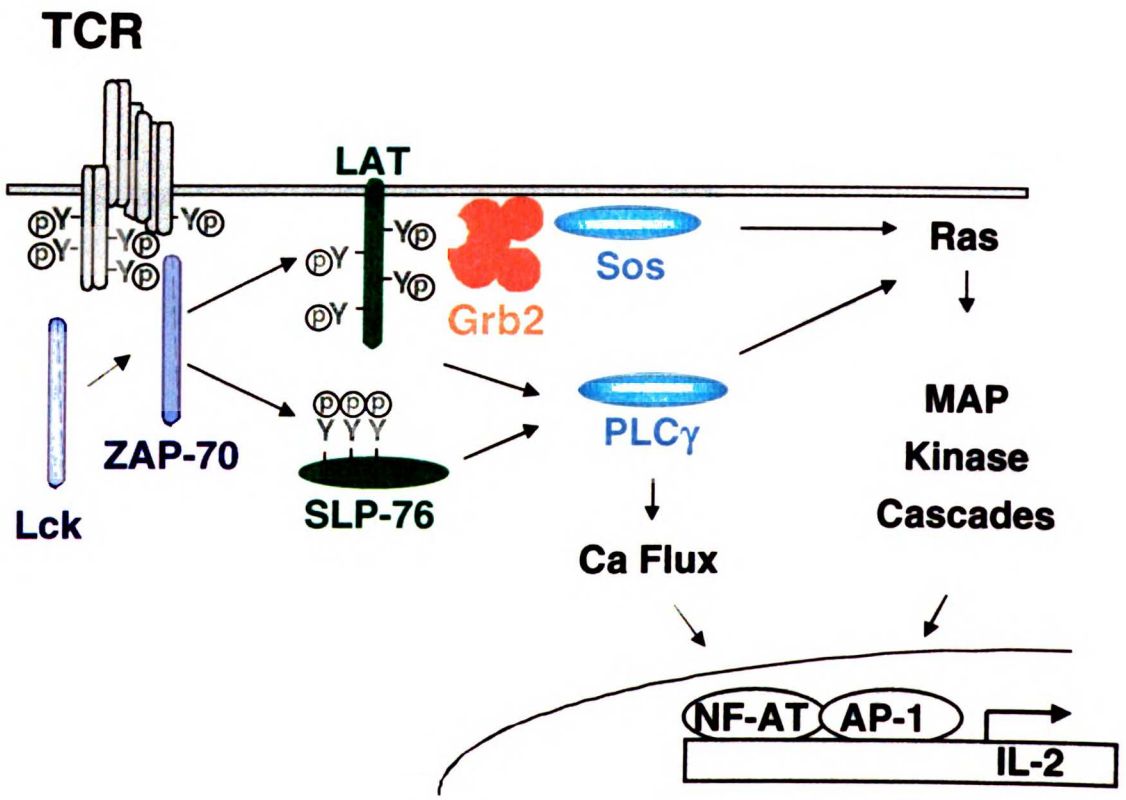
T cell receptor signal transduction

T lymphocytes are central to the immune system. Following T cell antigen receptor (TCR) recognition of antigen presented by the major histocompatibility complex (MHC), a series of signaling events are initiated that culminate in cell proliferation, transcriptional activation, cytoskeleton reorganization, and cell differentiation (Kane et al., 2000). TCR signaling is also critical for thymocyte maturation. It is believed that the strength of signaling determines whether the thymocyte will follow a path leading to positive selection or negative selection (Mariathasan et al., 1999). Patients that fail to activate their T lymphocytes are immunodeficient. Therefore, it is crucial to understand how engagement of the TCR leads to the various signaling responses in T cells.

Activation of protein tyrosine kinases (PTKs) is the initial signaling event following the engagement of the TCR (Figure 1). Activated Src family PTKs such as Lck and Fyn phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domains of CD3 chains. ZAP-70, a Syk family PTK, is then recruited to phosphorylated ITAMs, and subsequently becomes activated via its phosphorylation by Lck. Activated Lck and ZAP-70 phosphorylate their substrates such as the adaptor proteins LAT and SLP-76 as well as phospholipase C-γ1 (PLC-γ1). Recruitment of signaling proteins to tyrosine phosphorylated LAT and the activation of PLC-γ1 are essential for the activation of Ras, mitogen-activated protein (MAP) kinase cascades, and calcium responses. These signaling events lead to the activation of transcription factors including NFAT and AP-1 which are important for the upregulation of interleukin-2 (IL-2), a hallmark of T cell activation (van Leeuwen and Samelson, 1999; Weiss and Littman, 1994).

Figure 1: T-cell antigen receptor signal transduction

Following TCR stimulation, protein tyrosine kinases Lck and ZAP-70 are first activated. They, in turn, phosphorylate the adaptor proteins LAT and SLP-76. Tyrosine phosphorylation of LAT permits the recruitment of downstream effectors Grb2/Sos and PLCγ to the plasma membrane and the activation of the small GTPase Ras, MAP kinase cascades and calcium responses. These signaling events lead to the activation of transcription factors NFAT and AP-1, which are important for IL-2 production.


TCR
LAT
Grb2
Sos
Ras
ZAP-70
Lck
SLP-76
PLCγ
MAP
Kinase
Cascades
Ca Flux
NF-AT
AP-1
IL-2

Assembly of T cell signalosomes in GEMs by tyrosine phosphorylated LAT

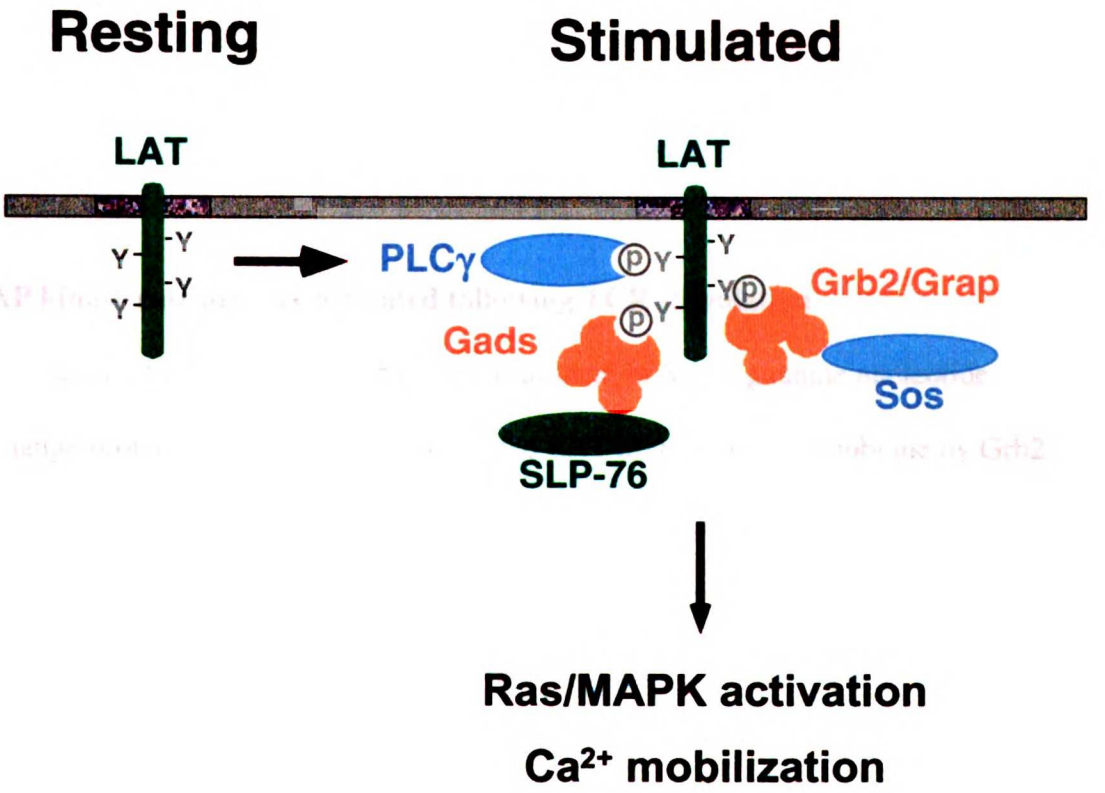
LAT is a hematopoietic cell-specific transmembrane protein with a short extracellular domain and a long cytoplasmic tail consisting of nine potential tyrosine phosphorylation sites (Zhang et al., 1998). In J.CaM2, a LAT-deficient Jurkat T leukemia cell line, Ras activation and calcium mobilization are defective (Finco et al., 1998). Furthermore, targeted gene disruption of LAT in mice results in a block in thymocyte development at the $CD4^-/CD8^-$ double negative stage (Zhang et al., 1999). Therefore, LAT is a critical component of TCR and pre-TCR signaling.

Targeting of LAT to glycolipid-enriched microdomains (GEMs) in the plasma membrane is central to its function in TCR signaling (Lin et al., 1999; Zhang et al., 1998). Mutations at palmitoylation sites C26 and C29 of LAT abrogate its localization in GEMs and inducible tyrosine phosphorylation. Tyrosine phosphorylated LAT can recruit SH2 domain-containing proteins such as Grb2-like adaptor proteins, and PLC-γ1 into GEMs, and thereby relay signals from the TCR to downstream pathways (Figure 2). Mutations of four tyrosine residues of LAT responsible for binding of PLC-γ1 and Grb2-like adaptors disrupt the function of LAT (Zhang et al., 2000). Thus, LAT can nucleate signaling complexes, and promote efficient functional responses for T cell activation.

Grb2 is a prototypic adaptor protein. It was first identified by its binding to tyrosine phosphorylated residues in the cytoplasmic tail of the EGF receptor (EGFR) (Lowenstein et al., 1992). Grb2 consists of an SH2 domain flanked by two SH3 domains. Therefore, Grb2 can link proteins containing proline-rich sequences via its SH3 domains to tyrosine phosphorylated proteins via its SH2 domain. Tyrosine phosphorylation of LAT following TCR stimulation provides docking sites for the Grb2 SH2 domain.

Figure 2: Recruitment of effector proteins to LAT following TCR stimulation

Following TCR stimulation, tyrosine phosphorylation of the adaptor protein LAT, present in glycolipid-enriched microdomains in the plasma membrane, allows the recruitment of SH2 domain-containing effector and adaptor proteins such as PLCγ1, Grb2, Grap, and Gads to LAT. Since Grb2, Grap, and Gads contain both SH2 and SH3 domains, they can link LAT to their SH3 domain-binding partners such as Sos and SLP-76. These recruitment events are important for the induction of Ras/MAP kinase activation and calcium mobilization.


Resting
Stimulated
LAT
LAT
Y
Y
Y
Y
PLCγ
P Y
P Y
Y
Y P
Grb2/Grap
Gads
Sos
SLP-76
Ras/MAPK activation
Ca2+ mobilization

Several Grb2-associated proteins in T cells such as Sos, and Cbl have been shown to contribute to positive or negative regulation of T cell activation (Clements et al., 1999). Furthermore, two Grb2-like adaptor proteins present exclusively in hematopoietic cells, Grap and Gads, have also been shown to recruit signaling proteins to LAT following TCR stimulation (Feng et al., 1996; Liu et al., 1999; Trüb et al., 1997). While the SH2 domains of all three adaptors seem to bind to similar targets, the SH3 domain of Gads displays a distinct binding specificity from that of Grb2 and Grap (Liu and McGlade, 1998). It is thought that Gads functions in linking SLP-76 to LAT.

MAP kinase cascades are activated following TCR stimulation

Current models suggest that the recruitment of Sos, a guanine nucleotide exchange protein, to tyrosine phosphorylated LAT in the plasma membrane by Grb2 leads to the activation of the small GTPase Ras. Activated Ras can trigger the activation of MAP kinase cascades and therefore induce transcriptional responses. The activities of MAP kinases are regulated through a three-tiered cascade consisting of a MAP kinase (MAPK), a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Figure 3). Since MAP kinases function to phosphorylate and regulate transcription factors including AP-1 and NFAT, which have been implicated in regulating cytokine production and cell proliferation, it is of great interest to understand the mechanisms by which TCR stimulation leads to the regulation of MAP kinases (Chang and Karin, 2001; Chow et al., 1997).

Figure 3: MAP kinase cascades

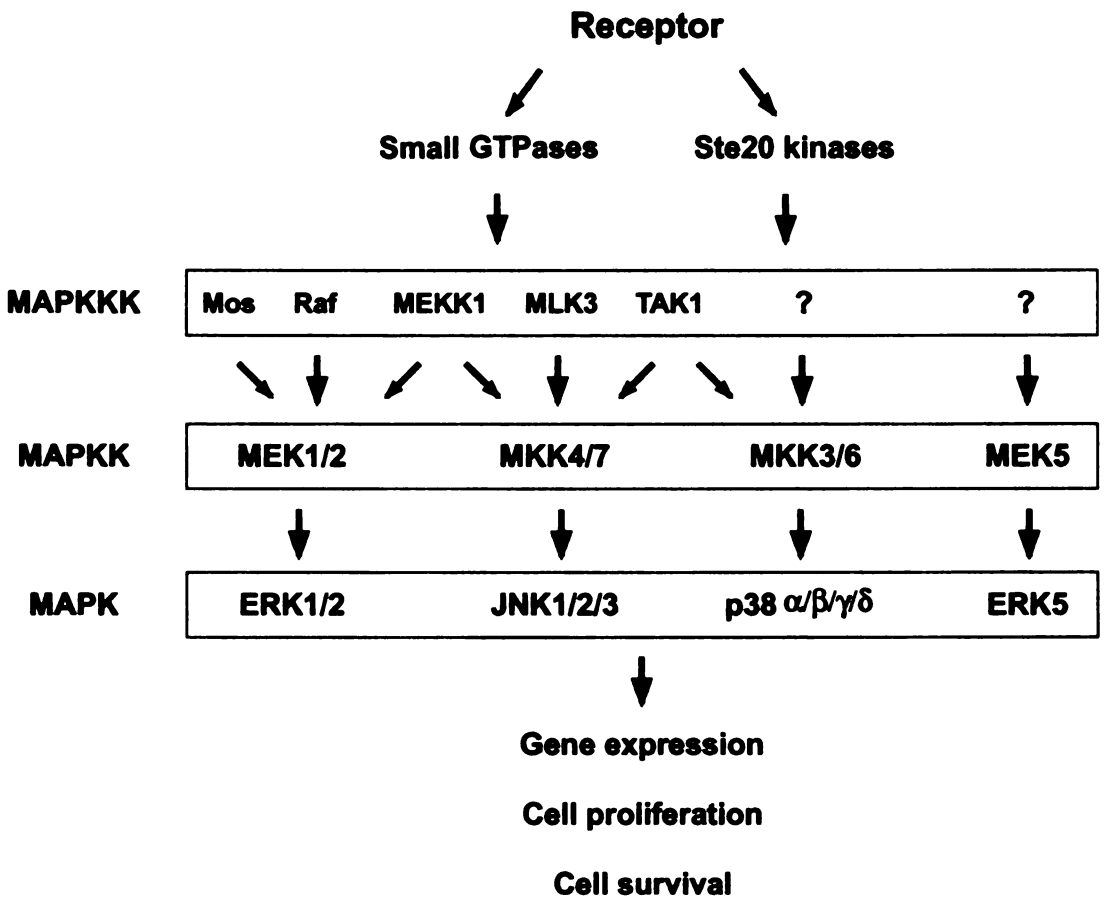
MAP kinases, ERK1/2, JNK1/2/3, p38α/β/γ/δ, and ERK5, are regulated through a three-tiered cascade containing a MAP kinase (MAPK), a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). These cascades can be regulated by cell surface receptors through small GTPases or Ste20 kinases. Activation of MAP kinases can lead to gene expression, cell proliferation, and cell survival.

d through a

PKK), and

e

can lead



Ste20 group kinases are upstream regulators of MAP kinase cascades

In addition to small GTPases, MAP kinase cascades can also be regulated by Ste20 kinases. Due to the ease of genetic analysis, this regulation is best understood in budding yeast *Saccharomyces cerevisiae* (Gustin et al., 1998). In the yeast mating pathway, the serine/threonine kinase Ste20p is activated by the binding of the GTP-bound small G-protein Cdc42p, and recruited to the Gβ subunit released by a heterotrimeric G protein following pheromone stimulation (Figure 4). Ste20p then phosphorylates the MAPKKK Ste11p, which is in a MAP kinase module also recruited to Gβ, and initiates the activation of the MAP kinase cascade. Activated MAPK Fus3p translocates into the nucleus to activate transcription factors, which in turn regulates genes involved in the mating reaction.

More than 30 Ste20-related kinases have been discovered in higher organisms (Dan et al., 2001). They are divided into the p21-activated kinase (PAK) family of kinases and the germinal center kinase (GCK) family of kinases by the location of their kinase domains. The kinase domain of PAKs are located at their C-termini, whereas those of GCKs are at their N-termini. Ste20 group kinases share a signature sequence "(V/I)GTPYWMAPE" in subdomain VIII of their kinase domains. Since subdomain VIII is primarily involved in the recognition of substrates, Ste20 group kinases may have similar substrate specificity.

Recently, the PAK and GCK families are further divided into two and eight subfamilies, respectively (Dan et al., 2001). A phylogenic analysis of these kinases is depicted in Figure 5. Kinases in each subfamily have distinct structural features, and may

Figure 4: Ste20 is an upstream regulator of MAP kinase signaling cascade

In the mating pathway in the haploid budding yeast *Saccharomyces cerevisiae*, the serine/threonine kinase Ste20p is activated by the binding of the GTP-bound small G-protein Cdc42p, and recruited to the Gβ subunit released by a heterotrimeric G protein following pheromone stimulation. Ste20p then phosphorylates the MAPKKK Ste11p, which is in a MAP kinase module also recruited to Gβ, and initiates the activation of the MAP kinase cascade.

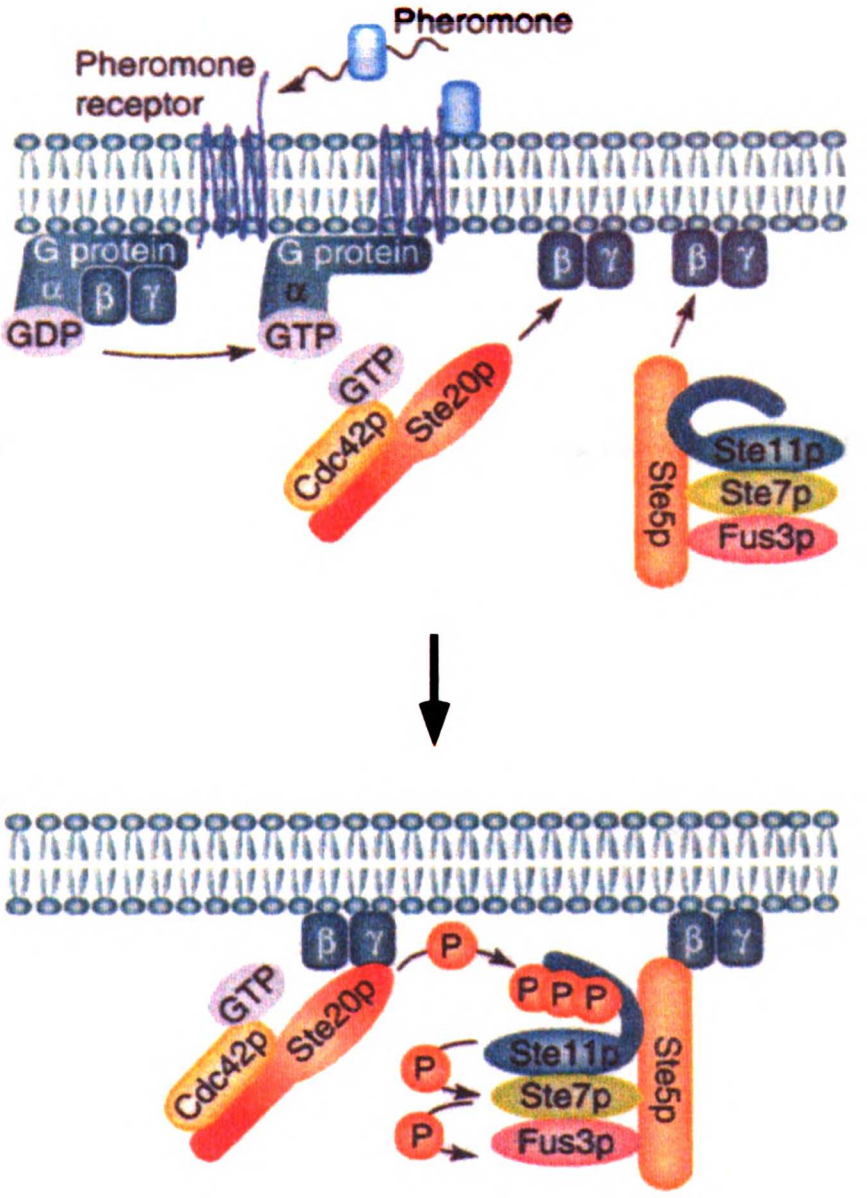
the

nall G-

protein

Ste11p,

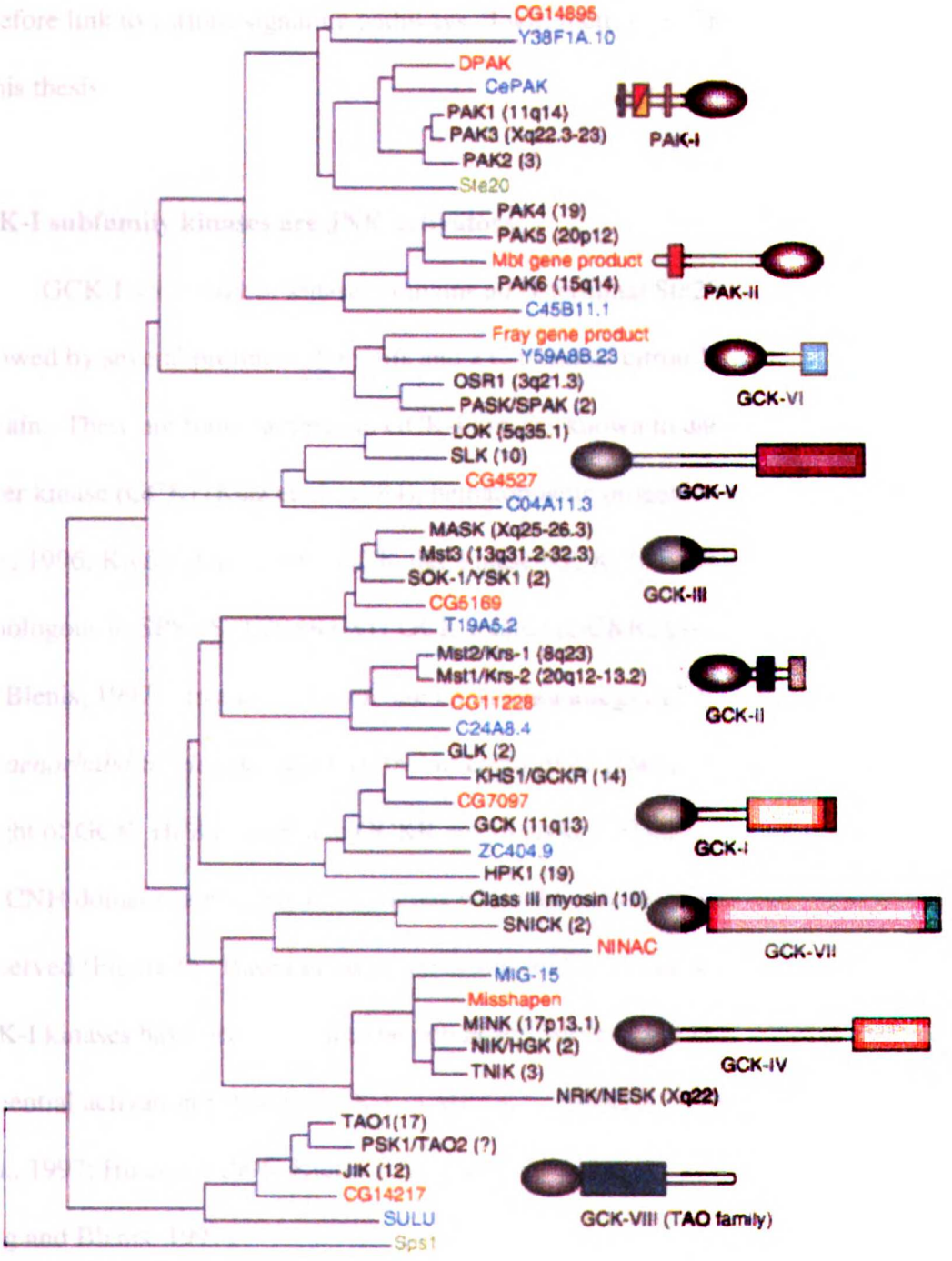
on of the



From Dan et al., TREND in Cell Biology 11:220 (2001)

Figure 5: Phylogenic relationships among Ste20 group kinases and their schematic structures

Mammalian Ste20 kinases are in black. Drosophila Ste20 kinases are in orange. C. elegans Ste20 kinases are in blue. Yeast proteins are in green. The chromosome location of each human kinase is indicated in parentheses.



From Dan et al., TREND in Cell Biology 11:220 (2001)

therefore link to various signaling pathways. I will focus on GCK-I subfamily of kinases in this thesis.

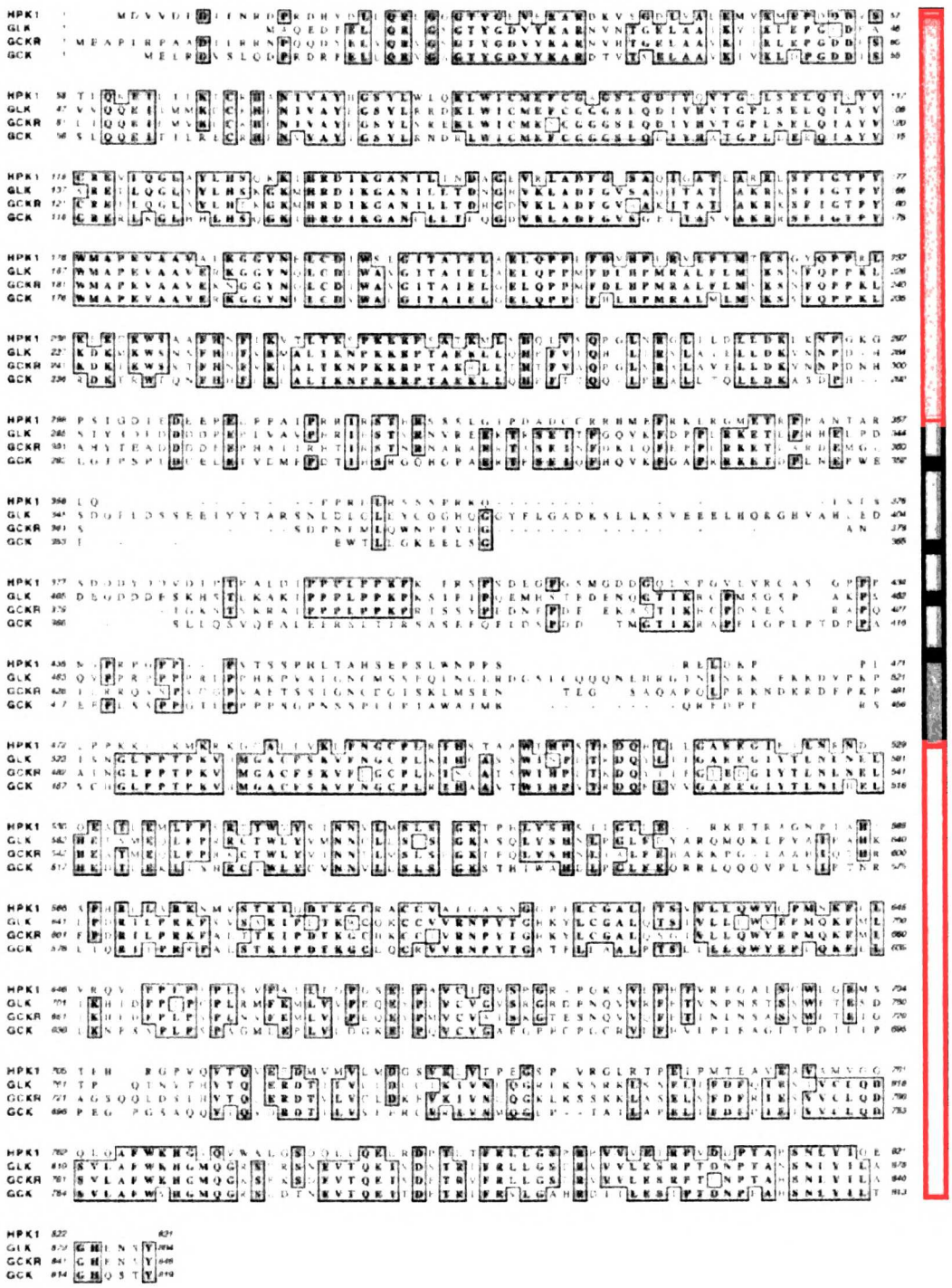
GCK-I subfamily kinases are JNK activators

GCK-I subfamily of kinases contains an N-terminal Ste20-like kinase domain followed by several proline-rich motifs and a C-terminal citron-homology (CNH) domain. There are four mammalian GCK-I kinases known to date. They are germinal center kinase (GCK) (Katz et al., 1994), hematopoietic progenitor kinase-1 (HPK1) (Hu et al., 1996; Kiefer et al., 1996), GCK-like kinase (GLK) (Diener et al., 1997), and kinase homologous to SPS1/STE20 (KHS) / GCK-related (GCKR) (Shi and Kehrl, 1997; Tung and Blenis, 1997). In addition, there are GCK-I homologs in lower organisms-ZC404.9 in *Caenorhabditis elegans*, and CG7097 in *Drosophila* (Dan et al., 2001). The molecular weight of GCK, HPK1, GLK and GCKR are all about 100 kDa. Their kinase domains and CNH domains are highly homologous while the central proline-rich regions are less conserved (Figure 6). Based on overexpression studies in fibroblasts, all four mammalian GCK-I kinases have been shown to be potent activators of the JNK pathway and a sequential activation pathway GCK-I -> MEKK1 -> MKK4 -> JNK is suggested (Diener et al., 1997; Hu et al., 1996; Kiefer et al., 1996; Pombo et al., 1995; Shi and Kehrl, 1997; Tung and Blenis, 1997).

The CNH domain is about 300 amino acids long (Schultz et al., 1998). In addition to GCK-I and GCK-IV subfamilies of kinases, it is also present in the mouse Rho-GTP/Rac-GTP binding protein Citron (Madaule et al., 1995), and *Saccharomyces*

Figure 6: Sequence conservation of GCK-I family kinases

The amino acid sequences of all four mammalian GCK-I kinases- HPK1, GLK, GCKR, and GCK, are aligned using "ClustalW Alignment" of the MacVector software (version 6.5.3). Identical residues are highlighted. Note the conservation of the kinase domains and the citron-homology domains.


Ser/Thr kinase
Proline-rich
Citron-homology

cerevisiae RhoGEF (guanine-nucleotide exchange factor) Rom1p and Rom2p (Ozaki et al., 1996) (Figure 7). The function of this domain remains unclear but it is noteworthy that citron and Rom1/2p are effectors of small G-protein Rho.

HPK1 - a hematopoietic-specific GCK-I kinase

GCK, GLK and GCKR are present in all tissues. The expression of HPK1 is widespread in embryonic tissues, then becomes restricted to hematopoietic organs in adults. HPK1 can be detected as a 97 kDa protein in bone marrow, spleen, lymph node, thymus, and several myeloid and lymphoid cell lines including MOLT-4 (lymphoblastic leukemia), Daudi (Burkitt lymphoma), Raji (Burkitt lymphoma), KG1a (myelogenous leukemia), TF-1 (factor-dependent immature erythroleukemia), and Jurkat (T-cell leukemia) (Hu et al., 1996; Kiefer et al., 1996).

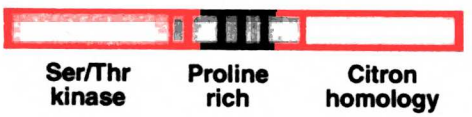
Murine HPK1 was first cloned in a search of genes differentially expressed in individual cells at defined hematopoietic differentiation stages (Kiefer et al., 1996). It was named as hematopoietic progenitor kinase 1 for its expression in mouse hematopoietic progenitor cells. Human HPK1 was later cloned using murine HPK1 cDNA as a probe to screen a human fetal liver cDNA library (Hu et al., 1996). The sequences of murine and human HPK1 are 85% identical (Figure 8).

The regulation of HPK1 is not well understood. In contrast to GCK, GLK and GCKR, HPK1 is not regulated by TNF-α (Hu et al., 1996). Notably, HPK1 is the most distantly related of this subfamily of kinases (Figure 5). HPK1 is also the only one containing four proline-rich motifs. It is possible that HPK1 has evolved to be specifically regulated by receptors in hematopoietic cells.

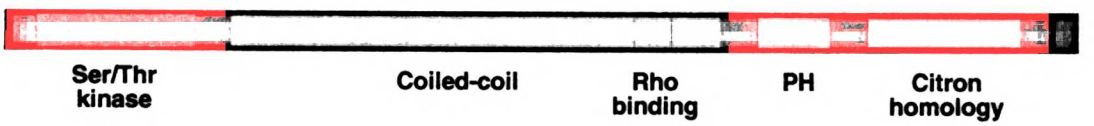
Figure 7: Schematic representation of citron-homology domain-containing proteins

In addition to GCK family kinases, the citron-homology domain is also present in the mouse Rho-GTP/Rac-GTP binding protein Citron, and two budding yeast Rho guanine-nucleotide exchange factors Rom1p and Rom2p. Note that citron and Rom1/2p are all effectors of small G-protein Rho.

GCK-I kinases



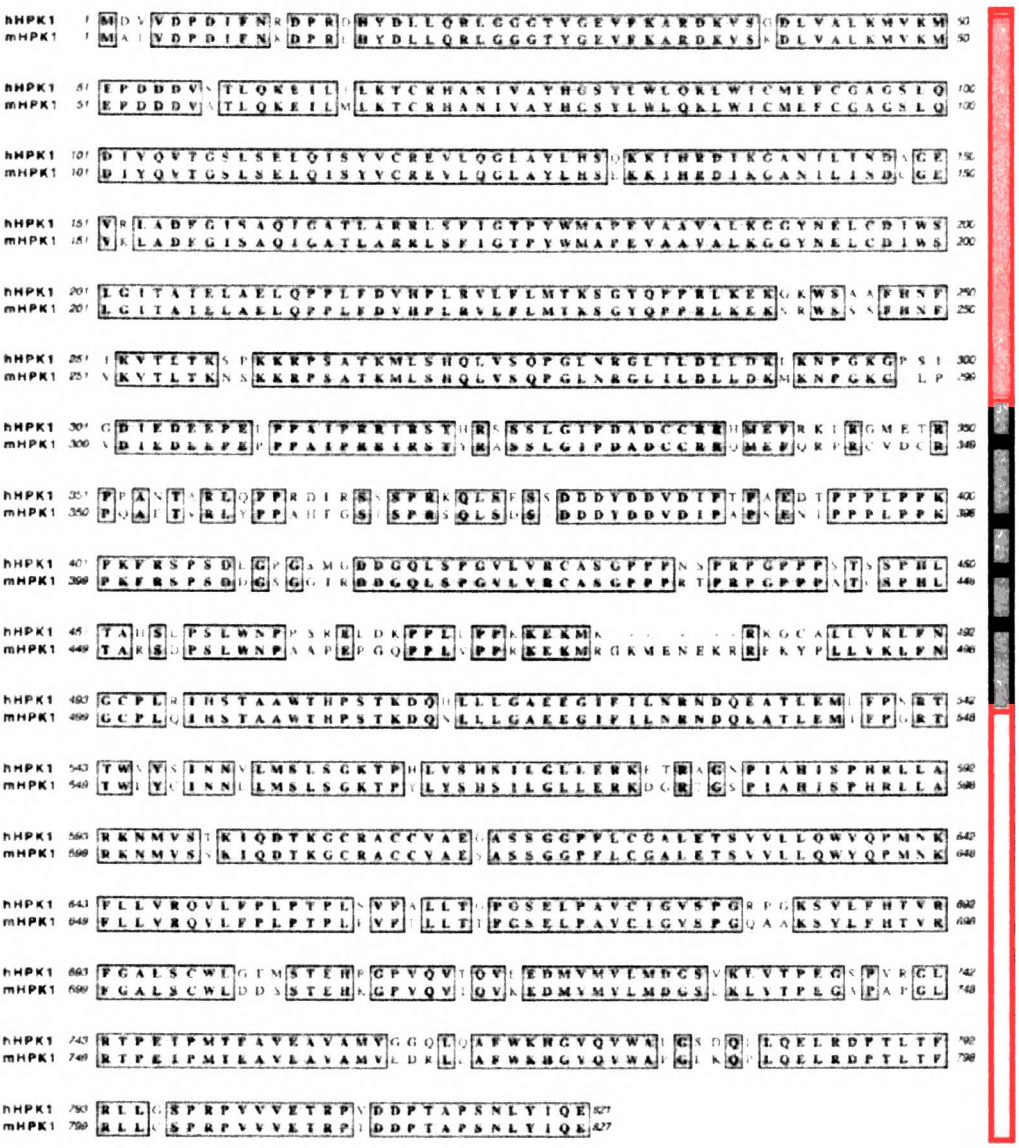
Citron



Rom1/2p

Figure 8: Sequence conservation of human and murine HPK1

The amino acid sequences of human and murine HPK1 are aligned using "ClustalW Alignment" of the MacVector software (version 6.5.3). Identical residues are highlighted. The sequences of murine and human HPK1 are 85% identical.


Ser/Thr kinase
Proline-rich
Citron-homology

HPK1 may be linked to tyrosine kinases by SH2/SH3 adaptor proteins

The proline-rich motifs of HPK1 are potential binding sites for SH3 domain-containing proteins. Several SH2/SH3 domain-containing proteins are capable of binding to HPK1. Grb2 interacts with HPK1 *in vitro* and in a transfection model (Anafi et al., 1997). In addition, Nck can recognize a fragment of HPK1 proline-rich region in a yeast two-hybrid assay (Anafi et al., 1997). Moreover, HPK1 is found to interact with the first SH3 domain of the Crk family of adaptors, c-Crk and CrkL (Oehrl et al., 1998).

Antigen receptor signaling is initiated by PTKs which phosphorylate multiple proteins such as LAT. LAT, in turn, can recruit SH2/SH3 adaptor proteins. By binding to tyrosine phosphorylated proteins following antigen receptor, SH2/SH3 adaptor proteins can link the activation of protein tyrosine kinases to effectors containing proline-rich motifs and thus regulate downstream signaling events including the MAP kinase cascades. HPK1 is an upstream regulator of MAP kinases and is capable of binding to SH2/SH3 adaptor proteins. Therefore, HPK1 may be involved in antigen receptor signal transduction.

General organization of the thesis

Its specific expression in hematopoietic cells, the presence of a Ste20-like kinase domain and proline-rich motifs, and its ability to interact with Grb2 suggest that HPK1 may be involved in antigen receptor signaling. In Chapter 2, I demonstrate the activation of HPK1 by antigen receptors, and the molecular requirements for this activation in T and B lymphocyte lines. I also provide functional evidence supporting a negative regulatory role of HPK1 in TCR-mediated AP-1 activation. In Chapter 3, I present evidence for the regulation of HPK1 by its association with the adaptor protein SLP-76 in T cells. In Chapter 4, I discuss the observations in Chapter 2 and 3, and propose a mechanistic model for HPK1 activation by the TCR. Additionally, I discuss the functional effects of HPK1 on TCR signaling as examined by overexpression studies and by analysis of HPK1-deficient mice. Future directions of this work are also suggested. The Appendix includes diagrams of HPK1 mutant and fusion constructs generated for the characterization of HPK1 regulation and function.

References

Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N., and Pawson, T. (1997). SH2/SH3 adaptor proteins can link tyrosine kinases to a Ste20-related protein kinase, HPK1. J Biol Chem *272*, 27804-11.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37-40.

Chow, C. W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R. J. (1997). Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. Science *278*, 1638-41.

Clements, J. L., Boerth, N. J., Lee, J. R., and Koretzky, G. A. (1999). Integration of T cell receptor-dependent signaling pathways by adapter proteins. Annual Review of Immunology *17*, 89-108.

Dan, I., Watanabe, N. M., and Kusumi, A. (2001). The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol *11*, 220-30.

Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Keesler, G., Zukowski, M., Tan, T. H., and Yao, Z. (1997). Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. Proc Natl Acad Sci U S A *94*, 9687-92.

Feng, G. S., Ouyang, Y. B., Hu, D. P., Shi, Z. Q., Gentz, R., and Ni, J. (1996). Grap is a novel SH3-SH2-SH3 adaptor protein that couples tyrosine kinases to the Ras pathway. Journal of Biological Chemistry *271*, 12129-32.

Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998). LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity *9*, 617-26.

Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev *62*, 1264-300.

Hu, M. C., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T. H. (1996). Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes and Development *10*, 2251-64.

Kane, L. P., Lin, J., and Weiss, A. (2000). Signal transduction by the TCR for antigen. Current Opinion in Immunology *12*, 242-9.

Katz, P., Whalen, G., and Kehrl, J. H. (1994). Differential expression of a novel protein kinase in human B lymphocytes. Preferential localization in the germinal center. Journal of Biological Chemistry *269*, 16802-9.

Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996). HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. Embo Journal *15*, 7013-25.

Lin, J., Weiss, A., and Finco, T. S. (1999). Localization of LAT in glycolipid-enriched microdomains is required for T cell activation. Journal of Biological Chemistry *274*, 28861-4.

Liu, S. K., Fang, N., Koretzky, G. A., and McGlade, C. J. (1999). The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. Current Biology *9*, 67-75.

Liu, S. K., and McGlade, C. J. (1998). Gads is a novel SH2 and SH3 domain-containing adaptor protein that binds to tyrosine-phosphorylated Shc. Oncogene *17*, 3073-82.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*, 431-42.

Madaule, P., Furuyashiki, T., Reid, T., Ishizaki, T., Watanabe, G., Morii, N., and Narumiya, S. (1995). A novel partner for the GTP-bound forms of rho and rac. FEBS Lett *377*, 243-8.

Mariathasan, S., Jones, R. G., and Ohashi, P. S. (1999). Signals involved in thymocyte positive and negative selection. Seminars in Immunology *11*, 263-72.

Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998). The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene *17*, 1893-901.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y., and Takai, Y. (1996). Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in Saccharomyces cerevisiae. Embo Journal *15*, 2196-207.

Pombo, C. M., Kehrl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T., and Kyriakis, J. M. (1995). Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. Nature *377*, 750-4.

Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America *95*, 5857-64.

Shi, C. S., and Kehrl, J. H. (1997). Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF-kappaB, by the tumor necrosis factor (TNF) receptor 1

through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. J Biol Chem *272*, 32102-7.

Trüb, T., Frantz, J. D., Miyazaki, M., Band, H., and Shoelson, S. E. (1997). The role of a lymphoid-restricted, Grb2-like SH3-SH2-SH3 protein in T cell receptor signaling. Journal of Biological Chemistry *272*, 894-902.

Tung, R. M., and Blenis, J. (1997). A novel human SPS1/STE20 homologue, KHS, activates Jun N-terminal kinase. Oncogene *14*, 653-9.

van Leeuwen, J. E., and Samelson, L. E. (1999). T cell antigen-receptor signal transduction. Current Opinion in Immunology *11*, 242-8.

Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263-74.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell *92*, 83-92.

Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., and

Samelson, L. E. (1999). Essential role of LAT in T cell development. Immunity *10*, 323-32.

Zhang, W., Trible, R. P., and Samelson, L. E. (1998). LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity *9*, 239-46.

Zhang, W., Trible, R. P., Zhu, M., Liu, S. K., McGlade, C. J., and Samelson, L. E. (2000). Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling. Journal of Biological Chemistry *275*, 23355-61.

CHAPTER 2

HPK1 IS ACTIVATED BY ANTIGEN RECEPTORS AND NEGATIVELY REGULATES AP-1

Summary

The serine/threonine kinase HPK1 is a member of the germinal center kinase (GCK) family that has been implicated in the regulation of MAP kinase pathways. Here, we demonstrate the involvement of HPK1 in TCR-mediated AP-1 activation. Engagement of the TCR or the BCR resulted in a marked induction of HPK1 catalytic activity. Subsequent analysis revealed that Src and Syk/ZAP-70 tyrosine kinases and the adaptor proteins LAT, SLP-76, BLNK, Grb2, and Grap are involved in HPK1 activation. Overexpression of HPK1 inhibited TCR activation of AP-1 and ERK2, whereas the kinase-inactive mutant of HPK1 potentiated these responses. Neither form of HPK1 affected PMA or v-Ras activation of AP-1 and ERK2. Thus, HPK1 is a downstream target of antigen receptor signaling and negatively regulates the TCR-induced AP-1 response pathway. Work in this chapter has been published in Immunity:12(4):399-408, April, 2000.

Introduction

The stimulation of lymphocyte antigen receptors initiates a series of intracellular biochemical events leading to the production of cytokines, cell proliferation, and differentiation (Weiss and Littman, 1994). In T lymphocytes, engagement of the T-cell receptor (TCR) activates Src and Syk/ZAP-70 families of protein tyrosine kinases (PTKs). Tyrosine phosphorylation of downstream substrates such as the adaptor proteins LAT (linker for activation of T cells) and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), and the enzyme phospholipase Cγ1 (PLC γ1) by PTKs contributes to the activation of Ras and the elevation of intracellular calcium (van Leeuwen and Samelson, 1999). These responses promote the transcriptional induction of multiple genes including interleukin 2 (IL-2), a key growth and differentiation factor for T cells (Weiss and Littman, 1994). The transcription factor AP-1 plays a critical role in the transactivation of IL-2 gene by binding to multiple regulatory elements in the IL-2 promoter (Jain et al., 1995). In anergic T cells, which fail to respond to antigen and produce IL-2, there is a specific block in AP-1-dependent transactivation (Kang et al., 1992). Therefore, the regulation of the AP-1 pathway plays an important role in coordinating immune responses.

AP-1 has been identified as a target of mitogen-activated protein (MAP) kinases (Karin, 1995). Among MAP kinases, extracellular signal-regulated kinases 1/2 (ERK1/2) are activated following TCR engagement alone, whereas c-Jun N-terminal kinase (JNK) activation requires both TCR and CD28 stimulation (Su et al., 1994). Recently, protein serine/threonine kinases related to germinal center kinase (GCK) have been implicated as

upstream regulators of MAP kinase signaling pathways (Kyriakis, 1999). The GCK family kinases (GCKs) include GCK (Katz et al., 1994), hematopoietic progenitor kinase-1 (HPK1) (Hu et al., 1996; Kiefer et al., 1996), Nck-interacting kinase (NIK) / HPK1/GCK-like kinase (HGK) (Su et al., 1997; Yao et al., 1999), GCK-like kinase (GLK) (Diener et al., 1997), kinase homologous to SPS1/STE20 (KHS) / GCK-related (GCKR) (Shi and Kehrl, 1997; Tung and Blenis, 1997), and Drosophila Misshapen (Su et al., 1998). All GCKs contain N-terminal STE20-like kinase domains, and long C-terminal regulatory domains with proline-rich motifs. STE20 is thought to function downstream of a G protein and upstream of the MAP kinase cascade in yeast (Herskowitz, 1995). Hence, STE20-like proteins may link receptor activation to the regulation of MAP kinase modules. GCKs have been shown to be potent activators of the JNK pathway, although they may well have additional biological functions.

Among GCKs, HPK1 is of particular interest because it is exclusively expressed in hematopoietic cells. Furthermore, proline-rich motifs of HPK1 were shown to interact with the SH3 domains of Grb2 (growth factor receptor binding protein 2) *in vitro* and in a transfection model (Anafi et al., 1997). Grb2 is an adaptor protein comprising a single SH2 domain flanked by two SH3 domains. It has been implicated in coupling PTKs to the Ras pathway by its association with SOS, a guanine nucleotide exchange factor (GEF) for Ras (Olivier et al., 1993; Simon et al., 1993). In addition, Grb2 interacts with several regulators of TCR signaling including LAT, SLP-76, Cbl, and Shc (Clements et al., 1999). Grb2 can also interact with the cytoplasmic tail of the T-cell costimulatory receptor CD28 (Schneider et al., 1995). Therefore, the potential interaction between Grb2 and HPK1 raises the possibility that HPK1 may be involved in T cell signaling.

In this report, we demonstrate that HPK1 is activated following TCR and B-cell receptor (BCR) stimulation. The signaling pathways linking receptor stimulation to the activation of HPK1 in T and B cells are strikingly similar. Furthermore, HPK1 suppresses TCR activation of AP-1 and ERK2 while a kinase-inactive mutant of HPK1 potentiates these responses. This report is the first to characterize the activating pathway and the involvement of a germinal center kinase in antigen receptor signal transduction.

Results

Activation of HPK1 by the TCR

To determine whether HPK1 plays a role in T cell activation, we first examined the effect of TCR and CD28 stimulation on HPK1 kinase activity. Using an immune complex kinase assay, we measured the kinase activity of HA-tagged HPK1 (HPK1:HA) following transient transfection into Jurkat T cells. As shown in Figure 1A, HPK1 kinase activity was markedly induced by TCR stimulation. However, neither the autophosphorylation of HPK1 nor the phosphorylation of an exogenous substrate, histone H2A, was observed with the kinase-inactive HPK1, HPK1(K46E), demonstrating the specificity of the kinase assay. CD28 stimulation failed to activate HPK1 or modulate HPK1 activity induced by the TCR (data not shown). An investigation of the time course of HPK1 activation showed that HPK1 activity was evident 1 min following TCR stimulation and decreased thereafter (Figure 1B). This rapid and transient kinetics suggests that HPK1 activation may be a relatively proximal event in the TCR signaling pathway.

We also examined the activation of HPK1 by the TCR in freshly isolated mouse lymph node T cells. Consistent with the observations in Jurkat cells, the kinase activity of endogenously expressed HPK1 was rapidly induced by CD3 cross-linking in purified mouse lymph node T cells, although the magnitude of this response was lower (Figure 1C). Together, these observations indicate that HPK1 is involved in the signal transduction by the TCR.

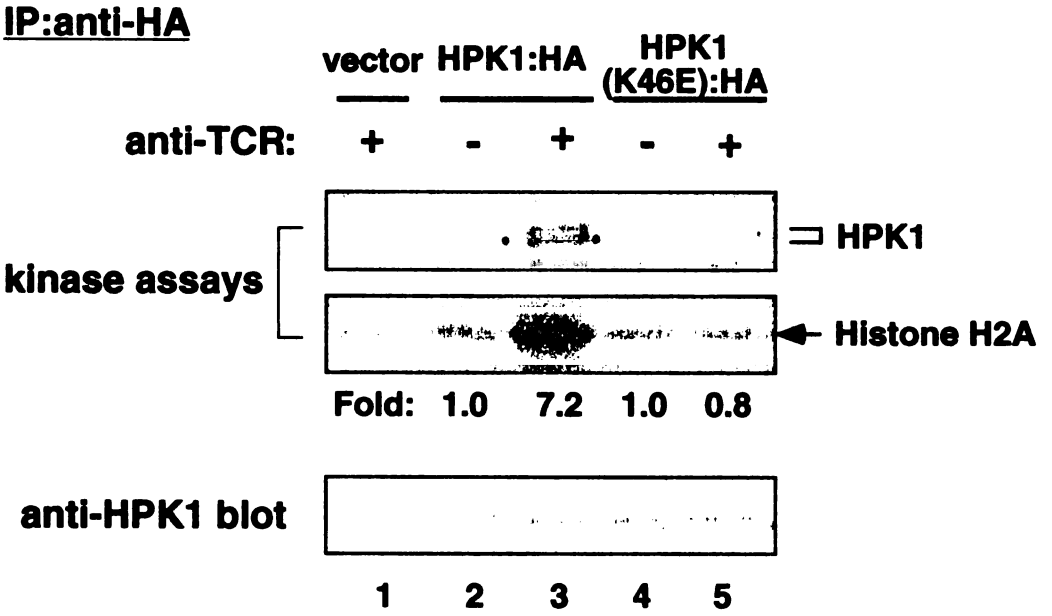
Figure 1. Activation of HPK1 Kinase Activity by the TCR.

(A) HPK1 activation by TCR stimulation. Jurkat cells transfected with empty vector (lane 1), HPK1:HA (lane 2 and 3), or HPK1(K46E):HA (lane 4 and 5), were stimulated with anti-TCR monoclonal antibody (MAb) (+) or a buffer control (-) for 1 min. Cells were then lysed and anti-HA immune complexes were assayed for kinase activity using histone H2A as the exogenous substrate. Phosphorylation was visualized by autoradiography (top and middle panels). The fold stimulation of HPK1 activity, measured by quantitating histone H2A phosphorylation relative to that with mock-stimulated cells, is indicated. The amount of HA-tagged HPK1 and HPK1(K46E) proteins immunoprecipitated was detected by immunoblotting with an anti-HPK1 antibody (bottom panel).

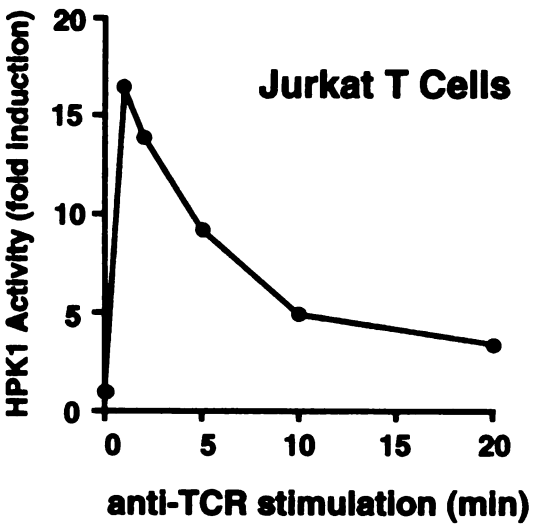
(B) Time-course of TCR-induced HPK1 activation in Jurkat T cells. HPK1:HA-transfected Jurkat cells were stimulated with anti-TCR MAb for 0-20 min as indicated. HPK1 kinase assays were performed as described in **(A)**. The data presented here are representative of at least two independent experiments.

(C) Time-course of TCR-induced HPK1 activation in mouse lymph node T cells. T cells purified from freshly isolated mouse lymph nodes were stimulated with anti-CD3 MAb for 0-15 min as indicated. Cells were then lysed, and anti-mouse HPK1 immune complexes were assayed for kinase activity using histone H2A as the substrate. The data are representative of two independent experiments.

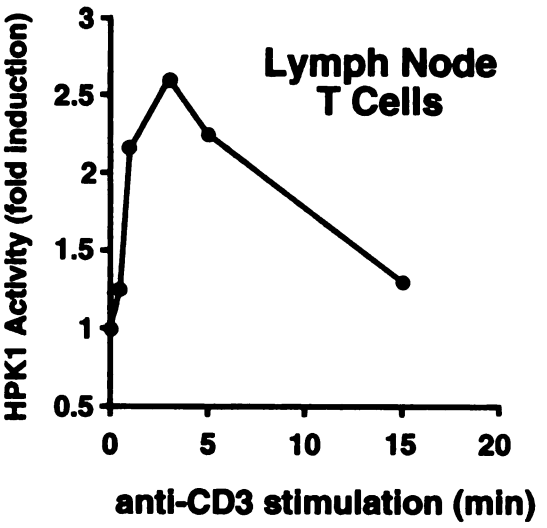
A


IP:anti-HA
vector
HPK1:HA
HPK1 (K46E):HA
anti-TCR:
+
-
+
-
+
kinase assays
HPK1
Histone H2A
Fold:
1.0
7.2
1.0
0.8
anti-HPK1 blot
1
2
3
4
5

B


Jurkat T Cells
HPK1 Activity (fold induction)
20
15
10
5
0
0
5
10
15
20
anti-TCR stimulation (min)

C


Lymph Node
T Cells
HPK1 Activity (fold induction)
3
2.5
2
1.5
1
0.5
0
5
10
15
20
anti-CD3 stimulation (min)

HPK1 Activation by the TCR Is Dependent on Lck, ZAP-70, LAT, and SLP-76

Signaling responses following TCR stimulation are initiated by tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the ζ and CD3 chains of the TCR by the Src family kinase Lck. The Syk family kinase ZAP-70 is then recruited to the phosphorylated ITAMs and upon phosphorylation becomes activated. Subsequent phosphorylation of downstream substrates by Lck and ZAP-70 leads to the activation of Ras and release of calcium from intracellular stores, eventually resulting in transcriptional activation of target genes (Weiss and Littman, 1994).

We utilized multiple Jurkat signaling mutants to determine which components of the TCR signaling pathway are involved in mediating HPK1 activation. Since Lck and ZAP-70 are the most upstream components in TCR signaling, we first investigated HPK1 activation in J.CaM1, an Lck-deficient Jurkat derivative (Straus and Weiss, 1992), and P116, a ZAP-70-deficient Jurkat line (Williams et al., 1998). Jurkat, J.CaM1, and P116 cells were transfected with HPK1:HA, stimulated with anti-TCR MAb or a buffer control, then subjected to HPK1 kinase assays. As expected, based on the importance of Lck and ZAP-70 in TCR signal transduction, inducible HPK1 activation was not detected in either J.CaM1 or P116 cells (Figure 2A). These results demonstrate that Lck and ZAP-70 are required for HPK1 activation upon TCR stimulation.

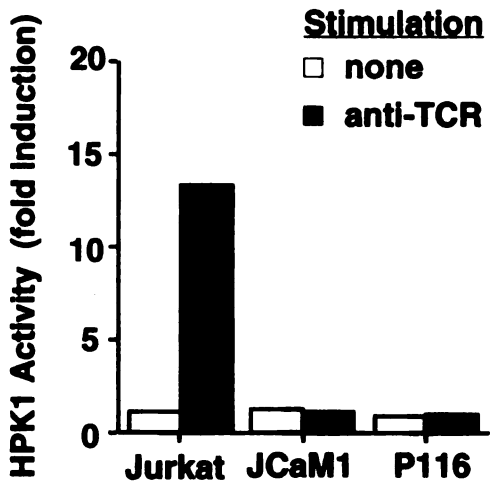
LAT is a transmembrane adaptor protein that becomes tyrosine phosphorylated following stimulation of the TCR (Zhang et al., 1998). It has been shown to couple the activation of PTKs to downstream signaling pathways based on the Jurkat variant J.CaM2, in which LAT expression is greatly reduced (Finco et al., 1998). In J.CaM2

Figure 2. TCR-Mediated HPK1 Activation Requires Lck, ZAP-70, LAT, and SLP-76.

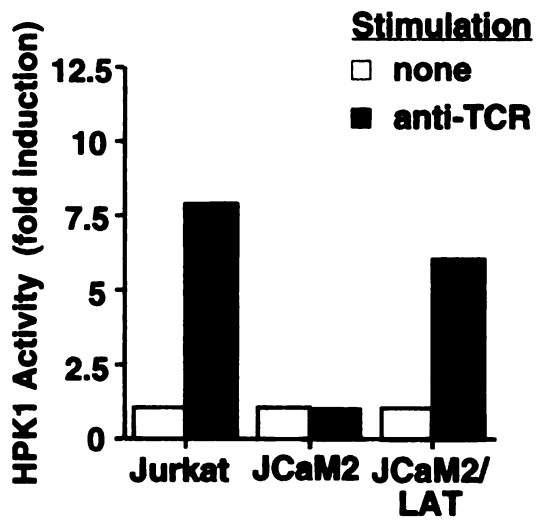
(A-C) Jurkat, J.CaM1 (Lck-deficient) and P116 (ZAP-70-deficient) cells **(A)**, or J.CaM2 and LAT-reconstituted J.CaM2 (J.CaM2/LAT) cells **(B)**, or J14 and SLP-76-reconstituted J14 (J14-76) cells **(C)**, were transfected with HPK1:HA and treated with anti-TCR MAb or a buffer control for 1 min. Cells were then lysed and anti-HA immunoprecipitates were assayed for HPK1 kinase activity using histone H2A as the substrate. The data are representative of at least two independent experiments. In all experiments, there were equivalent amounts of HPK1 protein immunoprecipitated as determined by immunoblotting (data not shown). Cell surface expression of TCR on all Jurkat lines was comparable.

(D) HPK1 is not activated by ionomycin or by PMA. HPK1:HA-transfected Jurkat T cells were stimulated for 1 min with anti-TCR MAb, or ionomycin (1 μM), and/or PMA (50 ng/ml). HPK1 catalytic activity was measured as described above. The data are representative of at least two independent experiments.

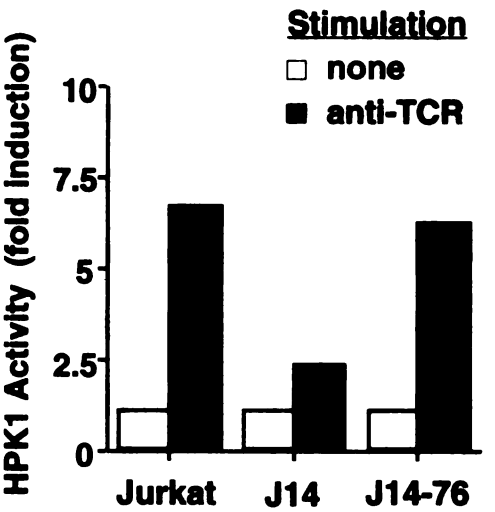
A


Stimulation
none
anti-TCR
HPK1 Activity (fold Induction)
20
15
10
5
0
Jurkat
JCaM1
P116

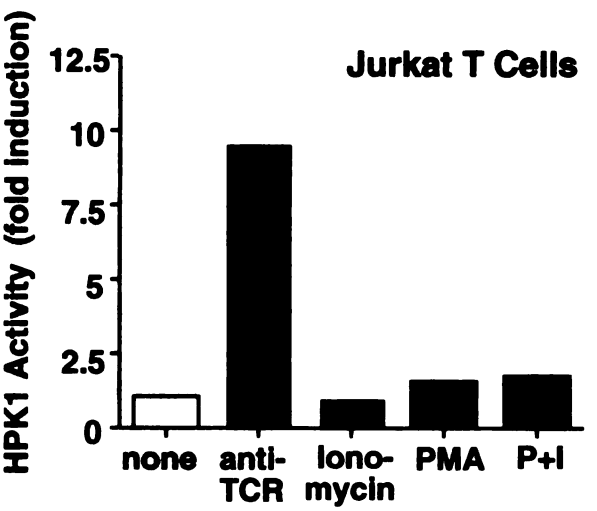
B


Stimulation
none
anti-TCR
HPK1 Activity (fold Induction)
12.5
10
7.5
5
2.5
0
Jurkat
JCaM2
JCaM2/
LAT

C


Stimulation
none
anti-TCR
HPK1 Activity (fold Induction)
10
7.5
5
2.5
0
Jurkat
J14
J14-76

D


Jurkat T Cells
HPK1 Activity (fold Induction)
12.5
10
7.5
5
2.5
0
none
anti-TCR
Iono-mycin
PMA
P+I

cells, HPK1 failed to be activated by TCR stimulation (Figure 2B). Importantly, reconstitution of LAT in J.CaM2 cells restored HPK1 activation following engagement of the TCR (Figure 2B). Another inducibly tyrosine phosphorylated adaptor protein, SLP-76, has also been implicated in linking upstream PTKs to the Ras and calcium pathways (Yablonski et al., 1998). To investigate whether HPK1 activation by the TCR depends on SLP-76, HPK1 kinase assays were performed in J14, a SLP-76-deficient Jurkat T cell line. HPK1 activation was substantially reduced in J14 cells, whereas in J14 cells stably transfected with SLP-76 (J14-76), HPK1 was efficiently activated (Figure 2C). Together, the data indicate that optimal HPK1 activation by TCR stimulation is dependent on the presence of adaptor proteins LAT and SLP-76.

Furthermore, we tested whether stimulation of the Ras and calcium pathways are sufficient to induce HPK1 activation. PMA is a phorbol ester capable of activating Ras and protein kinase C (PKC). Treatment of T cells with PMA and ionomycin, a calcium ionophore, can mimic TCR stimulation to induce downstream responses, including JNK activation and IL-2 gene transcription, while bypassing proximal signaling events such as tyrosine phosphorylation. We found that neither of these stimuli, alone or in combination, caused significant HPK1 activation (Figure 2D). These results suggest that HPK1 activation by the TCR may occur upstream or independent of Ras, PKC, and calcium signaling. Moreover, the activation of JNK, induced by the combination of PMA and ionomycin, is not dependent on HPK1.

HPK1 Interacts with Grb2 in T Cells

To further define the signaling pathway mediating HPK1 activation by the TCR, HPK1-interacting proteins in T cells were examined. We metabolically labeled untransfected and HPK1:HA-transfected cells with [^{35}S]-methionine and cysteine. Subsequently, anti-HA immunoprecipitations were performed, resolved by SDS-PAGE, transferred onto a membrane, and subjected to autoradiography. Such analysis revealed several proteins specifically associated with HPK1:HA (Figure 3A, ^{35}S labeling). Two specific proteins reproducibly detected were 100 kDa and 28 kDa. The band of approximately 100 kDa was determined to be HPK1:HA by immunoblotting (Figure 3A, anti-HA blot). One candidate for the 28 kDa band is the adaptor protein Grb2. Grb2 has been demonstrated to interact with HPK1 *in vitro* and upon ectopic coexpression in fibroblasts (Anafi et al., 1997). We therefore immunoblotted the membrane with an anti-Grb2 antibody and found that Grb2 was present specifically in the immunoprecipitates with HPK1:HA (Figure 3A, anti-Grb2 blot). The Grb2 signal obtained by immunoblotting aligned exactly with the 28 kDa band on the autoradiograph. Nevertheless, we can not exclude the possibility that the 28 kDa band may represent more than one HPK1-interacting protein.

The proline-rich motifs in HPK1 have been shown to bind to the SH3 domains of Grb2 (Anafi et al., 1997). Consistent with this previous observation, Grb2 was present in anti-HA immunoprecipitates from both resting and TCR-activated Jurkat cells transfected with HPK1:HA (Figure 3B, lanes 3 and 4). Furthermore, reciprocal immunoprecipitations performed using an antibody to Grb2 showed that endogenously expressed Grb2 and HPK1 associated in Jurkat cells regardless of TCR stimulation (Figure 3C, lanes 1 and 2), consistent with the results presented in Figure 3B. To ensure

Figure 3. HPK1 Association with Grb2 and Grap in T Cells.

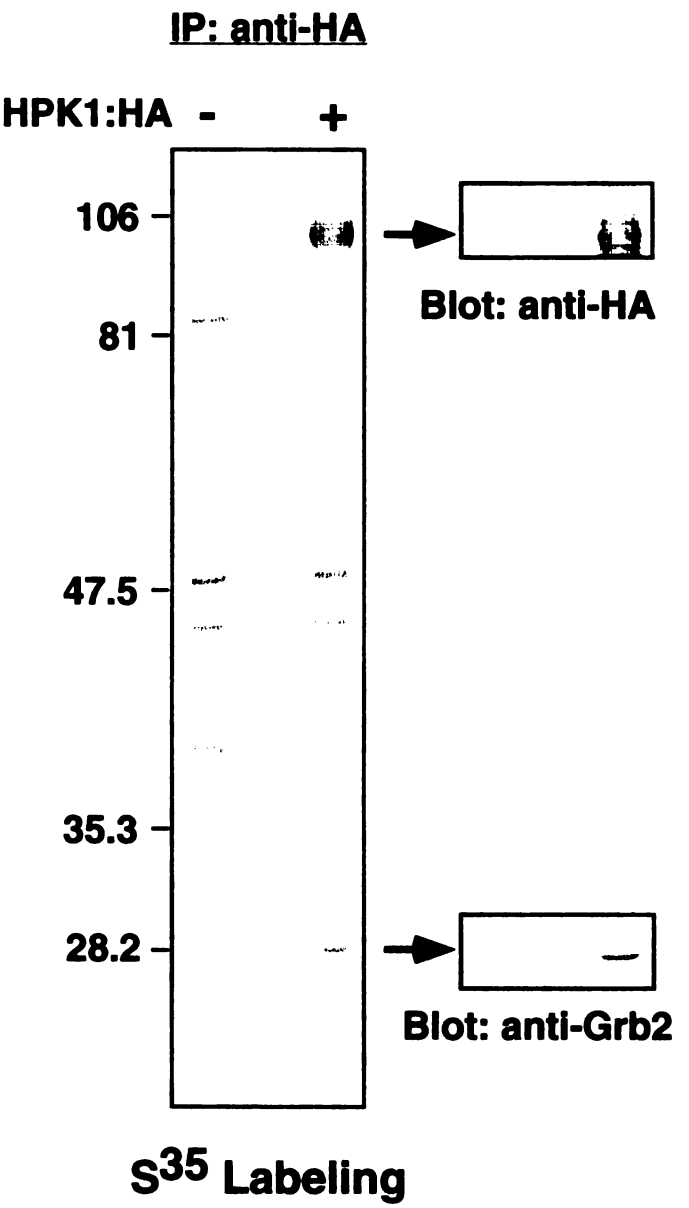
(A) Examination of HPK1-interacting proteins in T cells. Untransfected and HPK1:HA-transfected Jurkat cells were labeled with [^{35}S]-methionine/cysteine, and stimulated with anti-TCR MAb for 1 min prior to lysis. Anti-HA immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and visualized by autoradiography. The blot was subsequently probed with anti-HA and anti-Grb2 antibodies. Positions of molecular mass markers are indicated in kilodaltons on the left.

(B) HPK1 associates with Grb2 constitutively in T cells. Untransfected (lanes 1 and 2) and HPK1:HA-transfected (lanes 3 and 4) Jurkat cells were treated with a buffer control (-) or anti-TCR MAb (+) for 1 min. Cells were then lysed, immunoprecipitated with anti-HA MAb, and immunoblotted with anti-HA (top panel) or anti-Grb2 (bottom panel) antibodies.

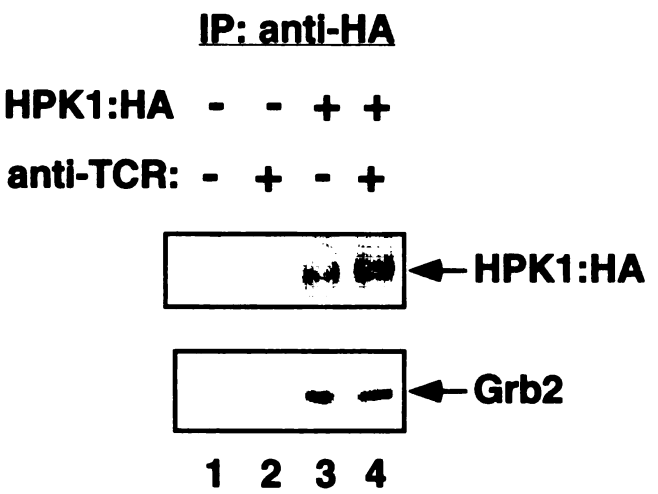
(C) The interaction between endogenously expressed HPK1 and Grb2. Jurkat cells were treated with a buffer control (-) or anti-TCR MAb (+) for 1 min, lysed, immunoprecipitated with an anti-Grb2 antibody in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the blocking peptide, then immunoblotted with anti-HPK1 (top panel) or anti-Grb2 (bottom panel) antibodies.

(D) HPK1 associates with Grap constitutively in T cells. Untransfected (lanes 1 and 2) and HPK1:HA-transfected (lanes 3 and 4) Jurkat cells were treated with a buffer control (-) or anti-TCR MAb (+) for 1 min. Cells were then lysed, immunoprecipitated with anti-HA MAb, and immunoblotted with anti-HA (top panel) or anti-Grap (bottom panel) antibodies.

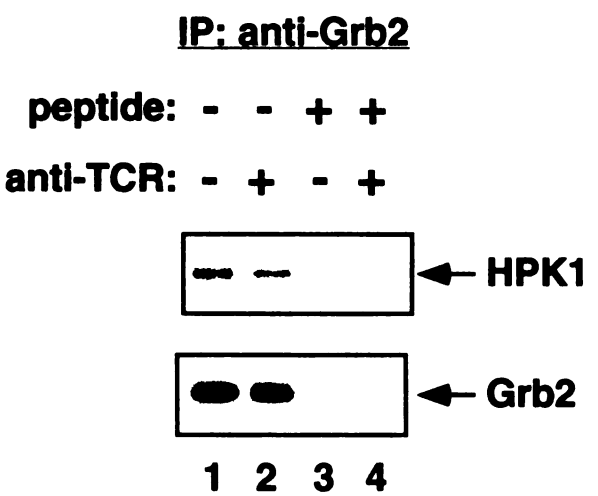
A


IP: anti-HA
HPK1:HA - +
106
81
47.5
35.3
28.2
Blot: anti-HA
Blot: anti-Grb2
S35 Labeling

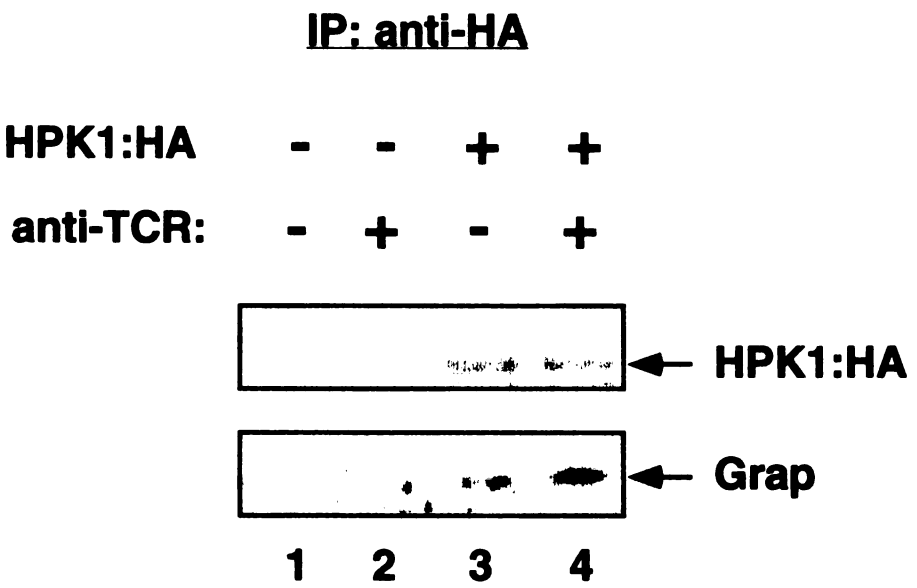
B


IP: anti-HA
HPK1:HA - - + +
anti-TCR: - + - +
HPK1:HA
Grb2
1 2 3 4

C


IP: anti-Grb2
peptide: - - + +
anti-TCR: - + - +
HPK1
Grb2
1 2 3 4

D



that the association of HPK1 with Grb2 is specific, a control immunoprecipitation was performed in the presence of an excess of the peptide epitope recognized by the anti-Grb2 antibody. Under these conditions, neither Grb2 nor HPK1 was detected in the immune complexes (Figure 3C, lanes 3 and 4). Similarly, a closely-related member of Grb2, Grap, was found to interact with HPK1 constitutively in T cells (Figure 3D). Taken together, these results demonstrate the constitutive association between Grb2 and HPK1 in T cells.

Activation of HPK1 by the BCR

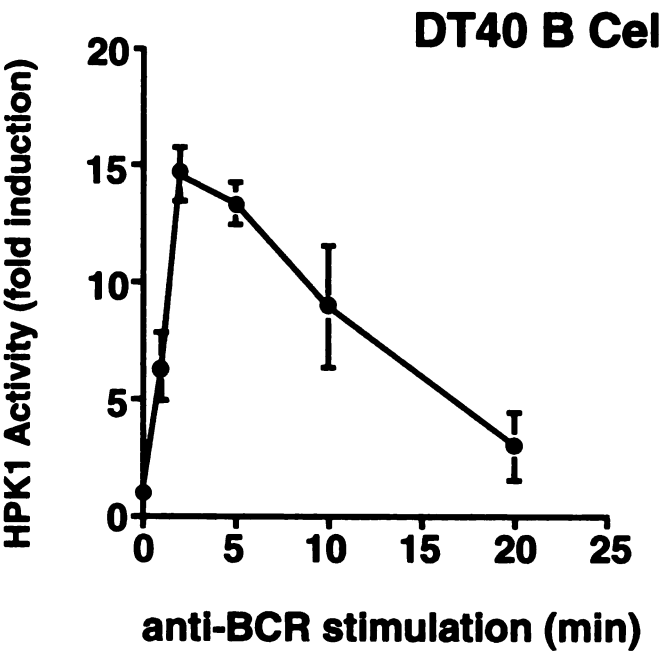
To further explore the role of Grb2 in HPK1 activation, we took advantage of a genetic system developed in the avian B cell line DT40. Many mutants of this cell line have been established by homologous recombination, including the Grb2-deficient DT40 line (Hashimoto et al., 1998). The signal transduction events following ligation of the TCR or the BCR are quite similar. Since HPK1 appears to be a downstream target in TCR signaling and is expressed in B cells (Kiefer et al., 1996), we examined whether BCR stimulation induces HPK1 activation in DT40 cells. As shown in Figure 4A, stimulation of the BCR evoked a potent induction of HPK1 kinase activity with kinetics similar to that with TCR stimulation. This demonstrated that both B and T lymphocyte antigen receptors can induce HPK1 activation upon stimulation.

The availability of many null mutants of the DT40 cell line allowed us to investigate the functional participation of multiple signaling effectors in BCR-mediated HPK1 activation. We first focused on DT40 mutants lacking protein tyrosine kinases, including Lyn, Syk, and Btk. We found that the level of HPK1 activation was partially

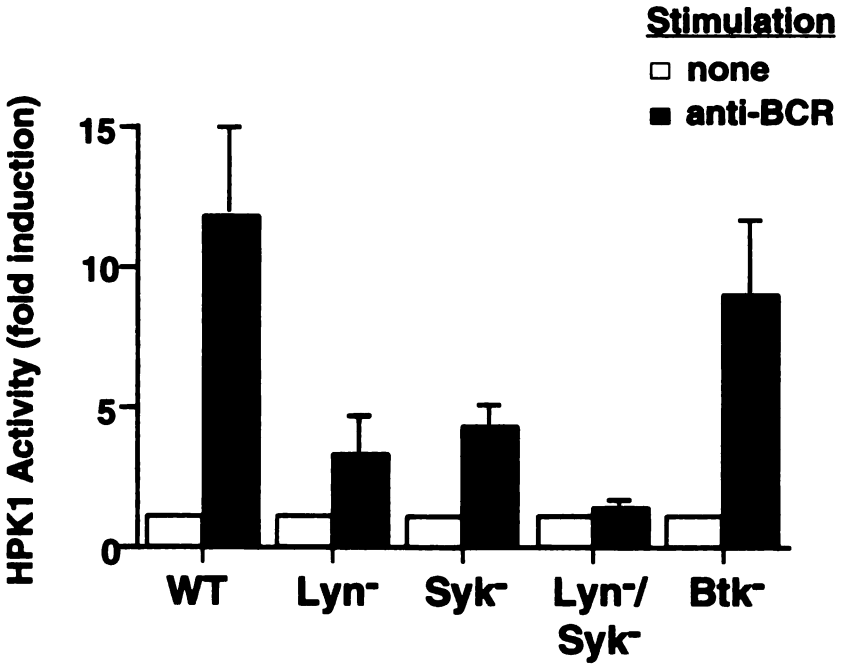
Figure 4. HPK1 Activation by the BCR.

(A) Time-course of BCR-mediated HPK1 activation. Wildtype DT40 B cells transfected with HPK1:HA were treated with anti-BCR MAb for 0-20 min as indicated. Anti-HA immune complexes were assayed for HPK1 kinase activity using histone H2A as the substrate. Shown is the fold induction of HPK1 activity measured by quantitating histone H2A phosphorylation relative to that with mock-stimulated cells. The average and standard error shown are representative of at least three independent experiments. **(B-E)** HPK1 activation in DT40 mutants. Various DT40 cells were transiently transfected with HPK1:HA, stimulated with anti-BCR MAb for 2 min, and HPK1 kinase assays were performed as described in **(A)**. The data are representative of a minimum of three independent experiments. Equivalent levels of HPK1 protein were immunoprecipitated in all kinase assays. BCR expression on the surface of various DT40 cell lines was comparable (data not shown).

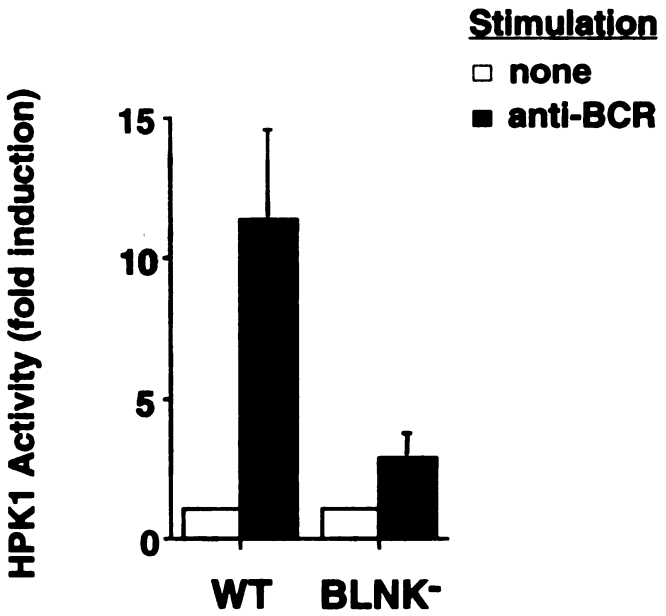
A


DT40 B Cells
HPK1 Activity (fold induction)
20
15
10
5
0
0
5
10
15
20
25
anti-BCR stimulation (min)

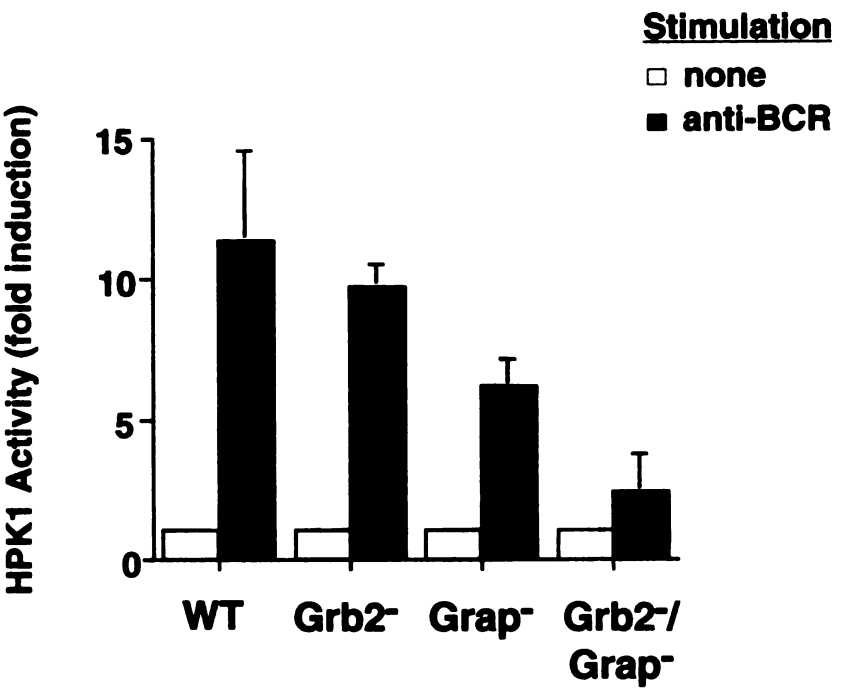
B


Stimulation
none
anti-BCR
HPK1 Activity (fold Induction)
15
10
5
0
WT
Lyn-
Syk-
Lyn-/ Syk-
Btk-

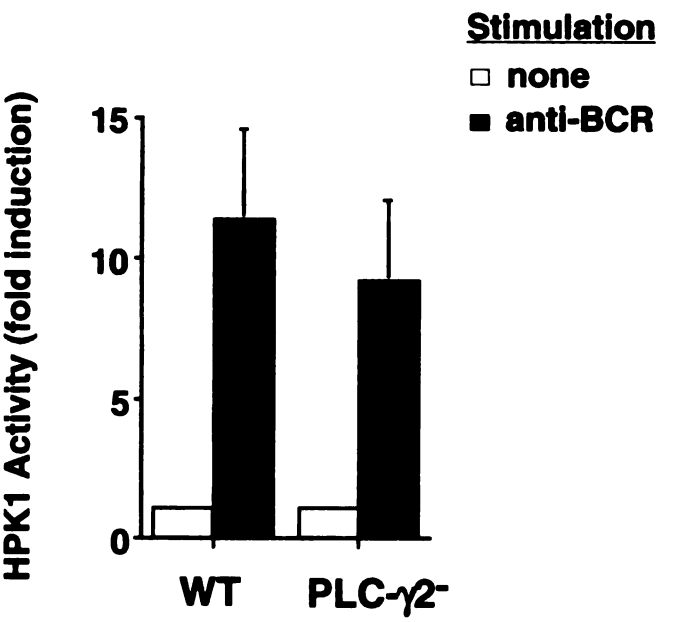
C


Stimulation
none
anti-BCR
HPK1 Activity (fold induction)
15
10
5
0
WT
BLNK-

D


Stimulation
none
anti-BCR
HPK1 Activity (fold induction)
15
10
5
0
WT
Grb2⁻
Grap⁻
Grb2⁻/
Grap⁻

E


Stimulation
none
anti-BCR
HPK1 Activity (fold Induction)
15
10
5
0
WT
PLC-γ2-

decreased in either Lyn- or Syk-deficient DT40 cells. Furthermore, HPK1 activation was completely abrogated in Lyn/Syk double-deficient DT40 cells (Figure 4B). Interestingly, BCR-mediated Btk activation is also partially impaired in Lyn and Syk single-deficient cells and is absent in Lyn/Syk double-deficient DT40 cells (Kurosaki and Kurosaki, 1997). Nevertheless, the activation of HPK1 in Btk-deficient DT40 cells was only slightly reduced when compared to wildtype cells (Figure 4B). These results indicate that Btk is not required for HPK1 activation, although both HPK1 and Btk utilize Src and Syk family kinases as upstream regulators. Together with the findings presented in Figure 2A, the data suggest that HPK1 activation by lymphocyte antigen receptors depends on Src and Syk family kinases but not Btk family kinases.

B cell linker protein (BLNK) is a SLP-76 homologue in B cells which, like SLP-76, links upstream tyrosine kinases to downstream signaling events. In addition, BLNK is capable of binding to Grb2 and other signaling effectors (Fu et al., 1998; Ishiai et al., 1999). Consistent with the results obtained with SLP-76-deficient T cells (Figure 2C), HPK1 activation was severely impaired by the loss of BLNK in DT40 cells (Figure 4C). These results demonstrate the importance of SLP-76-like adaptor molecules in HPK1 activation by B- and T-cell antigen receptors.

The Grb2 and Grap adaptor proteins share overall structural characteristics (Feng et al., 1996). Since they are both expressed in B and T lymphocytes, they may function redundantly in antigen receptor signal transduction. To test whether Grb2 or Grap is required for HPK1 activation, kinase assays were performed with Grb2-, Grap-, and Grb2/Grap double-deficient DT40 cells. Significant levels of HPK1 activation were still detected in Grb2 and Grap single-deficient cell lines; however, almost a complete

absence of HPK1 activation was observed in Grb2/Grap double-deficient cells (Figure 4D). These results indicate that both Grb2 and Grap participate and can play redundant roles in HPK1 activation.

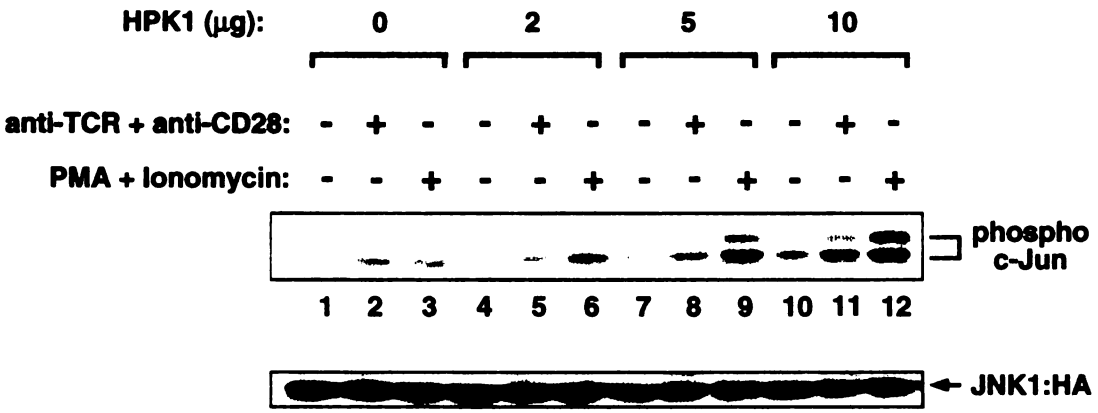
PLCγ2 is involved in the activation of the phosphatidylinositol pathway following BCR engagement. In PLCγ2-deficient DT40 cells, stimulation of the BCR failed to induce inositol 1,4,5-triphosphate (IP_3) production and calcium mobilization (Takata et al., 1995). We tested the activation of HPK1 by the BCR in PLCγ2-deficient DT40 cells. Normal levels of HPK1 catalytic activity were observed upon BCR stimulation in PLCγ2-deficient DT40 cells (Figure 4E). This result, together with the findings presented in Figure 2D, imply that calcium mobilization alone or in combination with the activation of PKC or Ras is neither necessary nor sufficient for HPK1 activation by antigen receptors.

Overexpression of HPK1 Activates JNK1 in T Cells

Previous reports have demonstrated that HPK1 activates JNK when HPK1 is ectopically expressed in fibroblasts (Hu et al., 1996; Kiefer et al., 1996). To examine whether HPK1 can activate JNK in T cells, we cotransfected a plasmid encoding JNK1 with the HPK1 expression vector into Jurkat cells. JNK1 activity was determined by the phosphorylation of the substrate GST-c-Jun. As shown in Figure 5, overexpression of HPK1 triggered the activation of JNK when expressed at a high dose (lane 10, Figure 5). Moreover, HPK1 further potentiated JNK activities induced by the costimulation of TCR and CD28 receptor, or PMA plus ionomycin (Figure 5). These results are consistent with the notion raised in previous studies that HPK1 is a potent activator for the JNK pathway.

Figure 5. Overexpression of HPK1 Activates JNK in T Cells.

Jurkat cells were cotransfected with 20 μg of HA-tagged JNK1 along with 0-10 μg of Myc-tagged HPK1 as indicated. Six hours later, cells were stimulated for 15 min with a buffer control (lanes 1, 4, 7 and 10), anti-TCR plus anti-CD28 antibodies (lanes 2, 5, 8, and 11), or PMA (50 ng/ml) plus ionomycin (1 μM) (lanes 3, 6, 9, and 12). JNK assays were performed on anti-HA immunoprecipitates using GST-c-Jun as the exogenous substrate. Phosphorylation of the substrate was measured by immunoblotting with an anti-phospho-c-Jun antibody (top panel). The amount of HA-tagged JNK1 protein immunoprecipitated was examined using an anti-JNK1 antibody (bottom panel).


HPK1 (μg):
0
2
5
10
anti-TCR + anti-CD28: - + - - + - - + - - + -
PMA + Ionomycin: - - + - - + - - + - - +
phospho c-Jun
1 2 3 4 5 6 7 8 9 10 11 12
JNK1:HA

HPK1 Suppresses AP-1 Activation by the TCR but Not by PMA

Downstream targets of JNK such as c-Jun and ATF2 are integral components of the AP-1 transcription factor complex (Karin et al., 1997). Since HPK1 potentiated JNK activation in T cells, it was of interest to test its effect on the AP-1 activity that is upregulated upon T cell activation. AP-1 transcription was monitored using an AP-1-driven luciferase reporter. Surprisingly, HPK1 inhibited AP-1 activation by the TCR in a dose-dependent manner (Figure 6A). We also used PMA, a potent PKC and Ras activator, to induce AP-1 activation. Although HPK1 suppressed AP-1 activation by the TCR, it exerted no effect on PMA-mediated AP-1 activation (Figure 6A). This selective inhibition of TCR but not PMA activation of AP-1 suggests that HPK1 either affects the TCR signaling pathway upstream of Ras or PKC activation or acts on a TCR-induced AP-1 activating pathway independent of Ras and PKC.

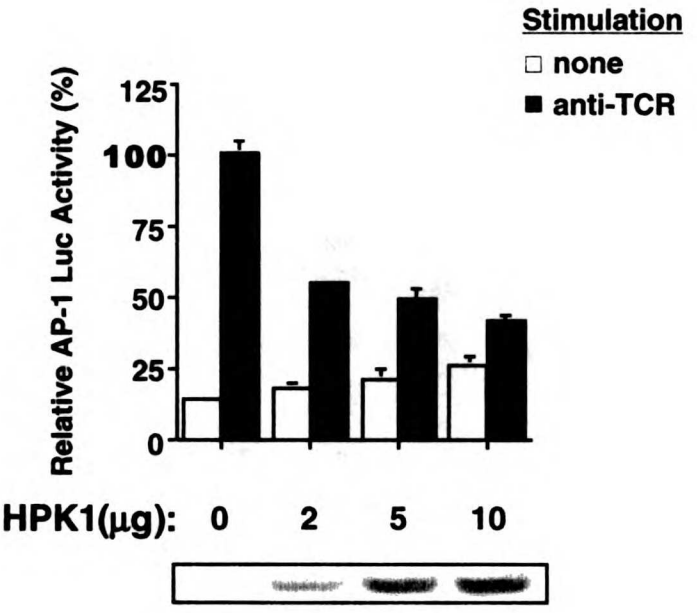
In contrast to the observations with wildtype HPK1, overexpression of the kinase-inactive mutant, HPK1(K46E), did not inhibit TCR-mediated AP-1 activation, but slightly potentiated it (Figure 6B). This result demonstrates that the kinase activity of HPK1 is essential for its negative regulatory effect on AP-1. Similar results were obtained with NFAT and REAP transcriptional reporters, which are dependent upon AP-1 binding, further confirming the inhibitory function of HPK1 on AP-1 (data not shown).

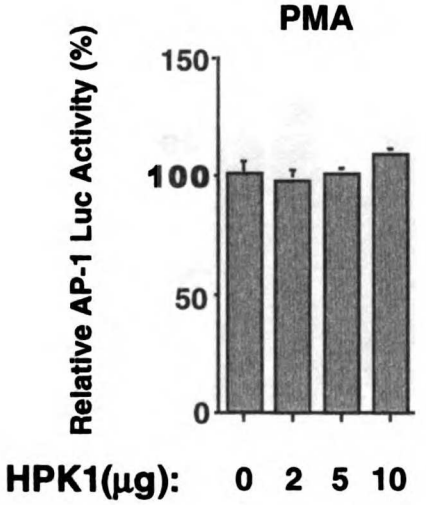
One possible explanation for this inhibitory effect is that persistent activation of JNK resulting from HPK1 overexpression could downregulate AP-1 through a negative feedback mechanism. To test this possibility, we expressed a constitutively-active form of JNK1, JNK1-MKK7 (Dang and Cobb, unpublished observation; Zheng et al., 1999), in Jurkat cells and assessed AP-1 activation. Unlike HPK1, JNK1-MKK7 upregulated both

Figure 6. HPK1 Selectively Inhibits TCR but not PMA Activation of AP-1.

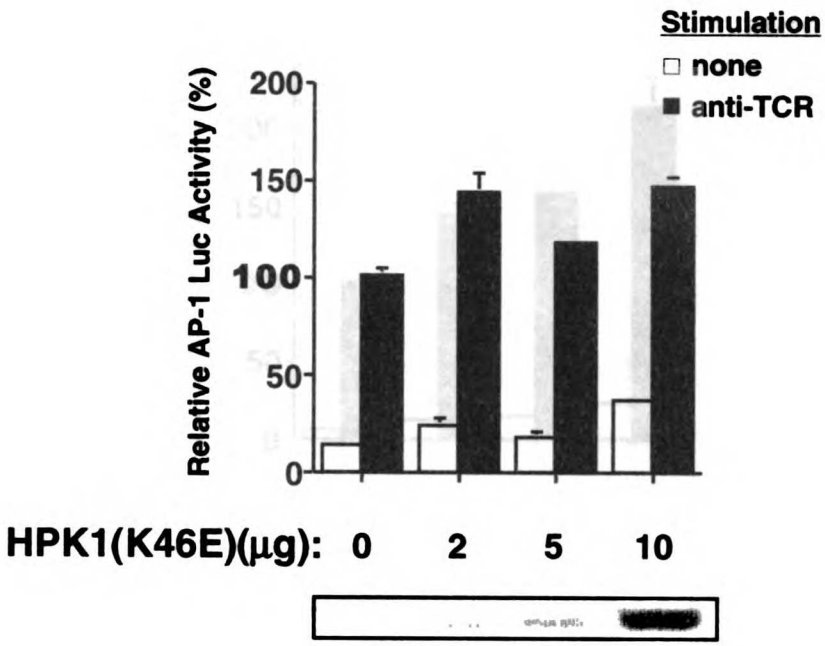
Jurkat cells were transfected with 0-10 μg of HPK1:HA (**A**), or HPK1(K46E):HA (**B**), or 0-25 μg of HA JNK1-MKK7 (**C**), as indicated together with 20 μg of AP-1 luciferase and 2 μg of β-galactosidase reporter constructs. Total DNA concentration was kept constant using empty vector. Sixteen hours after transfection, cells were stimulated with medium, anti-TCR MAb, or PMA for 6 hr. Cell lysates were then prepared and assayed for luciferase activity. β-galactosidase activity was used to normalize the differences in transfection efficiency. All data are presented as percentage induction of normalized luciferase activity obtained with the activating stimulus alone. The expression of HA-tagged proteins was examined by immunoblotting with anti-HA MAb. The data are representative from at least three independent experiments.

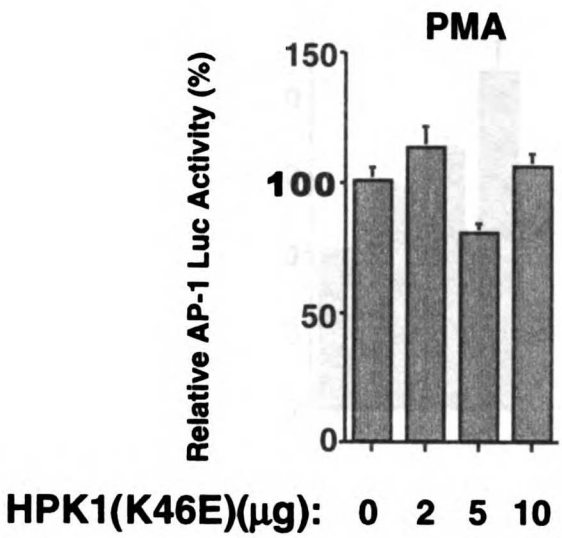
A


Stimulation
none
anti-TCR
Relative AP-1 Luc Activity (%)
125
100
75
50
25
0
HPK1(μg): 0 2 5 10

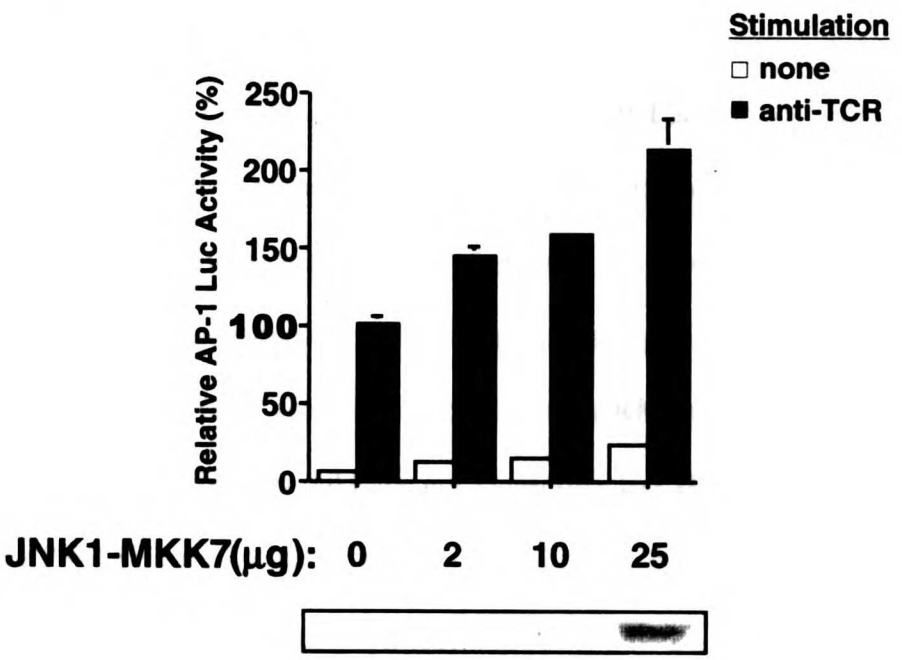

PMA
Relative AP-1 Luc Activity (%)
150
100
50
0
HPK1(μg): 0 2 5 10

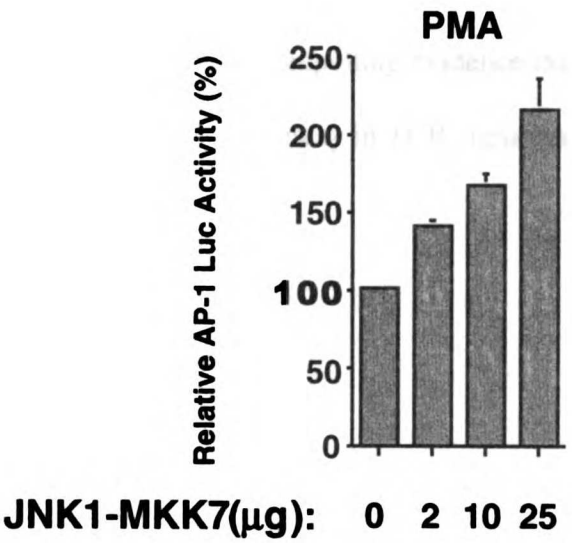
B


Stimulation
none
anti-TCR
Relative AP-1 Luc Activity (%)
200
150
100
50
0
HPK1(K46E)(μg): 0 2 5 10


PMA
Relative AP-1 Luc Activity (%)
150
100
50
0
HPK1(K46E)(μg): 0 2 5 10

C


Stimulation
none
anti-TCR
Relative AP-1 Luc Activity (%)
250
200
150
100
50
0
JNK1-MKK7(μg): 0 2 10 25


PMA
Relative AP-1 Luc Activity (%)
250
200
150
100
50
0
JNK1-MKK7(μg): 0 2 10 25

TCR and PMA activation of AP-1 (Figure 6C). These results suggested that HPK1 exerts an additional activity to inhibit AP-1, other than its effect on JNK activation.

HPK1 Inhibits ERK2 Activation by the TCR but Not by v-Ras

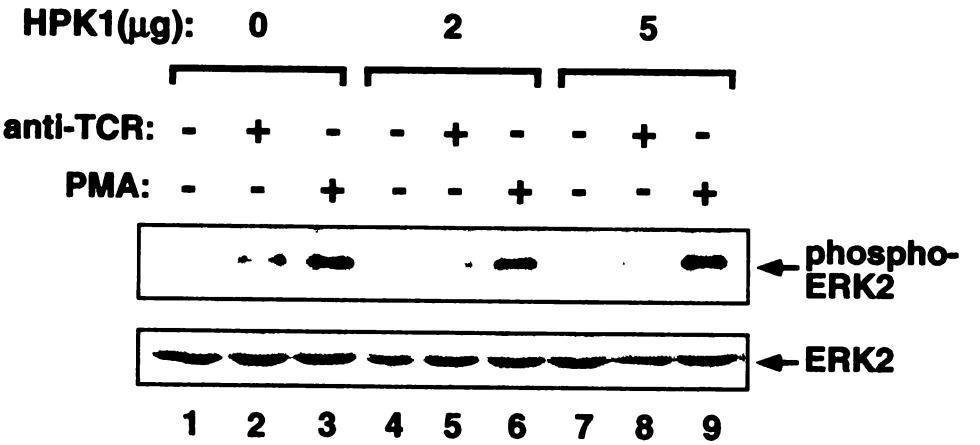
ERK, another MAP kinase capable of regulating AP-1 activity, is activated following TCR stimulation (Cantrell, 1996). We therefore examined the effect of HPK1 on ERK2 activation. Similar to the results obtained with AP-1 activation, TCR-mediated ERK2 phosphorylation was reduced in HPK1-overexpressing cells (Figure 7A). In contrast, kinase-inactive HPK1 significantly potentiated ERK2 phosphorylation induced by the TCR (Figure 7B). Neither wildtype nor kinase-inactive HPK1 affected ERK2 activation by PMA (Figure 7A and 7B). We also utilized an activated allele of Ras, v-Ha-Ras, which is mutated at codon 12 (Ser->Val) and 59 (Ala->Thr) resulting in a constitutively-active Ras, to activate ERK2. Activation of ERK2 by v-Ha-Ras was not affected by either wildtype or kinase-inactive HPK1 (Figure 7C). The data presented in Figure 6 and Figure 7 provide compelling evidence that HPK1 acts as a negative regulator of the ERK2/AP-1 pathway in TCR signal transduction.

Figure 7. HPK1 Suppresses ERK2 Activation by TCR Stimulation.

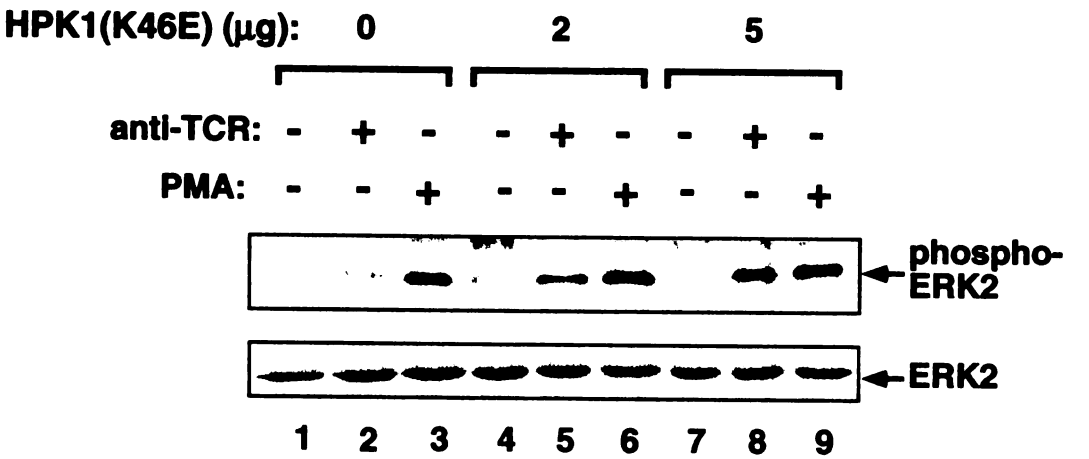
(A and B) Jurkat cells were transfected with 0-5 μg of plasmids encoding wildtype HPK1 **(A)**, or kinase-inactive HPK1(K46E) **(B)** as indicated together with 15 μg of expression vector encoding Myc-tagged ERK2. Six hours after transfection, cells were stimulated for 5 min with a buffer control (lanes 1, 4, and 7), anti-TCR MAb (lanes 2, 5, and 8), or PMA (50 ng/ml) (lanes 3, 6, and 9). ERK2 phosphorylation was assessed by anti-Myc immunoprecipitation followed by immunoblotting with an anti-phospho-ERK1/2 antibody (top panel). The expression level of Myc-tagged ERK2 protein was examined using anti-Myc MAb (bottom panel). These experiments were repeated at least three times with similar results.

(C) HPK1 does not affect ERK2 activation by v-Ras. Jurkat T cells were cotransfected with 15 μg of Myc-tagged ERK2 and 5 μg of HPK1:HA or HPK1(K46E) expression vectors with or without 10 μg of v-Ha-ras. Total DNA concentration was kept constant using empty vectors. Six hours later, cells were lysed and ERK2 phosphorylation was assessed as described above (top panel). The amount of Myc-tagged ERK2 protein immunoprecipitated was detected using an anti-ERK2 antibody (bottom panel).

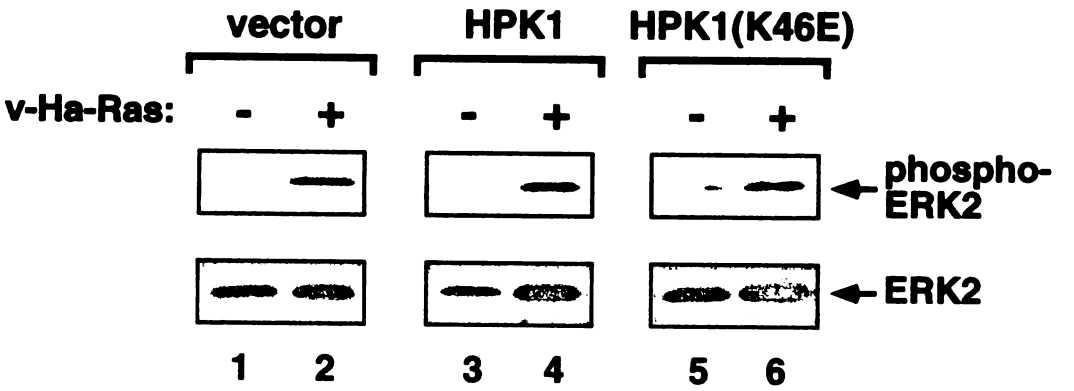
A


HPK1(μg): 0 2 5
anti-TCR: - + - - + - - + -
PMA: - - + - - + - - +
phospho-ERK2
ERK2
1 2 3 4 5 6 7 8 9

B


HPK1(K46E) (μg): 0 2 5
anti-TCR: - + - - + - - + -
PMA: - - + - - + - - +
phospho-ERK2
ERK2
1 2 3 4 5 6 7 8 9

C



Discussion

GCK family kinases (GCKs) are recently identified constituents of MAP kinase pathways and are therefore potential regulators for AP-1. The hematopoietic tissue-restricted expression pattern and the ability to interact with Grb2 led us to focus on the role of HPK1 in T cell signaling. Here, we show that HPK1 is activated following engagement of lymphocyte antigen receptors. We further demonstrate that HPK1 inhibits AP-1 and ERK2 activation by TCR stimulation. Hence, HPK1 is the first reported GCK family kinase that participates in antigen receptor signaling and functions as a negative regulator for the TCR-mediated AP-1 activation.

In this study, we have characterized a pathway, conserved in T and B lymphocytes, which relays signals from receptor crosslinking to the induction of HPK1 kinase activity. Previously, HPK1 has been shown to be activated by erythropoietin (Epo) (Nagata et al., 1999) and TGFβ (Zhou et al., 1999). However, the signaling pathway linking receptor stimulation to HPK1 activation was not defined. Using cell lines deficient in Src and Syk/ZAP-70 families of PTKs, we found that Lck and ZAP-70 are essential for HPK1 activation in T cells. Similarly, Lyn and Syk participate in HPK1 activation in B cells. Src and Syk/ZAP-70 PTKs may play a role by phosphorylating HPK1 directly or by activating upstream regulators of HPK1. Since we have not been able to detect tyrosine phosphorylation of HPK1 upon TCR stimulation (data not shown), it is likely that these tyrosine kinases may activate HPK1 indirectly.

Next, we identified inducibly tyrosine phosphorylated adaptor proteins LAT, SLP-76, and BLNK as critical components for antigen receptor-mediated HPK1

activation. Tyrosine phosphorylation of these three molecules provides docking sites for SH2 domain-containing proteins, thus permitting the phosphorylation and activation of downstream signaling events. Since LAT is a transmembrane protein, it also promotes relocalization of SH2 domain-containing proteins to the plasma membrane upon receptor ligation. However, HPK1 does not possess SH2 domains. One possibility is that LAT, SLP-76, and BLNK contribute to HPK1 activation indirectly by facilitating antigen receptor signaling. Another explanation is that these linker proteins are coupled to HPK1 by SH2/SH3 domain-containing proteins, thereby recruiting HPK1 to the receptor signaling complexes where it can be activated

Indeed, we found that the SH2/SH3 domain-containing proteins Grb2 and Grap are also involved in the signal transduction process leading to HPK1 activation. Grb2 was identified as the 28 kDa protein associated with HPK1 in metabolically-labeled T cells and interacted with HPK1 constitutively. These biochemical data were complemented by genetic data obtained with the avian DT40 cell line and its mutants. Although a normal level of HPK1 activation was observed in Grb2-deficient DT40 cells, this activation was greatly diminished in Grb2/Grap double-deficient cells. These results indicate that Grb2 and Grap play redundant roles in BCR-mediated HPK1 activation. Since Grb2 and Grap are each 217 amino acids long, it is of interest to determine whether the 28 kDa band in the ^{35}S labeling of Jurkat cells also contains Grap. Grb2 and Grap have been shown to associate with LAT upon TCR stimulation (Trüb et al., 1997). Therefore, they may function to couple HPK1 to tyrosine phosphorylated proteins in the activated receptor complex.

Based the above observations, we postulate the following mechanism of HPK1 activation in T cells. TCR ligation activates Lck resulting in the activation of ZAP-70. ZAP-70 in turn phosphorylates LAT, which recruits Grb2-HPK1 and/or Grap-HPK1 complexes to the TCR signal transduction machinery. SLP-76 may facilitate signaling events within the machinery, thereby participating in HPK1 activation. It is possible that HPK1 is activated either by membrane localization to the proximity of an activator, or by oligomerization leading to trans-autophosphorylation. A similar mechanism appears to be employed by the BCR to activate HPK1.

Other mechanisms may also play a role in HPK1 regulation. Recently, a novel domain designated as citron-homology (CNH) domain has been identified in the C-terminal regulatory regions of GCKs (Schultz et al., 1998). Interestingly, the CNH domain is also present in several proteins involved in the signaling of the small G protein Rho. These proteins include citron kinase, a target of Rho (Madaule et al., 1998), and ROM1/2 , two GEFs that activate Rho1 in yeast (Ozaki et al., 1996). The presence of CNH domains in GCKs suggests that there may exist a crosstalk between GCKs and Rho GTPases. The role of the CNH domain in regulating HPK1 kinase activity and mediating downstream effects of HPK1 remains to be investigated. It is likely that the CNH domain is important for HPK1 activation since we failed to detect TCR induction of HPK1 kinase activity in an immune complex kinase assay using an antibody raised against an epitope within the CNH domain of HPK1 (data not shown). Future experiments such as the identification of the regulatory domains within HPK1 will hopefully address the detailed mechanism of HPK1 activation.

In addition to HPK1, other GCKs including GCK and GCKR are also expressed in lymphocytes. It is not known whether lymphocyte antigen receptors can activate GCK family kinases other than HPK1. GCK and GCKR have been shown to be activated by TNFα (Pombo et al., 1995; Shi and Kehrl, 1997); however, HPK1 does not respond to TNFα stimulation (data not shown; Hu et al., 1996). These results suggest that GCK family kinases may be differentially regulated by various receptors.

Ectopic expression of HPK1 in fibroblasts was shown to activate JNK (Hu et al., 1996; Kiefer et al., 1996). Although we observed the JNK stimulatory function of HPK1 in T cells, the kinase-inactive (K46E) or a kinase domain-deleted mutant of HPK1 failed to block JNK activation following TCR plus CD28 or PMA plus ionomycin stimulation (data not shown). These results indicate that either these two forms of HPK1 can not function as dominant-negative mutants for the JNK pathway, or that HPK1 is not involved in JNK activation upon T cell stimulation. Notably, the combination of ionomycin and PMA failed to activate HPK1, but potently activated JNK in T cells. Moreover, since stimulation of the TCR is sufficient to activate HPK1, but JNK activation is not activated by TCR stimulation alone, it is clear that at least some other signaling pathways are required to activate JNK in T cells.

Surprisingly, we found that HPK1 inhibited AP-1 and ERK2 activation upon TCR stimulation. One group recently reported that overexpression of two mutant forms of HPK1 resulted in a reduction of the IL-2 promoter activity in Jurkat cells. However, the upstream and downstream pathways of HPK1 in T cell signaling were not clearly defined (Ling et al., 1999). In this report, we demonstrated a signaling pathway mediating HPK1 activation by the T-cell antigen receptor. In addition, we examined the effect of HPK1

on TCR activation of AP-1 as well as ERK and JNK MAP kinases. We observed a dose-dependent inhibition of TCR-mediated AP-1 activation by wildtype HPK1. Whereas a kinase-inactive mutant of HPK1 not only failed to suppress AP-1 activation, but even exerted a stimulatory effect, perhaps due to the competition for substrates with endogenous HPK1 in T cells. Importantly, similar effects of HPK1 were observed with TCR-mediated ERK2 activation, suggesting that HPK1 may inhibit AP-1 through its effect on the ERK pathway. This inhibition seems to occur upstream or independent of PKC and Ras, since neither form of HPK1 affected PMA or v-Ras activation of AP-1 and ERK2. Such inhibition is unlikely to be the result of competition with SOS for available Grb2, because the SH3 domains of Grb2 should also interact with the HPK1 kinase-inactive mutant, yet this mutant potentiates ERK and AP-1 activation. Moreover, the Grb2/SOS complex may not represent the predominant means for antigen receptor-mediated Ras activation in lymphocytes. Instead, PLCγ and Ras GRP may play more important roles (Hashimoto et al., 1998; Ebinu et al., 1998; Yablonski et al., 1998). Taken together, it appears that the negative regulatory effect of HPK1 is associated with its catalytic activity.

One interpretation for the negative regulatory effect of HPK1 is that it may phosphorylate and thus downregulate the function of its target(s) in the TCR signal transduction pathway. The phosphorylation of SOS on serine and threonine residues has been shown to be a possible mechanism to terminate Ras signaling. In fibroblasts, this phosphorylation dissociates SOS from Grb2 and is dependent on MEK (Holt et al., 1996; Langlois et al., 1995; Waters et al., 1995). However, TCR-induced phosphorylation of SOS neither requires the activation of MEK/ERK pathway nor affects the association

between SOS and Grb2 (Zhao et al., 1997). Instead, this phosphorylation may modulate the ability of the Grb2/SOS complex to interact with LAT, thereby downregulating the Ras activation pathway (Buday et al., 1995). It will be of interest to test whether HPK1 is involved in the phosphorylation of SOS upon T cell stimulation and consequently suppresses ERK and AP-1 activation.

Alternatively, HPK1 may exert its function by activating inhibitory pathways for AP-1 and ERK2 activation. Recent data reveal a negative regulatory pathway for Ras activation in T cells involving the small G protein Rap1 (Boussiotis et al., 1997). Upon TCR stimulation, tyrosine phosphorylation of Cbl promotes its association with CrkL, an adaptor protein that complexes with the C3G guanine nucleotide exchange factor for Rap1. Activated Rap1 then antagonizes the function of Ras possibly through its ability to sequester Ras effectors (Boussiotis et al., 1997; Buday et al., 1996; Reedquist et al., 1996). HPK1 has been shown to interact with CrkL (Ling et al., 1999; Oehrl et al., 1998); however, it is not clear if this interaction contributes to the activation of Rap1. In addition, dual-specificity phosphatases such as MKP-1 and hematopoietic protein tyrosine phosphatase (HePTP) have been shown to dephosphorylate and inactivate ERK (Bokemeyer et al., 1996; Saxena et al., 1999). Since HPK1 specifically affected TCR but not PMA or v-Ras activation of ERK2, it is unlikely that HPK1 functions through these two phosphatases. Further investigations are required to identify the target(s) of HPK1 in the ERK2/AP-1 activation pathway.

The AP-1 pathway is specifically blocked in anergic T cells (Kang et al., 1992). Since HPK1 is activated by the TCR and negatively regulates AP-1 signaling in T cells, it is possible that HPK1 plays a role in the induction and/or the maintenance of the status of

T cell anergy. Antagonizing signals such as HPK1 activated by the TCR could orchestrate with positive signals to determine the magnitude and the outcome of TCR signaling. Therefore, it will be of interest to examine the kinase activity and expression of HPK1 in T cells at various stages of development and differentiation. Finally, generation of HPK1-deficient cell lines and animals should complement our study to understand the functions of HPK1.

Experimental Procedures

Reagents

The antibodies used for the stimulation of T cells were C305 (anti-Jurkat Ti β chain MAb) (Weiss and Stobo, 1984), 145-2C11 (anti-mouse CD3 ε chain MAb) (ATCC), and mouse MAb to human CD28 (CALTAG). M4 (anti-chicken B-cell receptor MAb) was a gift from M. Cooper and C. L. Chen, University of Alabama, Birmingham, AL. 12CA5 (anti-HA epitope MAb) was from Boehringer Mannheim. 9E10 (anti-Myc epitope MAb) was a gift from J. M. Bishop, UCSF, CA. 4G10 (anti-phosphotyrosine MAb) was from Upstate Biotechnology. Anti-Grb2 and anti-Grap antibodies were from Santa Cruz Biotechnology. Anti-phospho-ERK1/2 antibody was from New England Biolabs. Anti-ERK2 antibody was from Zymed. Anti-mouse HPK1 antibodies were previously described (Kiefer et al., 1996). pcdef3-HPK1:HA and pcdef3-HPK1(K46E):HA were constructed by subcloning HindIII-ScaI fragments containing HPK1 cDNA from pMT2-HPK1 and pMT2-HPK1(K46E):HA (Kiefer et al., 1996) into pcdef3 vector. pEF-BOS-HPK1-Myc was kindly provided by D. Qian, Sugen Inc., CA. The Myc-tagged ERK2 expression construct has been previously described (Qian et al., 1996). pSRα-HA-JNK1 and the LacZ reporter construct driven by the β-actin promoter (pRC-β-actin-LacZ) were gifts from M. Karin (University of California, San Diego, CA). pCEP4HA JNK1-MKK7 was constructed by A. Dang, and will be described in detail elsewhere. pEF v-Ha-ras was a gift from D. Cantrell, Imperial Cancer Research Fund, England. The 4XAP-1 luc reporter plasmid was described previously (Shapiro et al., 1996).

Cell Culture and Transfection

The Jurkat T cell line E6-1 (Weiss et al., 1984) and the signaling mutant derivatives J.CaM1 (Goldsmith et al., 1988), P116 (Williams et al., 1998), J.CaM2 (Goldsmith et al., 1988), J.CaM2/LAT (Finco et al., 1998), J14, and J14-76 (Yablonski et al., 1998) were maintained in RMPI 1640 supplemented with 5% fetal calf serum (FCS), penicillin, streptomycin and glutamine. DT40 cell lines deficient for Lyn (Takata et al., 1994), Syk (Takata et al., 1994), Lyn/Syk (Takata and Kurosaki, 1996), BTK (Takata and Kurosaki, 1996), BLNK (Ishiai et al., 1999), Grb2 (Hashimoto et al., 1998), Grap (Hashimoto et al., 1998), Grb2/Grap (generated by A. Hashimoto and will be described in detail elsewhere), and PLCγ2 (Takata et al., 1995) were maintained in the medium described above supplemented with 1% chicken serum. Transient transfections of Jurkat cells were performed by electroporating 2 x 10^7 cells resuspended in 400 µl serum-free RPMI 1640 medium with the indicated amount of DNA in a 0.4 cm cuvette using the Gene Pulser (Bio-Rad Laboratories) at a setting of 250 V and 960 µF. For DT40 transfections, 2 x 10^7 cells in 450 µl serum-free RPMI 1640 were electroporated at 350 V, 500 µF.

Cell Stimulation, Preparation of Lysates, Immunoprecipitation, and Immunoblotting

Cells were washed once with phosphate-buffered saline (PBS), then incubated at 37°C for 15 min. Subsequently, cells were either mock-stimulated with PBS (buffer control), or were stimulated for the time indicated with various antibodies, 4 β-phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), or ionomycin (1 µM). Cells were lysed in cold lysis buffer (1% Nonidet P-40, 10 mM Tris pH 7.6, 150 mM NaCl) supplemented with protease and

phosphatase inhibitors (10mM NaF, 2mM Sodium orthovanadate, 0.5mM EDTA, 2mM PMSF, 10μg/ml Aprotinin, 10μg/ml Leupeptin, and 1μg/ml Pepstatin) on ice for 20 min. Precleared lysates were incubated with antibody-precoupled Protein A- or G-sepharose beads for 2 hr at 4°C. The immunoprecipitates were then washed 4 times with lysis buffer, resuspended in SDS sample buffer, and heated at 95°C for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon-P membrane (Millipore), and probed with primary and secondary antibodies followed by enhanced chemiluminescence (ECL) detection (Amersham).

Immune Complex Kinase Assays

For HPK1 kinase assays, 5 x 10^6 Jurkat or DT40 cells transfected with 2 μg of pcdef3-HPK1:HA, or 16 x 10^6 purified mouse lymph node T cells were stimulated with anti-TCR, anti-BCR, or anti-CD3 MAb respectively, then lysed in cold lysis buffer A (Kiefer et al., 1996). Precleared lysates were immunoprecipitated with anti-HA or anti-mouse HPK1 antibody precoupled to Protein A-sepharose beads. Immunoprecipitates were assayed for kinase activities as described previously (Kiefer et al., 1996) with the addition of 5 μg of histone H2A as the exogenous substrate. Kinase reaction products were separated by SDS-PAGE, transferred to Immobilon-P membrane, and visualized by autoradiography and phosphorimaging. For JNK assays, Jurkat cells were cotransfected with 20 μg of pSRα-HA-JNK1 and 10 μg of other indicated plasmids. Six hours after transfection, 7 x 10^6 cells were stimulated for 15 min with PBS, a combination of anti-TCR and anti-CD28 antibodies, or a combination of PMA (50 ng/ml) and ionomycin (1

μM). Non-radioactive JNK assays were performed according to the instruction manual of the manufacturer (New England Biolabs).

Metabolic Labeling with [^{35}S]-Methionine/Cysteine

Cells were washed twice with methionine- and cysteine-free RPMI 1640 (ICN), then starved 30 min in the same medium supplemented with 5% dialyzed FCS. [^{35}S]-methionine/cysteine (Tran^{35}S-label; ICN) was added at 125 μCi/ml. After incubation at 37°C for 2 hr, cells were washed twice with PBS, stimulated with anti-TCR MAb for 1 min, and lysed. Precleared lysates were immunoprecipitated with preformed antibody complexes containing anti-HA MAb at 4°C for 2 hr. Washed immunoprecipitates were analyzed by SDS-PAGE, transferred to Immobilon-P membrane, and visualized by autoradiography.

Luciferase Assays

Jurkat cells were transiently cotransfected with 20 μg of AP-1 luciferase reporter plasmid, 2 μg of β-galactosidase reporter construct, and the plasmids indicated. Sixteen to twenty hours after transfection, cells were harvested and stimulated with medium, anti-TCR MAb, or PMA (50 ng/ml). After 6 hr, cells were lysed and assayed for luciferase activity as previously described (Shapiro et al., 1996). β-galactosidase activity was measured using the Galacto-Light Reporter Gene Assay System (TROPIX).

Acknowledgments

We thank D. Qian for providing the Myc-tagged human HPK1 construct. We thank J. Baker, M. Tomlinson, and J. Lin for critically reading of this manuscript, and the members of the Weiss laboratory for helpful discussions. This work was supported by a grant from National Cancer Institute (RO1 CA72531).

References

Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N., and Pawson, T. (1997). SH2/SH3 adaptor proteins can link tyrosine kinases to a Ste20-related protein kinase, HPK1. Journal of Biological Chemistry *272*, 27804-11.

Bokemeyer, D., Sorokin, A., Yan, M., Ahn, N. G., Templeton, D. J., and Dunn, M. J. (1996). Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not by extracellular signal-regulated kinase in fibroblasts. Journal of Biological Chemistry *271*, 639-42.

Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L., and Nadler, L. M. (1997). Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. Science *278*, 124-8.

Buday, L., Khwaja, A., Sipeki, S., Faragó, A., and Downward, J. (1996). Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. Journal of Biological Chemistry *271*, 6159-63.

Buday, L., Warne, P. H., and Downward, J. (1995). Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos. Oncogene *11*, 1327-31.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annual Review of Immunology *14*, 259-74.

Clements, J. L., Boerth, N. J., Lee, J. R., and Koretzky, G. A. (1999). Integration of T cell receptor-dependent signaling pathways by adapter proteins. Annual Review of Immunology *17*, 89-108.

Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Keesler, G., Zukowski, M., Tan, T. H., and Yao, Z. (1997). Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. Proc Natl Acad Sci U S A *94*, 9687-92.

Feng, G. S., Ouyang, Y. B., Hu, D. P., Shi, Z. Q., Gentz, R., and Ni, J. (1996). Grap is a novel SH3-SH2-SH3 adaptor protein that couples tyrosine kinases to the Ras pathway. Journal of Biological Chemistry *271*, 12129-32.

Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998). LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity *9*, 617-26.

Fu, C., Turck, C. W., Kurosaki, T., and Chan, A. C. (1998). BLNK: a central linker protein in B cell activation. Immunity *9*, 93-103.

Goldsmith, M. A., Dazin, P. F., and Weiss, A. (1988). At least two non-antigen-binding molecules are required for signal transduction by the T-cell antigen receptor. Proceedings of the National Academy of Sciences of the United States of America *85*, 8613-7.

Hashimoto, A., Okada, H., Jiang, A., Kurosaki, M., Greenberg, S., Clark, E. A., and Kurosaki, T. (1998). Involvement of guanosine triphosphatases and phospholipase C-gamma2 in extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. Journal of Experimental Medicine *188*, 1287-95.

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more. Cell *80*, 187-97.

Holt, K. H., Kasson, B. G., and Pessin, J. E. (1996). Insulin stimulation of a MEK-dependent but ERK-independent SOS protein kinase. Molecular and Cellular Biology *16*, 577-83.

Hu, M. C., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T. H. (1996). Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes and Development *10*, 2251-64.

Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A. C., and Kurosaki, T. (1999). BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. Immunity *10*, 117-25.

Jain, J., Loh, C., and Rao, A. (1995). Transcriptional regulation of the IL-2 gene. Current Opinion in Immunology *7*, 333-42.

Kang, S. M., Beverly, B., Tran, A. C., Brorson, K., Schwartz, R. H., and Lenardo, M. J. (1992). Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science *257*, 1134-8.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. Journal of Biological Chemistry *270*, 16483-6.

Karin, M., Liu, Z., and Zandi, E. (1997). AP-1 function and regulation. Current Opinion in Cell Biology *9*, 240-6.

Katz, P., Whalen, G., and Kehrl, J. H. (1994). Differential expression of a novel protein kinase in human B lymphocytes. Preferential localization in the germinal center. Journal of Biological Chemistry *269*, 16802-9.

Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996). HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. Embo Journal *15*, 7013-25.

Kurosaki, T., and Kurosaki, M. (1997). Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. Journal of Biological Chemistry *272*, 15595-8.

Kyriakis, J. M. (1999). Signaling by the germinal center kinase family of protein kinases. Journal of Biological Chemistry *274*, 5259-62.

Langlois, W. J., Sasaoka, T., Saltiel, A. R., and Olefsky, J. M. (1995). Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated p21ras activation. Journal of Biological Chemistry *270*, 25320-3.

Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrl, W., Feller, S. M., and Tan, T. H. (1999). Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. Molecular and Cellular Biology *19*, 1359-68.

Madaule, P., Eda, M., Watanabe, N., Fujisawa, K., Matsuoka, T., Bito, H., Ishizaki, T., and Narumiya, S. (1998). Role of citron kinase as a target of the small GTPase Rho in cytokinesis. Nature *394*, 491-4.

Nagata, Y., Kiefer, F., Watanabe, T., and Todokoro, K. (1999). Activation of hematopoietic progenitor kinase-1 by erythropoietin. Blood *93*, 3347-54.

Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998). The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene *17*, 1893-901.

Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993). A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell *73*, 179-91.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y., and Takai, Y. (1996). Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in Saccharomyces cerevisiae. Embo Journal *15*, 2196-207.

Pombo, C. M., Kehrl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T., and Kyriakis, J. M. (1995). Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. Nature *377*, 750-4.

Qian, D., Mollenauer, M. N., and Weiss, A. (1996). Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. Journal of Experimental Medicine *183*, 611-20.

Reedquist, K. A., Fukazawa, T., Panchamoorthy, G., Langdon, W. Y., Shoelson, S. E., Druker, B. J., and Band, H. (1996). Stimulation through the T cell receptor induces Cbl association with Crk proteins and the guanine nucleotide exchange protein C3G. Journal of Biological Chemistry *271*, 8435-42.

Saxena, M., Williams, S., Brockdorff, J., Gilman, J., and Mustelin, T. (1999). Inhibition of T cell signaling by mitogen-activated protein kinase-targeted hematopoietic tyrosine phosphatase (HePTP). Journal of Biological Chemistry *274*, 11693-700.

Schneider, H., Cai, Y. C., Prasad, K. V., Shoelson, S. E., and Rudd, C. E. (1995). T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. European Journal of Immunology *25*, 1044-50.

Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America *95*, 5857-64.

Shapiro, V. S., Mollenauer, M. N., Greene, W. C., and Weiss, A. (1996). c-rel regulation of IL-2 gene expression may be mediated through activation of AP-1. Journal of Experimental Medicine *184*, 1663-9.

Shi, C. S., and Kehrl, J. H. (1997). Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF-kappaB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. J Biol Chem *272*, 32102-7.

Simon, M. A., Dodson, G. S., and Rubin, G. M. (1993). An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. Cell *73*, 169-77.

Straus, D. B., and Weiss, A. (1992). Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell *70*, 585-93.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727-36.

Su, Y. C., Han, J., Xu, S., Cobb, M., and Skolnik, E. Y. (1997). NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. Embo Journal *16*, 1279-90.

Su, Y. C., Treisman, J. E., and Skolnik, E. Y. (1998). The Drosophila Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. Genes and Development *12*, 2371-80.

Takata, M., Homma, Y., and Kurosaki, T. (1995). Requirement of phospholipase C-gamma 2 activation in surface immunoglobulin M-induced B cell apoptosis [see comments]. Journal of Experimental Medicine *182*, 907-14.

Takata, M., and Kurosaki, T. (1996). A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. Journal of Experimental Medicine *184*, 31-40.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. Embo Journal *13*, 1341-9.

Trüb, T., Frantz, J. D., Miyazaki, M., Band, H., and Shoelson, S. E. (1997). The role of a lymphoid-restricted, Grb2-like SH3-SH2-SH3 protein in T cell receptor signaling. Journal of Biological Chemistry *272*, 894-902.

Tung, R. M., and Blenis, J. (1997). A novel human SPS1/STE20 homologue, KHS, activates Jun N-terminal kinase. Oncogene *14*, 653-9.

van Leeuwen, J. E., and Samelson, L. E. (1999). T cell antigen-receptor signal transduction. Current Opinion in Immunology *11*, 242-8.

Waters, S. B., Holt, K. H., Ross, S. E., Syu, L. J., Guan, K. L., Saltiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995). Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. Journal of Biological Chemistry *270*, 20883-6.

Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263-74.

Weiss, A., and Stobo, J. D. (1984). Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. Journal of Experimental Medicine *160*, 1284-99.

Weiss, A., Wiskocil, R. L., and Stobo, J. D. (1984). The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. Journal of Immunology *133*, 123-8.

Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998). Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. Molecular and Cellular Biology *18*, 1388-99.

Yablonski, D., Kuhne, M. R., Kadlecek, T., and Weiss, A. (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. Science *281*, 413-6.

Yao, Z., Zhou, G., Wang, X. S., Brown, A., Diener, K., Gan, H., and Tan, T. H. (1999). A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem *274*, 2118-25.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell *92*, 83-92.

Zhao, H., Li, Y. Y., Fucini, R. V., Ross, S. E., Pessin, J. E., and Koretzky, G. A. (1997). T cell receptor-induced phosphorylation of Sos requires activity of CD45, Lck, and protein kinase C, but not ERK. Journal of Biological Chemistry *272*, 21625-34.

Zheng, C., Xiang, J., Hunter, T., and Lin, A. (1999). The JNKK2-JNK1 fusion protein acts as a constitutively active c-Jun kinase that stimulates c-Jun transcription activity. Journal of Biological Chemistry *274*, 28966-71.

Zhou, G., Lee, S. C., Yao, Z., and Tan, T. H. (1999). Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. Journal of Biological Chemistry *274*, 13133-8.

CHAPTER 3

HPK1 IS REGULATED BY ITS INDUCIBLE ASSOCIATION WITH THE ADAPTOR PROTEIN SLP-76 IN T LYMPHOCYTES

Summary

Optimal HPK1 activation by the TCR is dependent on the presence of the adaptor protein SLP-76. However, the mechanism by which SLP-76 contributes to this process is unknown. Using a Syk-driven yeast two-hybrid approach, Karsten Sauer from Roger Perlmutter's group at Merck Research Laboratories isolated HPK1 as an interacting protein of the SH2 domain of BLNK, a B cell homolog of SLP-76. Additionally, he identified the tyrosine residue Y379 of murine HPK1 to be responsible for this association. Based on his observations, I tested the interaction between HPK1 and SLP-76 in T lymphocytes. Here, I demonstrate that HPK1 was inducibly associated with SLP-76 following TCR stimulation. This interaction was required for optimal HPK1 activation since the mutation of Y379 to phenylalanine, similar to the lack of SLP-76, resulted in a marked reduction of HPK activation by the TCR. Examination of HPK1 activation in SLP-76 deficient cells reconstituted with various SLP-76 mutants revealed that, both the SH2 domain and the Gads-binding motifs of SLP-76 are required for HPK1 activation. Thus, SLP-76 contributes to HPK1 activation by interacting with HPK1 following TCR stimulation. Results presented in this chapter will be combined with Karsten Sauer's work, and submitted for publication.

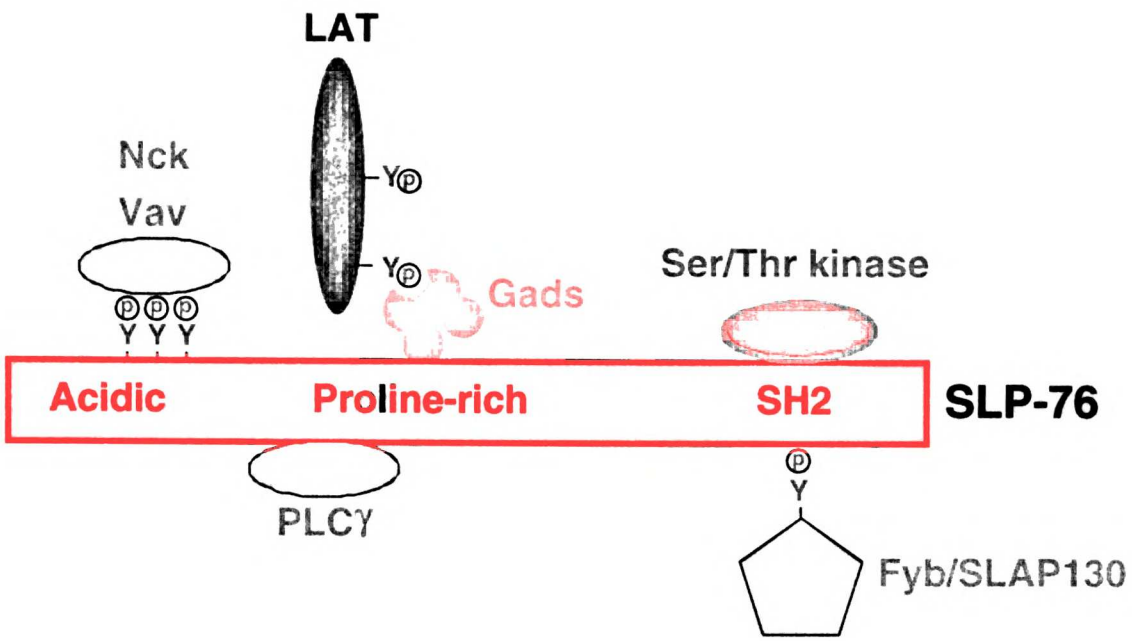
Introduction

In chapter 2, I demonstrated that HPK1 activation by the TCR requires the protein tyrosine kinases Lck and ZAP-70 as well as the adaptor proteins LAT and SLP-76. I also found that HPK1 is constitutively present in a complex with Grb2. From these results, a simple model of HPK1 activation can be proposed as follows: activation of Lck and ZAP-70 following TCR stimulation leads to the tyrosine phosphorylation of LAT. The Grb2/HPK1 complex can then be recruited to LAT, where it can be activated by oligomerization, or binding to an activator. However, this model fails to explain the requirement of SLP-76 for HPK1 activation. In B cells, the SLP-76 homolog BLNK is also necessary for HPK1 activation by the BCR. The mechanisms by which SLP-76-like proteins participate in HPK1 activation remain to be tested.

SLP-76 and BLNK each consist of an N-terminal acidic domain, a central proline-rich region, and a C-terminal SH2 domain (Jackman et al., 1995). Many signaling proteins have been shown to bind to SLP-76 in T cells (Figure 1). The Nck adaptor protein and Vav, an exchange factor for Rho-family GTPases, inducibly associate with tyrosine residues in the acidic domain (Fang and Koretzky, 1999; Wunderlich et al., 1999). The SH3 domain of PLC-γ1 binds to residues contained within 157-223, designated the P-1 domain, in the proline-rich region of SLP-76 (Yablonski et al, unpublished results). Moreover, the adaptor protein Gads binds to residues 224-244 of SLP-76, and mediates the recruitment of SLP-76 to LAT following TCR stimulation (Liu et al., 1999). Finally, the C-terminal SH2 domain interacts with the adaptor protein

Figure 1: SLP-76 binds to multiple signaling proteins in T cells

The adaptor protein SLP-76 contains an N-terminal acidic domain, a central proline-rich region, and a C-terminal SH2 domain. Many signaling proteins have been shown to bind to SLP-76 in T cells. The Nck adaptor protein and Vav, an exchange factor for Rho-family GTPases, inducibly associate with tyrosine residues in the acidic domain. The SH3 domain of PLC-γ1 binds to residues contained within 157-223, designated the P-1 domain, in the proline-rich region of SLP-76. Moreover, the adaptor protein Gads binds to residues 224-244 of SLP-76, and mediates the recruitment of SLP-76 to LAT following TCR stimulation. Finally, the C-terminal SH2 domain interacts with the adaptor protein SLAP-130/Fyb, and a putative 100 kDa serine/threonine kinase.


LAT
Nck
Vav
Gads
Ser/Thr kinase
Acidic
Proline-rich
SH2
SLP-76
PLCγ
Fyb/SLAP130

SLAP-130/Fyb (da Silva et al., 1997; Musci et al., 1997), and a putative 100 kDa serine/threonine kinase (Motto et al., 1996).

Similar to SLP-76, BLNK has been shown to bind to Nck, Vav, PLC-γ2, and Grb2-like adaptors (Fu et al., 1998). However, no ligands have been identified for the SH2 domain of BLNK. Karsten Sauer from Roger Perlmutter's group at Merck Research Laboratories employed a protein tyrosine kinase Syk-driven yeast two-hybrid approach to search for novel BLNK-interacting proteins using a murine spleen cDNA library. He isolated HPK1 as a phosphotyrosine-dependent BLNK-interacting partner. Three clones representing two distinct, yet overlapping cDNA fragments of murine HPK1 were obtained from this screening (Figure 2). The longer one contains most of the proline-rich region, and the shorter one includes sequences from the end of the kinase domain to the beginning of the second proline-rich motif. It is likely that tyrosine residues in HPK1, which can be phosphorylated by Syk, mediate the association with BLNK. In both HPK1 fragments isolated, a potential tyrosine phosphorylation site Y379 was identified. Mutation of Y379 to phenylalanine in murine HPK1 resulted in the loss of the interaction with BLNK (K. Sauer, unpublished results). Moreover, introduction of a point mutation into the SH2 domain of BLNK, which disrupts its ability to bind to phosphotyrosine residues, greatly diminished its ability to interact with HPK1 (K. Sauer, unpublished results). Therefore, the SH2 domain of BLNK and the Y379 residue of murine HPK1 are essential for their interaction.

The tyrosine residues of SLAP130/Fyb participating in the binding of SLP-76 SH2 domain following phosphorylation by Fyn-T have been defined (Geng et al., 1999). Interestingly, the surrounding sequences of these tyrosine residues are very similar to that

Figure 2: Isolation of HPK1 as a BLNK SH2 domain interactor

Structures of BLNK-interacting clones containing fragments of HPK1 isolated by Karsten Sauer. For comparison, the modular structures of full length HPK1 is depicted schematically above schemes of representative target isolates. Y: Tyrosine residues involved in SH2 domain interactions. Pro-rich, P_1-P_4: Proline-rich regions. CNH: citron homology domain.


HPK1
Kinase Domain
Y
clone group #1
Y
clone group #2
Y

of Y379 of murine HPK1 (Figure 3). It is noteworthy that this region is also well conserved in human HPK1. A consensus ligand motif for the SH2 domain of SLP-76/BLNK can thus be generated as (D/E) x x D (D/E) x Y D D V D.

The above observations made by Karsten Sauer inspired me to examine the interaction between SLP-76 and HPK1 in T cells. I found that endogenous HPK1 is associated with SLP-76 following TCR stimulation. Moreover, Y379 of HPK1, the SLP-76-binding motif, was required for the full activation of HPK1 by the TCR. I further demonstrated that the SH2 domain and Gads-binding site of SLP-76 are important for HPK1 activation. Taken together, these results identify HPK1 as a component in the SLP-76 complex assembled following T cell activation, and yield mechanistic insights into the regulation of HPK1 activation by the TCR.

Figure 3: HPK1 and SLAP-130 share a candidate ligand motif for SLP-76 SH2 domain

Alignment of regions from human (h) HPK1, murine (m) HPK1, and SLAP-130 sequences. Positions within the respective full-length sequences are indicated. All regions contain a Y (red) in an acidic sequence context. A putative consensus motif shared between HPK1 and SLAP130 is depicted underneath the alignment.

hHPK1	375	ESSDDD**Y**DDVD	385
mHPK1	373	SDSDDD**Y**DDVD	383
hSLAP130	589	EDDQEV**Y**DDVA	599
hSLAP130	645	DMGDEV**Y**DDVD	655
consensus:		**DxxDDxYDDVD** **E E**	

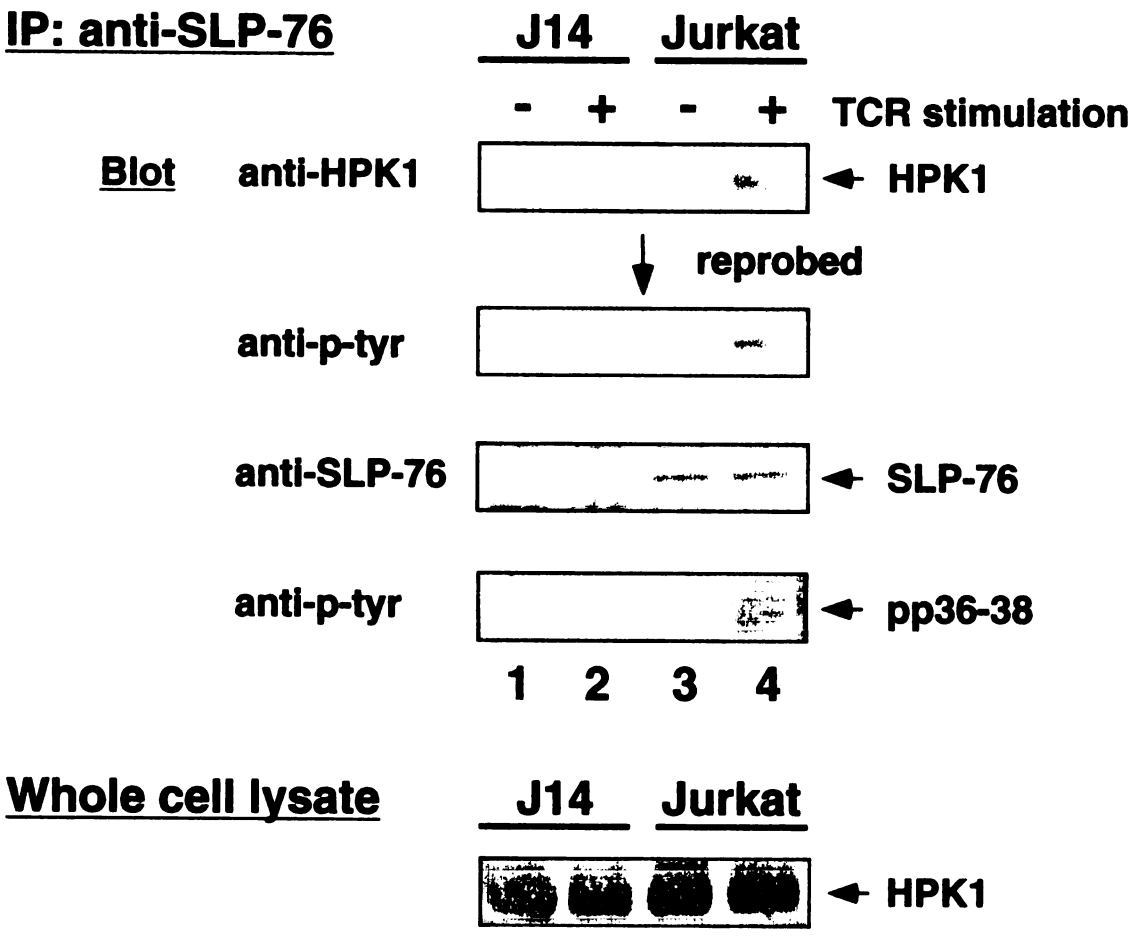
Results

Inducible association between HPK1 and SLP-76 in T cells

To test the interaction between HPK1 and SLP-76 in T cells, Jurkat T leukemia cells were stimulated with anti-TCR MAb for 2 minutes, lysed, and anti-SLP-76 immunoprecipitation (IP) was performed. As shown in Figure 4, endogenously expressed HPK1 was detected in anti-SLP-76 immunoprecipitates from stimulated but not unstimulated cells. With a prolonged exposure, a weak signal of HPK1 can be detected from non-stimulated cells, suggesting that there is a weak basal association between SLP-76 and HPK1. The HPK1 blot was stripped and reprobed with an anti-phosphotyrosine antibody. A band corresponding to HPK1 was observed in anti-SLP-76 IP from activated T cells. This result implicates HPK1 presence in a complex with SLP-76 and its tyrosine phosphorylation. It is also possible that the 100 kDa tyrosine-phosphorylated band corresponds to a different protein that comigrates with HPK1. Additionally, a tyrosine-phosphorylated protein at 36-38 kDa was also detected, which is likely to be LAT. As a specificity control, the SLP-76-deficient J14 cells (Yablonski et al., 1998) were used to show that the anti-SLP-76 antibody did not non-specifically precipitate HPK1. Finally, HPK1 was found to be expressed at equal levels in both J14 and Jurkat T cells. These results indicate that HPK1 is recruited to a complex with SLP-76 and LAT, and that it is tyrosine-phosphorylated in response to TCR stimulation.

Figure 4: TCR stimulation induces the association of SLP-76 and HPK1

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. Whole cell lysates representing $1x10^6$ cells , or IP-eluates representing $9x10^7$ cells with (+) or without (-) stimulation were separated by SDS-PAGE 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.


IP: anti-SLP-76
J14
Jurkat
- + - +
TCR stimulation
Blot
anti-HPK1
HPK1
reprobed
anti-p-tyr
anti-SLP-76
SLP-76
anti-p-tyr
pp36-38
1 2 3 4
Whole cell lysate
J14
Jurkat
HPK1

Mutation of Y379 in HPK1 impairs its activation by TCR

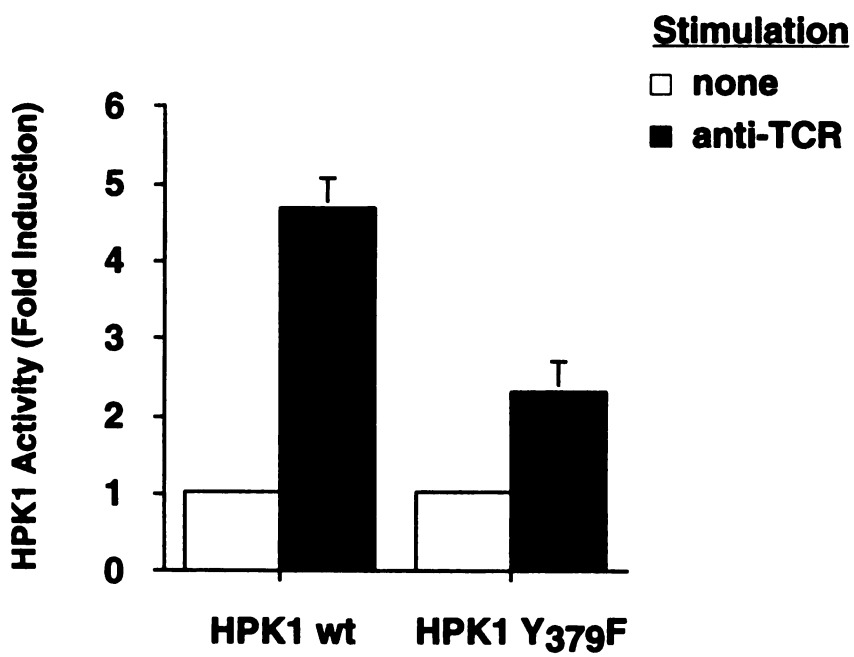
Previously I have observed that full activation of HPK1 in response to antigen receptor stimulation requires SLP-76 in T cells. The recruitment of HPK1 to SLP-76 provides a potential explanation for this observation. Therefore, I investigated the dependence of HPK1 activation on its interaction with SLP-76. Using an immune complex kinase assay, I measured the kinase activity of HA-tagged wildtype or a mutant form of murine HPK1 in which the tyrosine residue at position 379 was substituted with phenylalanine, following their transient transfection into Jurkat cells. I found that the activation of HPK1 is significantly reduced by the mutation of Y379 to phenylalanine (Figure 5), supporting the idea that the activation of HPK1 requires its binding to the SLP-76 SH2 domain which is mediated by the tyrosine-phosphorylated Y379 residue of HPK1. The expression levels of wildtype and Y379F HPK1 were similar in all experiments (data not shown).

Activation of HPK1 in various SLP-76 mutants reconstituted J14 cells

To further test the model described above, I measured the dependence of HPK1 activation on the various functional domains of SLP-76. Activation of wildtype HPK1 was measured in SLP-76-deficient J14 cells that were stably reconstituted with vector, or with constructs encoding wildtype or mutant forms of SLP-76. Whereas vector transfected cells only show a ~ 2-fold HPK1 activation in response to anti-TCR treatment, cells reconstituted with wildtype SLP-76 restored HPK1 activation to 6-fold above that of unstimulated controls (Figure 6). In contrast, the SLP-76 mutant in which the Gads/Grb2-binding region 224-244 was deleted (ΔGads) failed to rescue HPK1

Figure 5: Full HPK1 activation by the TCR requires Y379 of HPK1

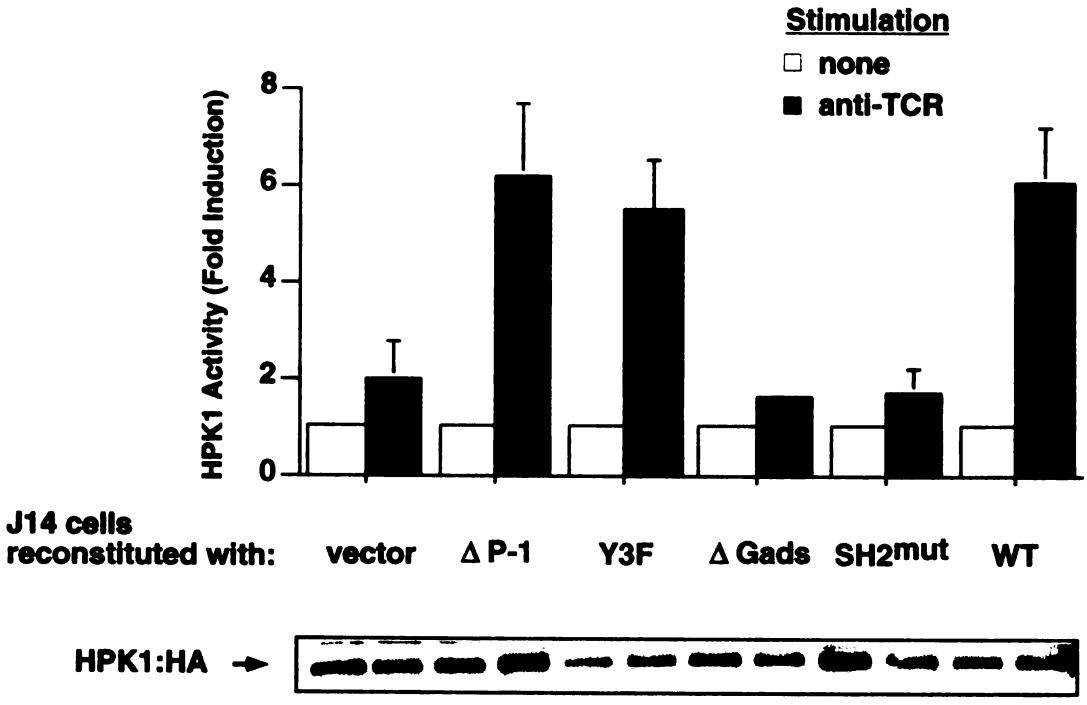
Jurkat cells were transiently transfected with HA-tagged wildtype or Y379F mutant HPK1 to equal expression levels (data not shown), and stimulated for 1 min with buffer or the anti-TCR antibody C305. Cells were then lysed, and anti-HA immunoprecipitates were assayed for HPK1 kinase activity. HPK1 catalytic activity was measured as described in Chapter 2, and is shown as the mean fold induction compared to unstimulated cells from 3 independent experiments. Error bars represent standard deviations.


Stimulation
none
anti-TCR
HPK1 Activity (Fold Induction)
0
1
2
3
4
5
6
T
T
HPK1 wt
HPK1 Y379F

activation, indicating that binding of SLP-76 to Grb2-adaptors is required for full HPK1 activation. In addition, the SLP-76 R448K mutant ($SH2^{mut}$), which represents a phosphotyrosine binding deficient SH2 domain, was unable to rescue HPK1 activation. Mutants in which residues 157-223 were deleted (ΔP-1), or in which critical tyrosine residues required for effector interactions with the SLP-76 N-terminus were changed to phenylalanine (Y3F) both rescued HPK1 activation similarly to wildtype SLP-76. Two stable clones of each mutant-reconstituted J14 cells were used to test HPK1 activation and consistent results were observed. These results suggest that binding of HPK1 via tyrosine-phosphorylated Y379 to the SH2 domain of SLP-76 is required for its activation. Furthermore, binding of adaptor proteins Gads and/or Grb2 to SLP-76 is critical for full HPK activation.

Figure 6: Full HPK1 activation by the TCR requires the Gads-binding and SH2 domains of SLP-76

SLP-76-deficient J14 cells reconstituted with vector, wildtype SLP-76, or one of the SLP-76 mutants ΔP-1, Y3F, ΔGads or $SH2^{mut}$ which harbors a phosphotyrosine binding deficient SH2 domain were transiently transfected with HA-tagged wildtype HPK1, 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. In all experiments, immunoprecipitates contained similar amounts of HPK1 protein as determined by immunoblotting with anti-HA antibodies. An immunoblot from one representative experiment is shown in the bottom panel. Cell surface expression of the TCR on all J14 lines was comparable. HPK1 catalytic activity was measured as described in Chapter 2 and are shown as the mean fold induction compared to unstimulated cells from 3 independent experiments. Error bars represent standard deviations.


Stimulation
none
anti-TCR
HPK1 Activity (Fold Induction)
0
2
4
6
8
J14 cells
reconstituted with:
vector
Δ P-1
Y3F
Δ Gads
SH2mut
WT
HPK1:HA

Discussion

HPK1 is a ligand for the SH2 domain of SLP-76

Karsten Sauer identified the SLP-76/BLNK SH2 domain-binding motif DDDYDDVD in HPK1. In this chapter, I demonstrated that HPK1 indeed binds to SLP-76 in T cells following TCR stimulation. Recently, Sauer performed transient transfection experiments, and found that only wildtype HPK1 and SLP-76, but not Y379F HPK1 or the SH2 mutant of SLP-76 could interact with each other. This interaction is readily seen without stimulation, and is further augmented by TCR stimulation. It is possible that overexpression of HPK1 may lead to its tyrosine phosphorylation. Future experiments are required to test this possibility.

Previously, Motto et al. detected a 100 kDa serine/threonine activity associated with the SH2 domain of SLP-76 following TCR stimulation (Motto et al., 1996). They also detected a 100 kDa tyrosine phosphorylated protein in SLP-76 IP from stimulated Jurkat T cells. Since the molecular weight of HPK1 is 97 kDa, our results suggest that the 100 kDa kinase and the 100 kDa SLP-76-associated tyrosine-phosphorylated protein may be HPK1.

HPK1 is an upstream regulator of MAP kinase cascades. The recruitment of HPK1 to SLP-76 may thus provide a link from SLP-76 to the regulation of MAP kinase pathways. Notably, the SH2 domain of SLP-76, following TCR stimulation, also interacts with the adaptor protein SLAP130/Fyb. Overexpression of SLAP-130/Fyb has been shown to suppress TCR- and SLP-76-mediated NFAT activation in Jurkat T cells (Musci et al., 1997), but also has been reported to potentiate IL-2 production in the

DC27.10 T cell hybridoma (da Silva et al., 1997). These conflicting results may reflect the differences in the expression levels of Fyn-T, which phosphorylates the SLP-76-binding motifs of SLAP-130/Fyb (Geng et al., 1999; Raab et al., 1999). Alternatively, the expression level of HPK1 might contribute to these opposing results. Since HPK1 can compete with SLAP-130/Fyb for the binding of SLP-76 following stimulation, the equilibrium between HPK1 and SLAP-130/Fyb may determine the outcome of signaling from SLP-76.

The role of SLP-76 in regulating HPK1 activation

The identification of the interaction between HPK1 and SLP-76 has shed light on how SLP-76 may contribute to HPK1 activation. In collaboration with Karsten Sauer, we demonstrated that the Y379 residue of HPK1 and the SH2 domain of SLP-76 are not only required for their interaction, but also important for HPK1 activation. These results indicate that the association between HPK1 and SLP-76 is necessary for the activation of HPK1. The interaction with SLP-76 may induce a conformational change or the dissociation from an inhibitor of HPK1, resulting in the full induction of the catalytic activity.

Notably, in SLP-76 deficient J14 cells, a two-fold activation of HPK1 was still observed. It is possible that SLP-76-like proteins, such as CLNK (Cao et al., 1999), may account for this weak activation. Alternatively, the recruitment of HPK1 to LAT via Grb2 may partially activate HPK1, and the interaction between SLP-76 and HPK provides additional steps for the full activation. Interestingly, I found that the Gads/Grb2-binding mutant of SLP-76 fails to reconstitute HPK1 activation in J14 cells.

This result indicates that the recruitment of SLP-76 to LAT via its binding to Gads or Grb2 is important for its activation of HPK1. Thus, I propose that following TCR stimulation HPK1 and SLP-76 are independently recruited to LAT by Grb2-like adaptor proteins. There, HPK1 becomes tyrosine phosphorylated by upstream protein tyrosine kinases. Tyrosine phosphorylation allows HPK1 to bind to the SH2 domain of SLP-76 present in close proximity. This induces its full activation. This model explains how mutations disrupting the binding to Gads/Grb2 or HPK1 abolish the HPK1 activating potential of SLP-76.

Experimental Procedures

Reagents

The antibodies used for the stimulation of T cells were C305 (anti-Jurkat Ti β chain MAb) (Weiss and Stobo, 1984). Sheep anti-human SLP-76 was a gift from G. Koretzky, University of Pennsylvania, PA. 4G10 (anti-phosphotyrosine MAb) was from Upstate Biotechnology. Anti-mouse HPK1 antibody (#7) was kindly provided by Friedemann Kiefer, Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany. pcdef3-HPK1(Y379F):HA was constructed by subcloning a fragment containing the Y379F mutation from pcDNA3-HPK1(Y379F) generated by Karsten Sauer into pcdef3-HPK1:HA.

Cell Culture and Transfection

Jurkat, J14, and various SLP-76 mutants reconstituted J14 stable lines (Yablonski et al., unpublished results) were maintained in RMPI 1640 supplemented with 5% fetal calf serum (FCS), penicillin, streptomycin, and glutamine. Transient transfections of Jurkat cells and various J14 lines were performed by electroporating 2 x 10^7 cells resuspended in 400 μl serum-free RPMI 1640 medium with 2μg of pcdef3-HPK1:HA or pcdef3-HPK1(Y379F):HA DNA in a 0.4 cm cuvette using the Gene Pulser (Bio-Rad Laboratories) at a setting of 250 V and 960 μF.

Cell Stimulation, Preparation of Lysates, Immunoprecipitation, and Immunoblotting

9 x 10^7 Jurkat or J14 cells were washed once with phosphate-buffered saline (PBS), then incubated at 37°C for 15 min. Subsequently, cells were either mock-stimulated with PBS (buffer control), or were stimulated for the time indicated with C305. Cells were lysed in cold lysis buffer (1% Nonidet P-40, 10 mM Tris pH 7.6, 150 mM NaCl) supplemented with protease and phosphatase inhibitors on ice for 20 min. Precleared lysates were incubated with anti-SLP-76 antibody-precoupled Protein G-sepharose beads for 2 hr at 4°C. The immunoprecipitates were then washed 4 times with lysis buffer, resuspended in SDS sample buffer, and heated at 95°C for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon-P membrane (Millipore), and probed with primary and secondary antibodies followed by enhanced chemiluminescence (ECL) detection (Amersham).

Immune Complex Kinase Assays

For HPK1 kinase assays, 5 x 10^6 Jurkat cells transfected with 2 μg of pcdef3-HPK1:HA, or pcdef3-HPK1(Y379F):HA were stimulated with anti-TCR MAb C305 for 2 minutes, then lysed in cold lysis buffer A (Kiefer et al., 1996). Precleared lysates were immunoprecipitated with anti-HA antibody precoupled to Protein A-sepharose beads. Immunoprecipitates were assayed for kinase activities as described previously (Kiefer et al., 1996) with the addition of 5 μg of histone H2A as the exogenous substrate. Kinase reaction products were separated by SDS-PAGE, transferred to Immobilon-P membrane, and visualized by autoradiography and phosphorimaging.

Acknowledgments

I would like to thank Karsten Sauer for collaborating on this project as well as Debbie Yablonski and Terri Kadlecek for providing various SLP-76 mutant-reconstituted J14 stable lines.

References

Cao, M. Y., Davidson, D., Yu, J., Latour, S., and Veillette, A. (1999). Clnk, a novel SLP-76-related adaptor molecule expressed in cytokine- stimulated hemopoietic cells. J Exp Med *190*, 1527-34.

da Silva, A. J., Li, Z., de Vera, C., Canto, E., Findell, P., and Rudd, C. E. (1997). Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain- containing leukocyte protein 76 and modulates interleukin 2 production. Proc Natl Acad Sci U S A *94*, 7493-8.

Fang, N., and Koretzky, G. A. (1999). SLP-76 and Vav function in separate, but overlapping pathways to augment interleukin-2 promoter activity. J Biol Chem *274*, 16206-12.

Fu, C., Turck, C. W., Kurosaki, T., and Chan, A. C. (1998). BLNK: a central linker protein in B cell activation. Immunity *9*, 93-103.

Geng, L., Raab, M., and Rudd, C. E. (1999). Cutting edge: SLP-76 cooperativity with FYB/FYN-T in the Up-regulation of TCR-driven IL-2 transcription requires SLP-76 binding to FYB at Tyr595 and Tyr651. J Immunol *163*, 5753-7.

Jackman, J. K., Motto, D. G., Sun, Q., Tanemoto, M., Turck, C. W., Peltz, G. A., Koretzky, G. A., and Findell, P. R. (1995). Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. J Biol Chem *270*, 7029-32.

Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996). HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. Embo Journal *15*, 7013-25.

Liu, S. K., Fang, N., Koretzky, G. A., and McGlade, C. J. (1999). The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. Current Biology *9*, 67-75.

Motto, D. G., Ross, S. E., Wu, J., Hendricks-Taylor, L. R., and Koretzky, G. A. (1996). Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. J Exp Med *183*, 1937-43.

Musci, M. A., Hendricks-Taylor, L. R., Motto, D. G., Paskind, M., Kamens, J., Turck, C. W., and Koretzky, G. A. (1997). Molecular cloning of SLAP-130, an SLP-76-associated substrate of the T cell antigen receptor-stimulated protein tyrosine kinases. J Biol Chem *272*, 11674-7.

Raab, M., Kang, H., da Silva, A., Zhu, X., and Rudd, C. E. (1999). FYN-T-FYB-SLP-76 interactions define a T-cell receptor zeta/CD3- mediated tyrosine phosphorylation

pathway that up-regulates interleukin 2 transcription in T-cells. J Biol Chem *274*, 21170-9.

Weiss, A., and Stobo, J. D. (1984). Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. Journal of Experimental Medicine *160*, 1284-99.

Wunderlich, L., Farago, A., Downward, J., and Buday, L. (1999). Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. Eur J Immunol *29*, 1068-75.

Yablonski, D., Kuhne, M. R., Kadlecek, T., and Weiss, A. (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76- deficient T cell. Science *281*, 413-6.

CHAPTER 4

HPK1 ACTIVATION AND FUNCTION IN TCR SIGNALING: DISCUSSION AND FUTURE EXPERIMENTS

Summary

HPK1 is a hematopoietic-specific Ste20-like kinase whose function and regulation had not been well characterized. I have demonstrated the activation of HPK1 by the TCR and BCR in lymphocytes, and identified signaling components linking these receptors to HPK1 using genetic and biochemical approaches. Overexpression studies with HPK1 suggest a negative regulatory role of HPK1 in TCR-mediated AP-1 activation, and this inhibition acts at a proximal step of TCR signaling. In this concluding chapter, I discuss these results and propose a mechanistic model of HPK1 activation by the TCR. I also discuss the functional effects of HPK1 on TCR signaling examined by overexpression studies using tetracycline-inducible HPK1 stable lines, and by HPK1 knockout mice. Future experiments based on this work are suggested.

Activation of HPK1 by antigen receptors

HPK1 is a hematopoietic-specific Ste20-like serine/threonine kinase whose proline-rich motifs can mediate its association with SH3 domain-containing proteins. Unlike its closely related GCK-I subfamily members, GCK, GLK and GCKR/KHS, HPK1 is not activated by tumor necrosis factor-α (TNF-α) (Hu et al., 1996). I discovered that HPK1 is activated by antigen receptors in T and B lymphocytes. Following antigen receptor crosslinking, the catalytic activity of HPK1 is increased by about 5 to 10 fold. The activity peaks between 1 to 3 minutes following stimulation, and decreases afterwards. CD28 crosslinking neither induces HPK1 activity nor modulates that activated by the TCR. Moreover, pharmacological agents PMA and Ionomycin fail to trigger HPK1 activation in T cells, suggesting that activation of Ras/PKC and calcium mobilization are not sufficient to activate HPK1.

Multiple Jurkat mutant lines were used to define components of the pathway required from the TCR to HPK1 activation. HPK1 failed to be activated in J.CaM1 (Lck-deficient), P116 (ZAP-70-deficient), and J.CaM2 (LAT-deficient) cells. In SLP-76-deficient J14 cells, HPK1 activation was greatly reduced, yet not completely abolished. Furthermore, a biochemical approach was used to detect HPK1-interacting proteins in activated T cells, as an approach to identifying molecules that link HPK1 to upstream signaling components. Immunoprecipitation of HPK1 from metabolically labeled T cells led to the identification of adaptor proteins Grb2 and Grap as HPK1-interacting partners. These associations are present in both resting and activated T cells. Since both Grb2 and Grap bind to the adaptor protein LAT following T cell activation, these results suggest that HPK1 can be recruited to LAT via Grb2 or Grap. Furthermore, I observed the

interaction of HPK1 with SLP-76 following TCR stimulation. This interaction appears to be essential for the full activation of HPK1. Disruption of the interacting sites in either protein results in a great reduction of HPK1 activation. Finally, I found that HPK1 activation by the TCR also depends on the ability of SLP-76 to bind to the adaptor protein Gads.

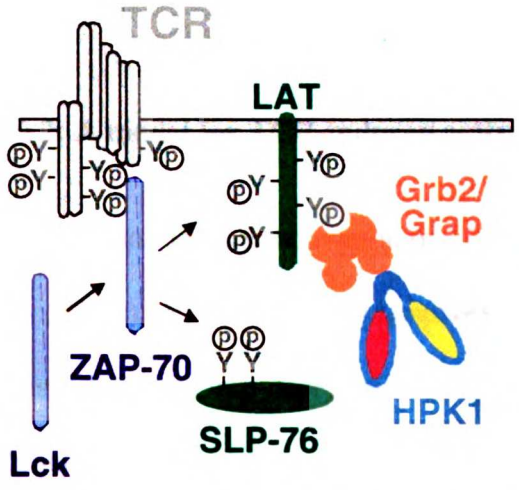
To explore additional components involved in HPK1 activation, I tested HPK1 activation in various gene-targeted DT40 B cells. Similar to TCR-mediated HPK1 activation, BCR-induced HPK1 activation is dependent on protein tyrosine kinases Lyn and Syk, as well as the adaptor protein BLNK. Additionally, I found that Grb2 and Grap are necessary for HPK1 activation by the BCR using Grb2/Grap doubly deficient DT40 cells. In contrast, PLC-γ2 and the protein tyrosine kinase BTK are dispensable for this activation. The molecular requirements for HPK1 activation by TCR and BCR are summarized in Figure 1.

A mechanistic model for HPK1 activation by the TCR

Based on the above findings, a mechanistic model for HPK1 activation by the TCR can be proposed (Figure 2). Following TCR stimulation, protein tyrosine kinases Lck and ZAP-70 are first activated. They, in turn, phosphorylate the transmembrane adaptor protein LAT localized in the glycolipid-enriched microdomains (GEMs) of the plasma membrane. Tyrosine phosphorylation of LAT permits the recruitment of the Grb2/HPK1 complex to close proximity with upstream protein tyrosine kinases Lck and ZAP-70. Following membrane translocation, HPK1 becomes tyrosine phosphorylated at

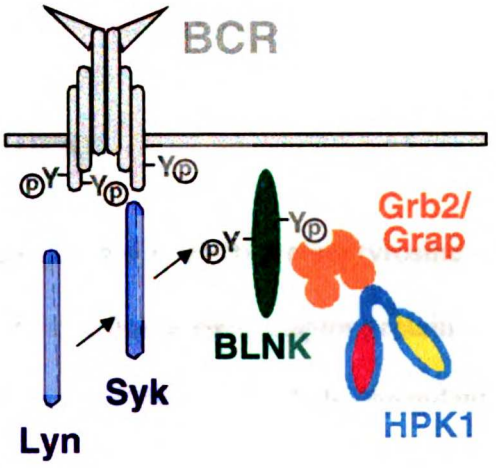
Figure 1: Conserved requirements of signaling proteins for HPK1 activation by the TCR and the BCR

Optimal HPK1 activation by the antigen receptors requires Src (Lck in T cells and Lyn in B cells, respectively) and Syk (ZAP-70 in T cells and Syk in B cells, respectively) families of protein tyrosine kinases, inducible tyrosine-phosphorylated adaptor proteins (LAT and SLP-76 in T cells and BLNK in B cells), as well as the SH2/SH3 adaptor proteins Grb2 and Grap.



Optimal HPK1 activation by TCR

requires:
- **Lck**
- **ZAP-70**
- **LAT**
- **SLP-76**



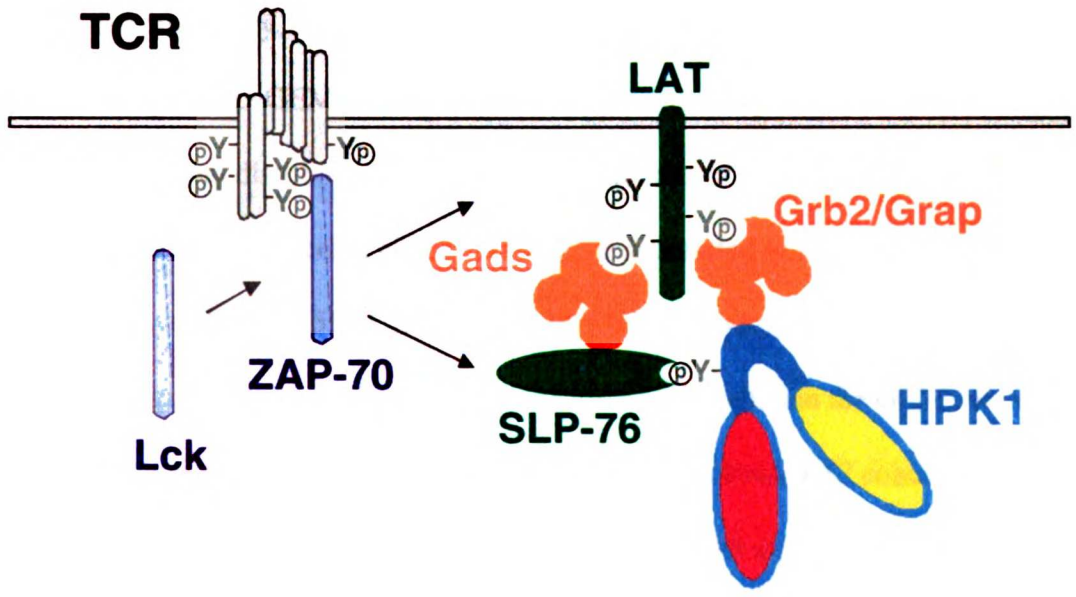
Optimal HPK1 activation by BCR

requires:
- **Lyn**
- **Syk**
- **BLNK**
- **Grb2/Grap**

Figure 2: A model for TCR-induced activation of HPK1

TCR-stimulation leads to activation of TCR-proximal Lck and ZAP-70 protein tyrosine kinases (PTKs). PTK-mediated phosphorylation of the transmembrane adaptor protein LAT permits Grb2/Grap-dependent membrane recruitment of HPK1 and Gads-dependent membrane recruitment of SLP-76. Membrane-recruited HPK1 then becomes tyrosine-phosphorylated, binds to the SLP-76 SH2 domain via its tyrosine residue 379, and is fully activated.

Model: HPK1 activation by the TCR



HPK1 activation upon TCR stimulation

1. is recruited to tyrosine phosphorylated LAT by Grb2-like adaptors

2. becomes tyrosine phosphorylated

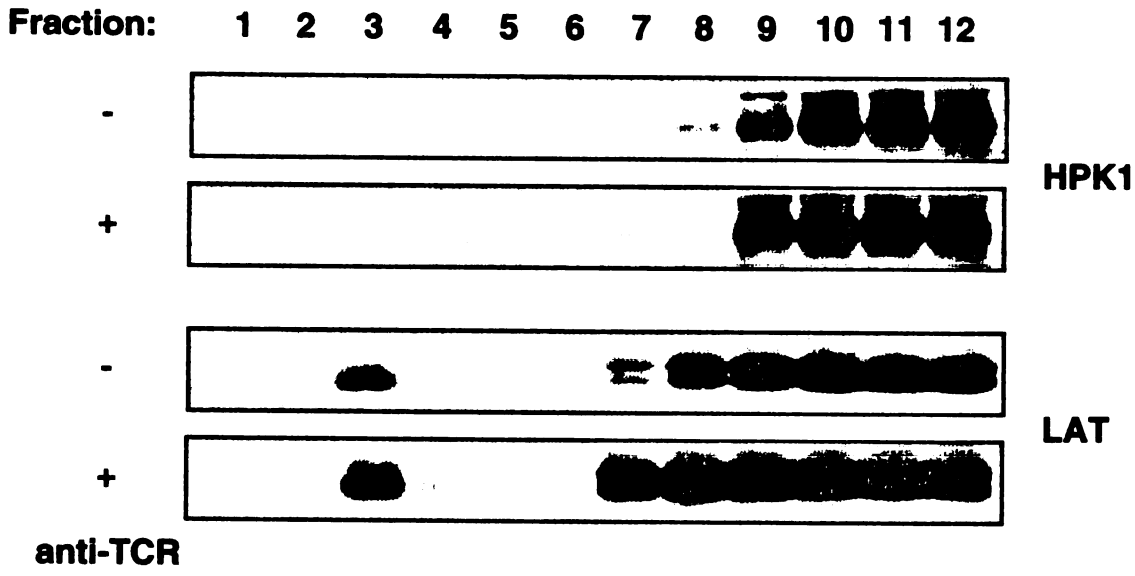
3. binds to the SH2 domain of SLP-76 via Y379

residue Y379, and presents a ligand for the SH2 domain of SLP-76. Meanwhile, the Gads/SLP-76 complex is also recruited to phosphorylated LAT. Finally, the interaction between HPK1 and SLP-76 in LAT signalosomes fully activates HPK1.

This model suggests that HPK1 is recruited to the plasma membrane, or the GEMs following TCR stimulation. In collaboration with Joseph Lin, a fellow graduate student in our laboratory, I tested the presence of HPK1 in the GEMs following TCR stimulation (Figure 3). GEMs are characterized by insolubility to Triton X-100 extraction at 4°C and distribution in low-density fractions of a sucrose gradient (Simons and Ikonen, 1997). Jurkat cells, either untreated or stimulated with anti-TCR MAb for 2 minutes, were lysed with 0.5% Triton, and lysates were subjected to ultracentrifugation over a sucrose gradient. A total of 12 fractions were collected, and the GEMs were contained in fraction 3 based on the presence of LAT. Fractions 9-12 contained the Triton-soluble proteins. LAT was probed as a control for the quality of fractionation. In resting cells, HPK1 was strictly present in the soluble fractions. Interestingly, in activated cells, weak signals of HPK1 were found in low-density fractions (fractions 5-7), indicating that a small portion of HPK1 appears to change its physical state following TCR stimulation. In this experiment, HPK1 is not found in fraction 3 (the GEMs) where LAT is located. However, I have observed a 36-38 kDa tyrosine phosphorylated protein co-precipitating with HPK1, which is likely to be LAT. It is possible that a low stoichiometry of HPK1 recruitment into the GEMs, which failed to be detected in this experiment. In a recent report, Ling et al. showed similar results, and a weak signal for HPK1 was seen in the GEM fraction isolated from activated T cells (Ling et al., 2001). Their findings suggest that TCR stimulation induces the translocation of a small amount

Figure 3: Distribution of HPK1 and LAT analyzed by sucrose gradient fractionation

Fractions from a sucrose gradient separating a 0.5% Triton lysate of unstimulated or C305-stimulated Jurkat cells were analyzed by SDS-PAGE, and immunoblotted by anti-HPK1 and anti-LAT antibodies. Twelve fractions were collected. Fraction 1 is from the light end and fraction 12 is from the dense end of the gradient.


Fraction:
1 2 3 4 5 6 7 8 9 10 11 12
-
+
HPK1
-
+
LAT
anti-TCR

of HPK1 into the GEMs.

An HPK1-GFP (green fluorescence protein) fusion construct has been generated for future experiments to monitor the translocation event of HPK1 following T cell activation (see Appendix for HPK1-GFP and other constructs generated). It will be interesting to examine the kinetics and the molecular requirements associated with HPK1 activation and presumed translocation. Moreover, mutant constructs of HPK1 fused to GFP will allow the characterization of the structural domains of HPK1 involved in this translocation. Furthermore, LAT-HPK1 fusion constructs have also been made to test the role of LAT recruitment in HPK1 activation. To test which proline-rich motifs in HPK1 are required for its interaction with Grb2 and its subcellular redistribution, I have generated several HPK1 mutant constructs (see Appendix). Meanwhile, Joseph Lin has made multiple J.CaM2 stable lines reconstituted with LAT mutants that fail to interact with Grb2 and other LAT effectors. These stable lines will be useful for future examination of our model for HPK1 activation.

Tyrosine phosphorylation of HPK1

The requirement of HPK1 Y379 phosphorylation for the interaction with the SLP-76 SH2 domain and its full catalytic activation indicates the importance of tyrosine phosphorylation of HPK1. The highly acidic sequence surrounding Y379 ($DDDY_{379}DDVD$) suggests that Y379 is a potential substrate for the Syk family of kinases which prefers substrate tyrosines neighboring acidic residues (Brunati et al., 1995; Schmitz et al., 1996). Indeed, Syk was used by Sauer and Perlmutter in a modified yeast 2-hybrid screening, in which HPK1 was identified as a BLNK SH2 domain-

interacting protein. Therefore, Syk and its family member ZAP-70 may contribute to HPK1 tyrosine phosphorylation in activated lymphocytes.

I attempted to detect the tyrosine phosphorylation of HPK1 following TCR stimulation. Due to the lack of an anti-human HPK1 antibody for the immunoprecipitation of endogenous HPK1, I examined the tyrosine phosphorylation of HPK1 by transfecting Jurkat T cells with an HPK1 construct fused to 3 tandem HA tags. A concern with this approach was that there are several potential tyrosine phosphorylation sites in the HA tag. I observed a basal tyrosine phosphorylation of the transfected HA-tagged HPK1; however, the signal was not significantly enhanced following TCR stimulation. In the meantime, Liu et al. reported that endogenous HPK1 is tyrosine phosphorylated following TCR crosslinking in a mouse T cell line (Liu et al., 2000). Recently, Ling et al. also observed that endogenous HPK1 becomes tyrosine phosphorylated following TCR stimulation in Jurkat T cells (Ling et al., 2001). Interestingly, the kinetics of HPK1 tyrosine phosphorylation are similar to that of its catalytic activation by the TCR. Furthermore, as shown in Chapter 3, residue Y379 of HPK1 appears to be important for its activation. Together, these observations support the notion that tyrosine phosphorylation of HPK1 is involved in its activation.

HPK1-interacting proteins

HPK1 has been shown to interact with multiple SH3 domain-containing proteins including Grb2, Grap, Gads, c-Crk, CrkL, Nck, HS1, and HIP-55 (Anafi et al., 1997; Ensenat et al., 1999; Liu et al., 2000; Nagata et al., 1999; Oehrl et al., 1998). Grb2, Grap, and Gads are involved in coupling HPK1 to activated TCR or BCR. The functional

significance of HPK1 binding to c-Crk and CrkL in hematopoietic cells is not clear. Nevertheless, c-Crk and CrkL can synergize with HPK1 to activate JNK in fibroblasts and can be phosphorylated by HPK1 (Ling et al., 1999). Furthermore, HPK1 associates with HS1, an adaptor protein functioning in B cell proliferation and apoptosis induced by BCR crosslinking, in hematopoietic cells (Fukuda et al., 1995; Nagata et al., 1999; Taniuchi et al., 1995). The significance of this interaction is not yet understood. HIP-55 is identified as an HPK1-interacting protein of 55 kDa (Ensenat et al., 1999). It contains an N-terminal actin-binding domain and a C-terminal SH3 domain. A kinase-inactive mutant of HPK1 blocks JNK activation by HIP-55 indicating that HPK1 is a downstream effector mediating HIP-55-induced JNK activation. The binding of HPK1 to SH3-domain containing adaptors other than Grb2-like proteins suggests the existence of alternative pathways linking HPK1 to the TCR complex, and the possible involvement of HPK1 in multiple signaling pathways in T cells.

Functional studies of HPK1 by overexpression

Overexpression of HPK1 in T cells results in a strong activation of JNK even in the absence of stimuli. This result is consistent with the notion that HPK1 works as a MAPKKKK for JNK by binding to and phosphorylating MEKK1 and MLK3, two MAPKKK for JNK (Hu et al., 1996; Kiefer et al., 1996). Interestingly, HPK1 has also been found in a complex with JIP1, a scaffold protein for the JNK cascade (Whitmarsh et al., 1998). However, the kinase-inactive HPK1 fails to block JNK activation induced by TCR and CD28. This indicates that there are other signaling pathways, in addition to HPK1, sufficient for JNK activation in T cells.

Moreover, overexpression of wildtype but not the kinase-inactive HPK1 in Jurkat T cells induces transcriptional activation of NFκB. Consistently, HPK1 has been shown to activate IkB kinases (IKK)-α and -β (Hu et al., 1999). This activation is also dependent on the kinase activity of HPK1. It is possible that HPK1 exerts its effect on the NFκB pathway through its target MEKK1, since MEKK1 shares similar NFκB-activating function (Lee et al., 1997).

Based on its effects on JNK and NFκB, it was surprising to see that HPK1 inhibits the activation of AP-1, NFAT, and REAP transcriptional responses as well as the activation of the MAP kinase ERK. This inhibitory effect is not due to the toxicity introduced by overexpressing a kinase, since PMA and/or Ionomycin induced responses are not affected. Moreover, the kinase-dead form of HPK1, which contains only one point mutation in the ATP-binding site of the kinase domain, reverses the inhibition. In some experiments, overexpression of kinase-dead HPK1 readily induces ERK activation (Figure 7C in Chapter 2). This implies that HPK1 suppresses ERK activity in resting cells. Taken together, HPK1 is a negative regulator for TCR-mediated ERK/AP-1 response, and acts upstream of Ras signaling.

Another interesting finding with kinase-dead HPK1 is that it can synergize with PMA to induce the transcriptional activation of NFAT. This result suggests that HPK1 may also participate in antagonizing NFAT signaling. It has been reported that activated JNK can phosphorylate NFAT and inhibits its nuclear accumulation (Chow et al., 2000). It will be of interest to examine whether HPK1 affects the subcellular localization of NFAT via its JNK activating function.

In order to test the effects of wildtype and kinase-dead HPK1 on endogenous signaling proteins, tet-inducible Jurkat stable lines have been generated. A global reduction of protein tyrosine phosphorylation following TCR stimulation was observed in stable lines transfected with wildtype or kinase-dead HPK1, but not in non-transfected controls. One explanation is that these are artifacts caused by the high expression levels of wildtype and kinase-dead HPK1, since both constructs contain binding sites for SH3 domain proteins. A titration of Doxycycline should address the problem. These stable lines may be useful for future functional studies for HPK1.

HPK1 substrates

The kinase activity of HPK1 appears to be essential for its functional effects in T cells. Therefore, it is important to identify the substrates of HPK1. MEKK1 and MLK3 are two known substrates of HPK1 and function as MAPKKKs of the JNK kinase cascade (Hu et al., 1996; Kiefer et al., 1996). The JNK and NFκB activating functions of HPK1 may be mediated by these two direct targets. However, they can not explain the inhibitory effect of HPK1 on AP-1 and ERK. I consistently observed the presence of a 40 kDa and a 28 kDa band in HPK1 protein kinase assays performed with two different antibodies (anti-HA and anti-HPK1), and in two different types of cells (Jurkat T cells and purified mouse lymph node T cells). The phosphorylation of these two bands correlates with the kinase activity of HPK1. It will be interesting to identify these two potential substrates of HPK1. Candidates include Gads (42 kDa), LAT (36-38 kDa), CrkL (36 kDa), Grb2 (28 kDa), and Grap (28 kDa).

HPK1 knockout mice

HPK1 knockout mice were generated by Dr. Tak Mak and co-workers in collaboration with Dr. Friedemann Kiefer (personal communication). These mice are viable, and display normal thymocyte development. In addition, there is no defect observed in TCR-induced proliferation and mixed lymphocyte reactions (personal communication with Friedemann Kiefer). To test whether HPK1 negatively regulates TCR signaling *in vivo*, I obtained HPK1 knockout mice from Friedemann Kiefer and analyzed the activation of MAP kinases in thymocytes. Normal ERK and JNK activation induced by CD3 crosslinking were observed in heterozygous and knockout thymocytes.

Multiple groups have examined the *in vivo* roles of HPK1 using these mice. However, no phenotypes have been reported so far. One explanation is that the existence of redundantly functioning genes may compensate for the loss of HPK1 in these mice. At least three closely-related GCK-I subfamily kinases, GCK, GLK, and GCKR/KHS, are expressed in lymphocytes. Studies in fibroblasts indicate that they may share similar substrate specificity with HPK1, and may therefore substitute for its function.

To further explore the functional role of HPK1, I attempted to generate HPK1-deficient DT40 B cells. A degenerate PCR approach was used to clone chicken HPK1 from DT40 cDNA. I identified chicken homologs of GLK, GCKR/KHS, and a novel GCK-I subfamily kinase based on homology analysis. These results indicate that there are at least three chicken GCK-I family members in this avian B cell line. The concern of potential redundancy ended this line of experiments. Although there are 4 mammalian GCK-I family kinases, there is only one in C. elegans and in Drosophila. The phenotypes

of GCK-I mutants in C. elegans and Drosophila may yield insights into the physiological functions of this family of kinases.

With the HPK1 knockout mice, it is possible that we have not found the pathways that are disrupted by the loss of HPK1. Since HPK1 suppresses AP-1 activation by the TCR, it will be of interest to test whether T cell anergy induction and memory formation are affected by HPK1 deficiency. Furthermore, it will be important to examine B cell function in these mice.

Role of HPK1 in other signaling pathways

In addition to antigen stimulation, HPK1 is also activated by erythropoietin (Epo) and TGF-β (Nagata et al., 1999; Zhou et al., 1999). The kinetics of HPK1 activation by Epo are similar to that by antigen stimulation. It can be detected 3 minutes after the treatment of Epo. This activation is thought to be involved in Epo-induced growth and differentiation signals. In contrast, HPK1 activity induced by TGF-β peaks 6 hours following stimulation. The kinase-inactive form of HPK1 blocks JNK activation by TGF-β, suggesting a role of HPK1 in TGF-β signaling.

Interestingly, I found that a G-protein coupled receptor (GPCR) activates HPK1. In Jurkat derived cell line J.HM1.2.2 (Goldsmith et al., 1989), which stably expresses the human muscarinic receptor 1 (HM1R), HPK1 is activated following stimulation of the HM1R. The HM1R is a GPCR that employs distinct proximal signaling events from those in TCR signaling to activate PLCβ and therefore Ras and calcium pathways (Desai et al., 1990). HPK1 activation by HM1R is reminiscent of Ste20p activation by the pheromone receptor in yeast. It will be interesting to test whether HPK1 associates with

the Gβ subunit of a G protein. Furthermore, recent reports suggest that PTKs are effectors for heterotrimeric G proteins (Jiang et al., 1998; Ma et al., 2000). Future experiments are required to identify the HPK1-activating GPCRs in hematopoietic cells, and to address the dependence on PTK of GPCR-induced HPK1 activation.

Conclusion

I have identified the Ste20 group kinase HPK1 as a novel component in antigen receptor signal transduction. HPK1 is rapidly activated by the TCR. Interestingly, the activation of this serine/threonine kinase may involve its tyrosine phosphorylation. Moreover, HPK1 is recruited to a complex with the adaptor proteins LAT and SLP-76 following stimulation. It is plausible that HPK1 may phosphorylate proteins in this complex. Furthermore, the functions of HPK1 in TCR signaling have been studied by overexpression and knockout approaches. A negative role for HPK1 in AP-1 activation in T cells is suggested by the results. Taken together, I have identified a signaling pathway linking TCR stimulation to HPK1 activation. I also identified an inhibitory role for this serine/threonine kinase acting at a proximal step of the TCR signaling pathway. HPK1 is involved in regulating multiple downstream pathways including JNK, NFκB, ERK, and NFAT; however, the physiological roles of HPK1 remain to be resolved. Further analysis of T and B cell function in HPK1 knockout mice should help define the physiological roles of this kinase. Finally, HPK1 substrates in the TCR signaling pathway should be an area of future experimentation.

References

Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N., and Pawson, T. (1997). SH2/SH3 adaptor proteins can link tyrosine kinases to a Ste20-related protein kinase, HPK1. J Biol Chem *272*, 27804-11.

Brunati, A. M., Donella-Deana, A., Ruzzene, M., Marin, O., and Pinna, L. A. (1995). Site specificity of p72syk protein tyrosine kinase: efficient phosphorylation of motifs recognized by Src homology 2 domains of the Src family. FEBS Lett *367*, 149-52.

Chow, C. W., Dong, C., Flavell, R. A., and Davis, R. J. (2000). c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. Mol Cell Biol *20*, 5227-34.

Desai, D. M., Newton, M. E., Kadlecek, T., and Weiss, A. (1990). Stimulation of the phosphatidylinositol pathway can induce T-cell activation. Nature *348*, 66-9.

Ensenat, D., Yao, Z., Wang, X. S., Kori, R., Zhou, G., Lee, S. C., and Tan, T. H. (1999). A novel src homology 3 domain-containing adaptor protein, HIP-55, that interacts with hematopoietic progenitor kinase 1. J Biol Chem *274*, 33945-50.

Fukuda, T., Kitamura, D., Taniuchi, I., Maekawa, Y., Benhamou, L. E., Sarthou, P., and Watanabe, T. (1995). Restoration of surface IgM-mediated apoptosis in an anti-IgM-

resistant variant of WEHI-231 lymphoma cells by HS1, a protein-tyrosine kinase substrate. Proc Natl Acad Sci U S A *92*, 7302-6.

Goldsmith, M. A., Desai, D. M., Schultz, T., and Weiss, A. (1989). Function of a heterologous muscarinic receptor in T cell antigen receptor signal transduction mutants. J Biol Chem *264*, 17190-7.

Hu, M. C., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T. H. (1996). Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes and Development *10*, 2251-64.

Hu, M. C., Wang, Y., Qiu, W. R., Mikhail, A., Meyer, C. F., and Tan, T. H. (1999). Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates IkappaB kinases (IKK-alpha/beta) and IKK-beta is a developmentally regulated protein kinase. Oncogene *18*, 5514-24.

Jiang, Y., Ma, W., Wan, Y., Kozasa, T., Hattori, S., and Huang, X. Y. (1998). The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. Nature *395*, 808-13.

Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996). HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. Embo Journal *15*, 7013-25.

Lee, F. S., Hagler, J., Chen, Z. J., and Maniatis, T. (1997). Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. Cell *88*, 213-22.

Ling, P., Meyer, C. F., Redmond, L. P., Shui, J. W., Davis, B., Rich, R. R., Wange, R. L., and Tan, T. H. (2001). Involvement of hematopoietic progenitor kinase 1 (HPK1) in T-cell receptor (TCR) signaling. J Biol Chem *13*, 13.

Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrl, W., Feller, S. M., and Tan, T. H. (1999). Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. Mol Cell Biol *19*, 1359-68.

Liu, S. K., Smith, C. A., Arnold, R., Kiefer, F., and McGlade, C. J. (2000). The adaptor protein Gads (Grb2-related adaptor downstream of Shc) is implicated in coupling hemopoietic progenitor kinase-1 to the activated TCR. J Immunol *165*, 1417-26.

Ma, Y. C., Huang, J., Ali, S., Lowry, W., and Huang, X. Y. (2000). Src tyrosine kinase is a novel direct effector of G proteins. Cell *102*, 635-46.

Nagata, Y., Kiefer, F., Watanabe, T., and Todokoro, K. (1999). Activation of hematopoietic progenitor kinase-1 by erythropoietin. Blood *93*, 3347-54.

Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998). The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene *17*, 1893-901.

Schmitz, R., Baumann, G., and Gram, H. (1996). Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. J Mol Biol *260*, 664-77.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-72.

Taniuchi, I., Kitamura, D., Maekawa, Y., Fukuda, T., Kishi, H., and Watanabe, T. (1995). Antigen-receptor induced clonal expansion and deletion of lymphocytes are impaired in mice lacking HS1 protein, a substrate of the antigen- receptor-coupled tyrosine kinases. Embo J *14*, 3664-78.

Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998). A mammalian scaffold complex that selectively mediates MAP kinase activation. Science *281*, 1671-4.

Zhou, G., Lee, S. C., Yao, Z., and Tan, T. H. (1999). Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. J Biol Chem *274*, 13133-8.

APPENDIX

HPK1 MUTANT AND FUSION CONSTRUCTS

Figure 1: Schematic representation of HPK1 mutant constructs

Multiple HPK1 mutant constructs were generated and subcloned into the pcdef3 expression vector. They are names as K46E (kinase-dead), dK (kinase domain-deleted), dKP (kinase domain and proline-rich region-deleted), Ko (kinase domain only), dC (CNH domain-deleted), and Y379F. dP1/2/4/12/14/124 (proline-rich motif(s)-deleted), HPK1 constructs were obtained from Friedemann Kiefer. All constructs are C-terminally HA tagged.

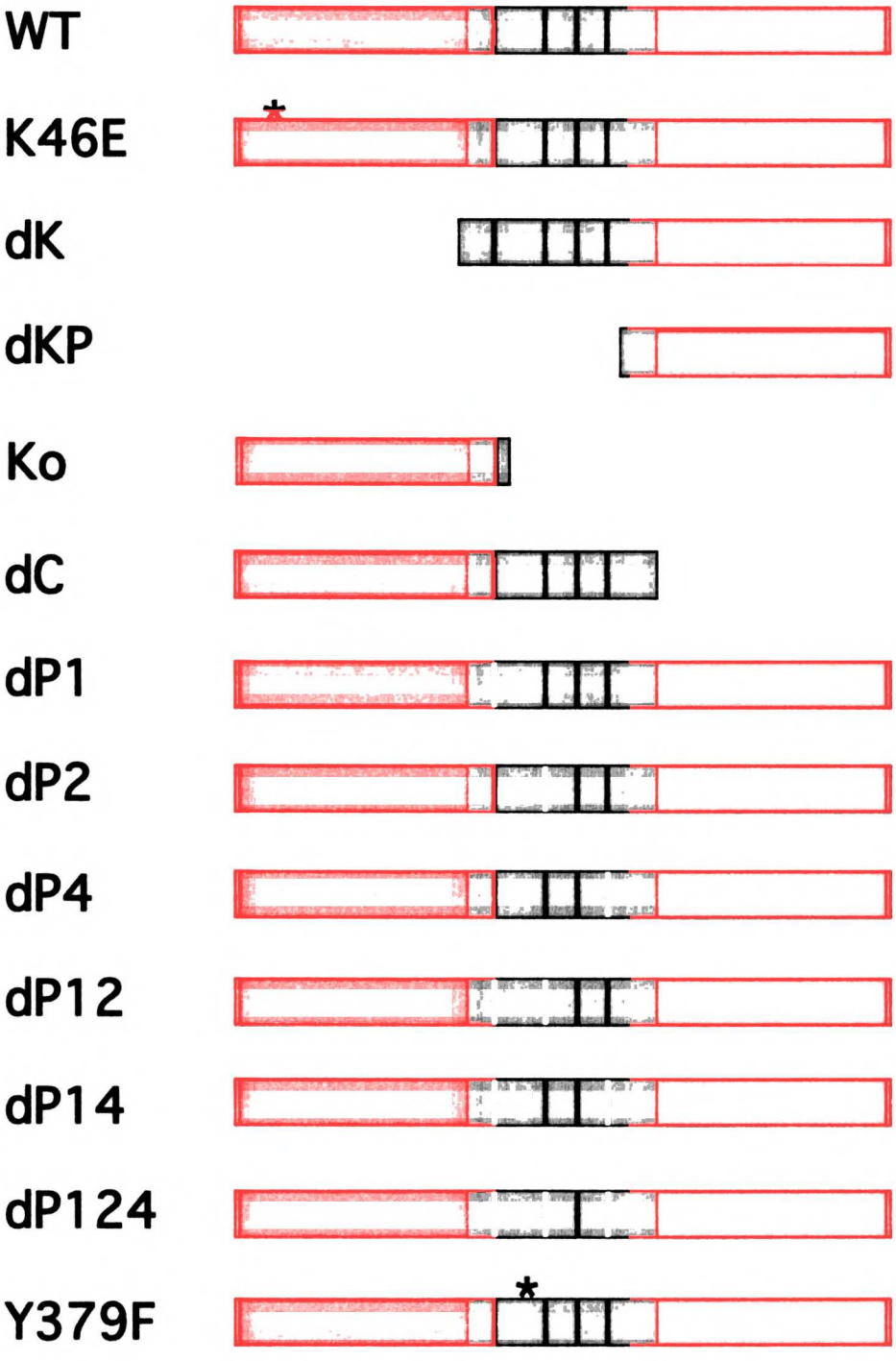
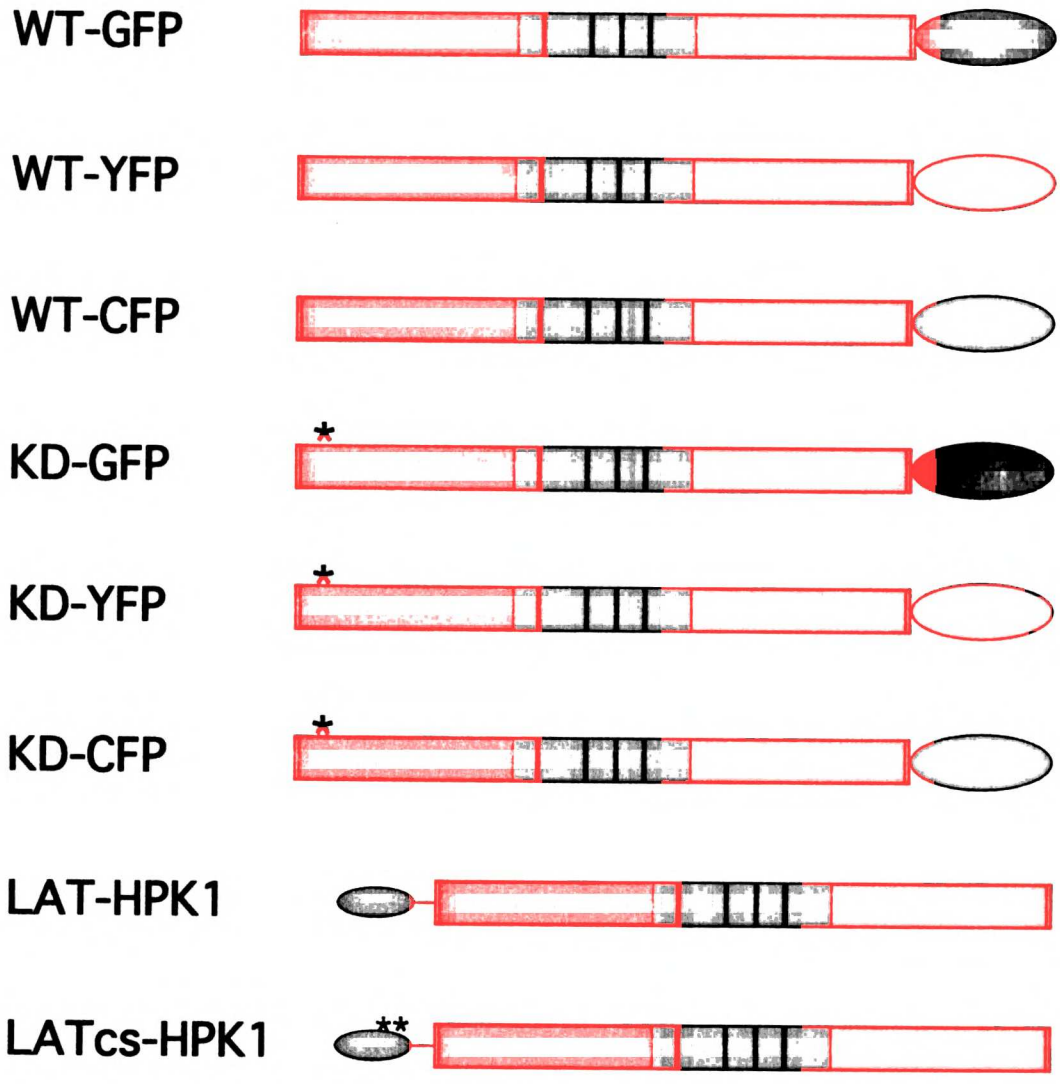

WT
K46E
dK
dKP
Ko
dC
dP1
dP2
dP4
dP12
dP14
dP124
Y379F

Figure 2: Schematic representation of HPK1 fusion constructs

Multiple HPK1 fusion constructs were generated in pcdef3 expression vectors. GFP, YFP and CFP were fused to the C-termini of wildtype and kinase-dead HPK1. The LAT-HPK1 construct contains the extracellular and transmembrane domains of LAT, a tandem Glycine linker, and the full-length HPK1. A similar construct was generated with the introduction of two Cysteine to Serine mutations in the residue 26 and 29 of LAT. LAT containing these mutations failed to localize into the GEMs.


WT-GFP
WT-YFP
WT-CFP
KD-GFP
KD-YFP
KD-CFP
LAT-HPK1
LATcs-HPK1
*
*
*
**