HIP-55 (hematopoietic progenitor kinase 1 (HPK1)-interacting protein of 55 kDa, also called SH3P7 and mAbp1) is a novel SH3 domain-containing protein. HIP-55 binds to actin filaments both in vitro and in vivo. HIP-55 activates HPK1 and c-Jun N-terminal kinase (JNK), which are two important lymphocyte signaling molecules. Until now, the regulation and function of HIP-55 in T cell receptor (TCR) signaling were unknown. We found that HIP-55 was recruited to glycolipid-enriched microdomains upon TCR stimulation, which indicates that HIP-55 is regulated by TCR signaling. HIP-55 interacted with ZAP-70, a critical protein-tyrosine kinase in TCR signaling, and this interaction was induced by TCR signaling. ZAP-70 phosphorylated HIP-55 at Tyr-334 and Tyr-344 in vitro and in vivo, and the HIP-55 mutant (Y334F/Y344F) was not tyrosine-phosphorylated in stimulated T cells. To study its function in T cell activation, HIP-55-deficient Jurkat T cells were established using the RNA interference approach. In the HIP-55-deficient cells, TCR (but not UV)-stimulated JNK activation was decreased. Furthermore, the activation of HPK1, a known JNK upstream activator and HIP-55-interacting protein, was also decreased in the HIP-55-deficient cells. Our data reveal the regulation of HIP-55 during TCR signaling, and using a genetic approach, we demonstrate for the first time that HIP-55 plays a functional role in TCR signaling.

The activation of T cells, which requires a primary signal from T cell antigen receptor (TCR)1 and a co-stimulatory signal from CD28, is a critical step for the optimal regulation of immune responses (1). Engagement of the TCR triggers a cascade of signaling events. First, it initiates the phosphorylation of TCR-associated CD3 by the Src family protein-tyrosine kinase (70-kDa zeta-associated protein-tyrosine kinase, ZAP-70) (2). Then the activated protein-tyrosine kinases transmit the TCR signals to the downstream molecules, including LAT, phospholipase Cγ, and SLP-76. LAT is localized in specific plasma membrane compartments known as glycolipid-enriched microdomains (GEMs), where the phosphorylated form of LAT acts as a docking protein for several adaptor proteins, including Gads and Grb2 (3, 4). The LAT-associated adaptors further recruit various signaling molecules to the GEMs, which are activated within GEMs. Thus, TCR signaling leads to multiple downstream signaling events, such as c-Jun N-terminal kinase (JNK) activation, ERK activation, the release of intracellular Ca2+, and increased interleukin-2 production.

JNK activation is an important downstream event after TCR stimulation. The JNK family belongs to the mitogen-activated protein kinase (MAPK) superfamily, which also comprises the ERK and p38 families (5). Activation of MAPKs goes through a conserved signaling cascade: MAPK kinases, MAPK kinase kinases, and sometimes MAPK kinase kinases (6). In T cells, JNK is implicated in the integration of TCR and CD28 signals, which is required for T cell activation and interleukin-2 production (7). Furthermore, both ERK and JNK activities are significantly reduced during T cell anergy (8). The JNK signaling cascade has also been shown to regulate the stabilization of interleukin-2 mRNA through its 5′-untranslated region (9). Studies from jnk1-null and jnk2-null mice indicate that both JNK1 and JNK2 are required for the differentiation of CD4+ T cells into effector Th1 cells (10, 11). These results highlight the importance of the JNK signaling cascade in T cell activation and differentiation.

Hematopoietic progenitor kinase 1 (HPK1) is a hematopoietic-specific mammalian Ste20-like protein serine/threonine kinase (12, 13) and a member of the MAPK kinase kinase family of kinases. HPK1 is a 97-kDa kinase with restricted expression in hematopoietic organs and cells. HPK1 is an upstream kinase of JNK and is involved in lymphocyte antigen receptor signaling (14, 15). HPK1-interacting protein of 55 kDa (HIP-55, also called SH3P7 and mAbp1) is a novel SH3 domain-containing protein that is conserved between humans (16) and mice (17). HIP-55 has an actin-binding domain at its N terminus and a SH3 domain at its C terminus (17). Mouse HIP-55 (SH3P7/mAbp1) co-localizes with actin filaments in vivo (17) and binds to filamentous actin in vitro (18). HIP-55 interacts with dynamin, a GTPase that is involved in endocytosis (19). HIP-55 is important for receptor-mediated endocytosis (20). HIP-55 has one caspase recognition site and is cleaved during apoptosis (21). HIP-55 interacts with HPK1 (16), a serine/threonine protein kinase involved in T cell receptor signaling (14, 15), and HIP-55 enhances the kinase activity of HPK1 and JNK in overexpression systems (16). Thus, the collective evidence suggests that HIP-55 may be involved in T lymphocyte signaling.

Until now, the role of HIP-55 in TCR signaling was unclear.
In this paper, we found that HIP-55 translocated into GEMs upon TCR stimulation. ZAP-70 interacted with and phospho-
rylated HIP-55 at tyrosine 334 and 344. Furthermore, we estab-
lished HIP-55-deficient Jurkat T cells using the RNA inter-
ference approach. In the HIP-55-deficient T cells, JNK activa-
tion upon TCR stimulation, but not UV irradiation, was par-
tially defective. In contrast, ERK activation by TCR stimu-
lation was unaffected in HIP-55-deficient T cells. Moreover, the activa-
tion of HPK1, a known JNK upstream activator, was also de-
creased in the HIP-55-deficient cells. Our results reveal the regu-
lation of HIP-55 during TCR signaling, and using a genetic ap-
proach, our data show for the first time that HIP-55 plays a func-
tional role in TCR signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stimulation**—Human embryonic kidney (HEK) 293T cells were maintained and transfected as previously described (22). Jurkat T cells were cultured in RPMI medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. For T cell stimula-
tion, Jurkat cells were resuspended in ice-cold RPMI medium without fetal calf serum and then mixed with anti-CD3 antibody (OKT3, 3 
\( \mu \text{g/ml} \)) at 4 °C for 10 min. Rabbit anti-mouse antibodies (3 μg/ml) were added and mixed for another 10 min at 4 °C to cross-link the primary antibody. The cells were then placed in a 37 °C water bath to allow stimulation and harvested at the indicated time points. For 6 mM pervanadate preparation, 20 μM of 10 mM Na3VO4, 33 μM of 30% H2O2, and 80 μl of H2O were mixed and incubated at room temperature for 5 min. The cells were lysed by Triton X-100 lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, freshly supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 3 μg/ml aprotinin).

**Antibodies**—The anti-HIP-55 antibody (Ab571), anti-JNK antibody (Ab102), and anti-HPK1 (Ab484) were described previously (16, 23). The anti-CD3 mAb (OKT3) was purified from hybridoma supernatants on a protein G affinity column using standard protocols. The anti-
ZAP-70 mAb was purchased from BD Transduction Lab (San Diego, CA). The anti-LAT antibody and anti-phosphotyrosine mAb (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-c-Myc (9E10), anti-ERK2 (C-14), and anti-Lck (3AS) mAbs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-FLAG mAb (M2) was purchased from Sigma. The anti-HA mAb (12C5) was purchased from Roche Applied Science. The anti-His-
mAb was purchased from Qiagen.

**Plasmids and Transformations**—The FLAG-tagged HIP-55, FLAG-tagged HIP-55 (Y334F/Y344F) mutants, HA-tagged HIP-55 (Y334P), HA-tagged HIP-55 (Y334F), HA-tagged SH2-mutated HIP-55 (W430K), and HIP-55 truncated (amino acids 1–144, tagged G418, and 145–300) were cloned into mammalian expression vector pcDNA3.1-TOPO. Myc-
tagged ZAP-70 and Lck constructs were gifts from Dr. R. L. Wange (National Institute of Aging, NIH, Baltimore, MD). For glutathione-
transferase (GST)-HIP-55 protein construction, HIP-55, HIP-55 (amino acids 1–144, tagged GST, and 145–300) were cloned into mammalian expression vector pcDNA3.1-TOPO. Myc-
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tagged ZAP-70 and Lck constructs were gifts from DNA sequencing (4, 25). Many critical signaling molecules are consti-
tutively enriched in or recruited to the GEMs upon TCR sig-
naling. Disruption of GEMs leads to the down-regulation of

**RESULTS**

**HIP-55 Translocates into GEMs upon TCR Stimulation**—It is known that GEMs are important lipid microdomains for TCR signaling (4, 25). Many critical signaling molecules are consti-
tutively enriched in or recruited to the GEMs upon TCR sig-
naling. Disruption of GEMs leads to the down-regulation of

**HIP-55 Interacts with ZAP-70**—Lck and ZAP-70 are two important tyrosine kinases in TCR signaling, and their activa-
tion is an early event in TCR signaling (2). After the activation of Lck and ZAP-70 by TCR signaling, phosphorylate the downstream signaling molecules, including LAT, SLP-76, and phospholipase Cγ1. Because HIP-55 may be involved in TCR signaling, it is possible that HIP-55 can interact with Lck or ZAP-70. To examine the interaction between HIP-55 and Lck or ZAP-70, FLAG-tagged HIP-55 was co-transfected into HEK293T cells with Myc-tagged ZAP-70, Lck, or both. HIP-55 was then immunoprecipitated, and its association with ZAP-70 or Lck was examined by Western blotting. From the anti-
HIP-55 immunoprecipitates, we detected the associated 
ZAP-70 (Fig. 2A). In a reciprocal experiment, HIP-55 was also

**GEM Fractionation**—GEM fractions preparation was previously de-
scribed (25). Briefly, Jurkat T cells (5 × 10^7) were either unstimulated or stimulated with an anti-CD3 mAb (OKT3, 10 μg/ml) cross-linked by a rabbit anti-mouse secondary antibody (5 μg/ml) for 2 min. The cells were collected and lysed in 1 ml of 1% Triton X-100 lysis buffer. The cell
lysates were well mixed with 1 ml of 80% sucrose and then transferred to ultracentrifuge tubes. These mixtures were overlaid with 2 ml of 30% sucrose and 1 ml of 5% sucrose sequentially and then subjected to ultracentrifugation for 16 h at 4 °C. Twelve fractions (0.4 ml/fraction) were collected from the top to the bottom of the sucrose gradient. The first nine fractions were subjected to immunoblotting.
found in the anti-ZAP-70 immunoprecipitates (Fig. 2B). These results indicate that HIP-55 may form a complex with ZAP-70 \textit{in vivo}. We did not detect any significant interaction between HIP-55 and Lck (data not shown). To study the interaction between endogenous ZAP-70 and HIP-55 during TCR signaling, we stimulated Jurkat cells with an anti-CD3 antibody, then immunoprecipitated HIP-55, and examined the associated ZAP-70. We found that there was a weak basal interaction between endogenous ZAP-70 and HIP-55 and that this interaction was greatly enhanced upon TCR stimulation (Fig. 2C). This finding was consistent with the results of the overexpression system, which showed that the activated form of ZAP-70 strongly interacted with HIP-55 as evidenced by the interaction between HIP-55 and ZAP-70 after ZAP-70 was activated by co-transfection with Lck (Fig. 2, A and B). The results from both the HEK293T and Jurkat T cells indicate that HIP-55 interacts with activated ZAP-70.

To map the region of HIP-55 responsible for its interaction with ZAP-70, the SH3 domain mutant and three truncated forms of HIP-55 (amino acids 1-144, 145-299, and 300-431) were examined for binding to ZAP-70. The SH3 domain mutant of HIP-55 still bound to active ZAP-70; however, all three truncated forms of HIP-55 failed to bind to ZAP-70, although they were detectable by Western blotting in cell lysates (Fig. 2D). Our results show that the tyrosine mutant (Fig. 2, A and B) and SH3 domain mutant (Fig. 2D) of HIP-55 are still able to bind to ZAP-70, but no truncated forms of HIP-55 (the N terminus, C terminus, or middle part) can bind to ZAP-70. Thus, it is likely that multiple regions of HIP-55 are required for its interaction with ZAP-70. It is noted that there is a difference between the predicted and apparent molecular weights for the three truncated forms of HIP-55. It is likely that the three truncated forms of HIP-55 have different
structural conformations, which causes the difference between the predicted and apparent molecular weights.

HIP-55 Is Tyrosine-phosphorylated by ZAP-70 at Tyr-334 and Tyr-344 in Vitro and in Vivo—To investigate the functional relevance of the interaction between HIP-55 and ZAP-70, we examined the ability of ZAP-70 to phosphorylate HIP-55 both in vitro and in vivo. We immunoprecipitated Myc-tagged ZAP-70 from transfected HEK293T cells and used purified GST-HIP-55 as a substrate for a ZAP-70 in vitro kinase assay. HIP-55 was phosphorylated after incubation with ZAP-70, which was activated by co-transfection with Lck (Fig. 3A, left panel). We also found that transfected ZAP-70 alone weakly phosphorylated HIP-55 in vitro (Fig. 3A, left panel), and this may be due to the weak activation of ZAP-70 induced by auto-phosphorylation in the overexpression system; activated/phosphorylated ZAP-70 was detected as the upper band of the ZAP-70 doublet bands by Western blotting (Fig. 2B). To map the phosphorylation sites of HIP-55 by ZAP-70, several truncated GST-HIP-55 constructs were generated and examined as substrates in an in vitro ZAP-70 kinase assay. We found that only HIP-55 (145–365) could be phosphorylated by ZAP-70 in vitro (Fig. 3A, left panel), although by Coomassie Blue staining, all of the GST fusion proteins were expressed (Fig. 3A, right panel). This indicates that the potential phosphorylation sites may lie within amino acids 145–365 of HIP-55.

Within the amino acids 145–365 of HIP-55, there are two tyrosine residues, Tyr-334 and Tyr-344, followed by two proline residues at the Y/H/HH10013 and Y/H/HH10014 positions, which are consensus phosphorylation motifs for tyrosine kinases (17). To test whether these two residues are possible phosphorylation targets of ZAP-70, we generated HIP-55 mutants, which carry either single or double tyrosine to phenylalanine mutation at residues 334 and 344. After co-transfection with ZAP-70 and Lck in HEK293T cells, both the wild-type and mutant HIP-55 were immunoprecipitated and examined by Western blotting using an anti-phosphotyrosine antibody. We found that a single mutation at Tyr-334 or Tyr-344 decreased the phosphorylation levels of HIP-55 by ZAP-70, and mutation of both sites abolished the phosphorylation of HIP-55 by ZAP-70 (Fig. 3, B and C). These results indicate that HIP-55 is tyrosine-phospho-
rylated by ZAP-70 at Tyr-334 and Tyr-344.

To further investigate the tyrosine phosphorylation of HIP-55 in T cells, FLAG-HIP-55 and FLAG-HIP-55-FF (Y334F/Y344F) stably transfectect Jurkat T cells were established. Transfected HIP-55 protein levels were examined by Western blotting using an anti-FLAG antibody. Both wild-type and mutant HIP-55 were expressed in these stably transfected Jurkat clones (Fig. 4A). To confirm the tyrosine phosphorylation sites of HIP-55 in T cells, these two stably transfected Jurkat clones were treated with pervanadate, which is a potent phosphatase inhibitor. After stimulation, the transfected wild-type and mutant HIP-55 were immunoprecipitated with an

FIG. 4. HIP-55 is tyrosine-phosphorylated by ZAP-70 at Tyr-334 and Tyr-344 in vivo. A, the FLAG-HIP-55 and FLAG-HIP-55-FF (Y334F/Y344F) stably transfected Jurkat T cells were lysed and examined by Western blotting (WB) using an anti-FLAG antibody. β-Actin serves as a control to show equal loading of protein. B, FLAG-HIP-55 (1 × 10⁷) and Y334F/Y344F (Flag-HIP-55-FF) (2 × 10⁷) stably transfected Jurkat T cells were treated with 100 μM pervanadate. After 2 min of pervanadate treatment, the cells were collected, and FLAG-HIP-55, Y334F/Y344F, and endogenous HIP-55 were immunoprecipitated (IP) with an anti-FLAG or anti-HIP-55 antibody and resolved by 10% SDS-PAGE. The tyrosine-phosphorylated HIP-55 was detected with an anti-phosphotyrosine antibody.

FIG. 5. HIP-55-deficient Jurkat cell lines. A, after establishing HIP-55-deficient Jurkat T cells, the cell lysate (50 μg) from vector transfected and HIP-55 siRNA transfected cells (Mixed population) were resolved by 10% SDS-PAGE, and the HIP-55 and β-actin protein levels were examined by Western blotting (WB, left panel). After establishing HIP-55-deficient single clones, the cell lysate (50 μg) from parental cells, vector-transfected cells, and HIP-55 siRNA transfected cells were resolved by 10% SDS-PAGE, and the HIP-55 and γ-tubulin protein levels were examined by Western blotting using anti-HIP-55 and anti-γ-tubulin antibodies (right panel). B, cell lysate from (50 μg) from parental cells, vector-transfected cells, and two independent HIP-55 siRNA stably transfected clones was resolved by 10% SDS-PAGE. The HIP-55, Lck, HPK1, LAT, and β-actin protein levels were examined by Western blotting using the indicated antibodies.
anti-FLAG antibody and examined by Western blotting using an anti-phosphotyrosine antibody. Phosphorylation was found in the wild-type HIP-55 transfected T cells but not in the FLAG-HIP-55-FF (Y334F/Y344F) mutant transfected cells or untransfected Jurkat cells (Fig. 4B). In summary, this result indicates that HIP-55 is phosphorylated on Tyr-334 and Tyr-344 in the stimulated Jurkat T cells.

JNK Activation Induced by TCR Signaling Is Defective in HIP-55-deficient Jurkat T Cells—To further study the function of HIP-55 in TCR signaling, we used the RNA interference approach (24) to stably knock down HIP-55 protein expression in Jurkat T cells. A HIP-55 siRNA construct was generated as described under “Experimental Procedures.” The empty vector or HIP-55 siRNA vector was transfected into Jurkat T cells by electroporation, and transfected cells were selected with puromycin for 4 weeks. In the puromycin-resistant mixed population, the protein level of HIP-55 was reduced (Fig. 5A, left panel). Then the puromycin-resistant single clones were established by the limiting dilution method. HIP-55 protein levels were examined by Western blotting in the parental, empty vector-transfected, and HIP-55 siRNA-transfected Jurkat T cells. As expected, HIP-55 expression was significantly reduced in Jurkat cells stably transfected with HIP-55 siRNA. HIP-55 levels in HIP-55 siRNA-transfected cells were reduced by 80–90% as compared with the levels in the vector-transfected or parental cells (Fig. 5A, right panel). Western blotting was performed using an anti-β-tubulin antibody as a control to show equal loading (Fig. 5A, right panel). The expression levels of other molecules involved in the TCR signaling were also examined by Western blotting. We found that the protein levels of Lck, HPK1, and LAT were not affected in two independent HIP-55-deficient clones (Fig. 5B), indicating that the RNA interference effects are specific.

HIP-55 was shown to activate JNK in an overexpression system (16), but its effect on JNK in physiological conditions was not clear. JNK is activated upon TCR stimulation, and this activation is an important event for T cell activation and cytokine production. To determine whether HIP-55 affects the downstream events of TCR signaling, JNK activation was studied in the empty vector-transfected and HIP-55 siRNA-transfected Jurkat T cells. As expected, HIP-55 expression was significantly reduced in Jurkat cells stably transfected with HIP-55 siRNA. HIP-55 levels in HIP-55 siRNA-transfected cells were reduced by 80–90% as compared with the levels in the vector-transfected or parental cells (Fig. 5A, right panel). Western blotting was performed using an anti-β-tubulin antibody as a control to show equal loading (Fig. 5A, right panel). The expression levels of other molecules involved in the TCR signaling were also examined by Western blotting. We found that the protein levels of Lck, HPK1, and LAT were not affected in two independent HIP-55-deficient clones (Fig. 5B), indicating that the RNA interference effects are specific.

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HIP-55 Mediates TCR Signaling

**Hip-55 Deficiency Affects JNK and ERK Activation**

In HIP-55-deficient Jurkat T cells, the induction of HPK1 kinase activity upon pervanadate treatment was not affected in the HIP-55-deficient cells (Fig. 7B). Our results suggest that HIP-55 plays a role in HPK1 activation induced by TCR signaling.

**Discussion**

HIP-55 is a novel SH3 domain-containing adaptor protein that was proposed to be involved in lymphocyte receptor signaling (16, 17). However, it was unclear whether and how HIP-55 is involved in TCR signaling. We investigated the involvement of HIP-55 in T cell signaling and found that upon TCR stimulation, HIP-55 was recruited to GEMs. We found an interaction between the tyrosine kinase ZAP-70 and HIP-55, which was induced by TCR stimulation. ZAP-70 phosphorylated HIP-55 at Tyr-334 and Tyr-344, which were shown to be the tyrosine phosphorylation sites of HIP-55 in stimulated T cells. When we stably knocked down HIP-55 expression in Jurkat T cells using the RNA interference approach, JNK activation induced by TCR stimulation, but not UV irradiation, was partially abolished. In contrast, TCR-induced ERK activation was not affected in HIP-55-deficient Jurkat T cells. Furthermore, the activation of HPK1, a known JNK upstream activator, was also decreased in the HIP-55-deficient Jurkat cells. Our results demonstrate for the first time that HIP-55 is an important adaptor protein for the JNK kinase cascade in TCR signaling.

GEMs are microdomains critical for TCR signaling. Many important molecules are recruited or constitutively localized to GEMs. The disruption of GEMs decreases the TCR signaling (4, 26). HIP-55 was also recruited to GEMs upon TCR stimulation, suggesting that HIP-55 plays a role in TCR signaling. We demonstrated that the activated form of ZAP-70 interacted with and phosphorylated HIP-55 in vitro and in vivo. The interaction sites between ZAP-70 and HIP-55 are unclear; although there are two SH2 domains in ZAP-70, the HIP-55 (Y334F/Y344F) mutant still interacted with ZAP-70 (Fig. 2A), suggesting that these two tyrosine residues are not critical for the binding. We found that the SH3 mutant of HIP-55 was still able to bind to ZAP-70. However, we did not detect any interaction between ZAP-70 and three truncated forms of HIP-55 (the N terminus, the C terminus, and the middle region). It is likely that multiple regions of HIP-55 may regulate its interaction with ZAP-70.

Tyro-334 and Tyr-344 of HIP-55, which are phosphorylated by ZAP-70, are conserved among some HIP-55 related molecules. HS1, another actin-binding protein, also contains two of the conserved tyrosine phosphorylation sites targeted by Syk (27, 28). These two tyrosine residues within HS1 are critical for B cell receptor-induced apoptosis (27), which may provide insight about the potential function of HIP-55 in TCR signaling. Cortactin is another actin-binding protein that can also be tyrosine-phosphorylated by the Src family kinases. The tyrosine phosphorylation of cortactin by Src kinases inhibits its actin cross-linking activity in vitro (29). It will be interesting to investigate whether HIP-55 has a similar regulatory mechanism to control the cytoskeleton reorganization upon TCR stimulation.

When overexpressed, HIP-55 activates JNK in HEK293 cells (16); however, it was unclear whether HIP-55 was involved in the JNK activation pathway under physiological conditions. Our data show that JNK activation induced by TCR stimulation was decreased in the HIP-55 knockdown T cells, suggesting that HIP-55 is an important adaptor for the JNK cascade in TCR signaling. However, the underlying mechanism by which HIP-55 regulates JNK activation induced by TCR stimulation remains unclear. We found that HPK1 activation induced by

**Fig. 7. HPK1 Activation Induced by TCR Stimulation Is Defective in HIP-55-deficient Jurkat T Cells.** A, empty vector and HIP-55 siRNA stably transfected Jurkat cells were stimulated by an anti-CD3 mAb, and the cells were collected at the indicated time points. Endogenous HPK1 was immunoprecipitated (IP) by an anti-HPK1 antibody (Ab484) from cell lysate (100 μg), and HPK1 kinase activity was examined by immunocomplex kinase assays using myelin basic protein as a substrate. B, empty vector and HIP-55 siRNA stably transfected Jurkat cells were stimulated by 100 μM pervanadate for 5 min. The cells were collected, and the HPK1 kinase assays were performed as described above.

**HIP-55 Mediates TCR Signaling**

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TCR signaling was also affected by HIP-55 deficiency. Potentially, HIP-55 may facilitate the activation of HPK1 or other upstream kinases, which in turn activate the JNK cascade. It is also possible that HIP-55 functions through ZAP-70 to affect downstream events in TCR signaling. We only observed partial defects in JNK activation; this may be because only 80–90% of the HIP-55 was knocked down by HIP-55 siRNA, and the remaining HIP-55 could still partially activate JNK, but we cannot exclude the possibility that there are other alternate pathways that could activate JNK, independent of HIP-55, in TCR signaling.

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REFERENCES
1. Wange, R. L., and Samelson, L. E. (1996) Immunity 5, 197–205
2. Kane, L. P., Lin, J., and Weiss, A. (2000) Curr. Opin. Immunol. 12, 242–249
3. Zhang, W., Trible, R. P., and Samelson, L. E. (1998) Immunity 9, 239–246
4. Zhang, W., and Samelson, L. E. (2000) Semin. Immunol. 12, 35–41
5. Chen, Y.-R., and Tan, T.-H. (2000) Int. J. Oncol. 16, 651–662
6. Chen, Y.-R., and Tan, T.-H. (1999) Gene Ther. Mol. Biol, 83–98
7. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994) Cell 77, 727–736
8. Li, W., Whaley, C. D., Mondino, A., and Mueller, D. L. (1996) Science 271, 1272–1276
9. Chen, C. Y., Del Gatto-Konczak, F., Wu, Z., and Karin, M. (1998) Science 280, 1945–1949
10. Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092–2096
11. Yang, D. D., Conze, D., Whitmarsh, A. J., Barret, T., Davis, R. J., Rincon, M., and Flavell, R. A. (1998) Immunity 9, 575–585
12. Hu, M.-C.-T., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T.-H. (1996) Genes Dev. 10, 2251–2264
13. Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Izcue, N. N. (1996) EMBO J. 15, 7013–7025
14. Ling, P., Meyer, C. F., Redmond, L. P., Shui, J.-W., Davis, R., Rich, R. R., Hu, M.-C.-T., Wange, R. L., and Tan, T.-H. (2001) J. Biol. Chem. 276, 18908–18914
15. Liu, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M. H., Karoski, T., and Weiss, A. (2000) Immunity 12, 399–408
16. Ensenat, D., Yao, Z., Wang, X. S., Kori, R., Zhou, G., Lee, S. C., and Tan, T.-H. (1999) J. Biol. Chem. 274, 33945–33950
17. Larbolette, O., Wollscheid, B., Schweikert, J., Nielsen, P. J., and Wienands, J. (1999) Mol. Cell. Biol. 19, 1539–1546
18. Kessels, M. M., Engvist-Goldstein, A. E., and Druhin, D. G. (2000) Mol. Biol. Cell 11, 303–412
19. Kessels, M. M., Engvist-Goldstein, A. E., Druhin, D. G., and Qualmann, B. (2001) J. Cell Biol. 153, 351–366
20. Mise-Omata, S., Montagne, B., Deckert, M., Wienands, J., and Acuto, O. (2003) Biochem. Biophys. Res. Commun. 301, 704–710
21. Chen, Y.-R., Kori, R., John, B., and Tan, T.-H. (2001) Biochem. Biophys. Res. Commun. 288, 981–989
22. Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehl, W., Feller, S. M., and Tan, T.-H. (1999) Mol. Cell. Biol. 19, 1359–1368
23. Chen, Y.-R., Wang, X., Templeton, D., Davis, R. J., and Tan, T.-H. (1996) J. Biol. Chem. 271, 31929–31936
24. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
25. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) Cell 92, 83–92
26. 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) Immunity 10, 323–332
27. Yamashita, Y., Fukuda, T., Nishizumi, H., Inazu, T., Higashi, K., Kitamura, D., Ishida, T., Yamamura, H., Watanabe, T., and Yamamoto, T. (1997) J. Cell Biol. 138, 1546–1556
28. Yamashita, Y., Okada, M., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., and Yamamoto, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3631–3635
29. Huang, C., Ni, Y., Wang, T., Gao, Y., Haudenschild, C. C., and Zhan, X. (1997) J. Biol. Chem. 272, 13911–13915