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J Immunol 2000; 165:1417-1426; doi: 10.4049/jimmunol.165.3.1417
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The Adaptor Protein Gads (Grb2-Related Adaptor Downstream of Shc) Is Implicated in Coupling Hemopoietic Progenitor Kinase-1 to the Activated TCR

Stanley K. Liu,*2 Christian A. Smith,2* Ruediger Arnold,† Friedemann Kiefer,† and C. Jane McGlade3*

The hemopoietic-specific Gads (Grb2-related adaptor downstream of Shc) adaptor protein possesses amino- and carboxyl-terminal Src homology 3 (SH3) domains flanking a central SH2 domain and a unique region rich in glutamine and proline residues. Gads functions to couple the activated TCR to distal signaling events through its interactions with the leukocyte-specific signaling proteins SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) and LAT (linker for activated T cells). Expression library screening for additional Gads-interacting molecules identified the hemopoietic progenitor kinase-1 (HPK1), and we investigated the HPK1-Gads interaction within the D011.10 murine T cell hybridoma system. Our results demonstrate that HPK1 indubitably associates with Gads and becomes tyrosine phosphorylated following TCR activation. HPK1 kinase activity is up-regulated in response to activation of the TCR and requires the presence of its proline-rich motifs. Mapping experiments have revealed that the carboxy-terminal SH3 domain of Gads and the fourth proline-rich region of HPK1 are essential for their interaction. Deletion of the fourth proline-rich region of HPK1 or expression of a Gads SH2 mutant in T cells inhibits TCR-induced HPK1 tyrosine phosphorylation. Together, these data suggest that HPK1 is involved in signaling downstream from the TCR, and that SH2/SH3 domain-containing adaptor proteins, such as Gads, may function to recruit HPK1 to the activated TCR complex. The Journal of Immunology, 2000, 165: 1417–1426.

The activation of tyrosine kinases is central to the initiation of signaling pathways from diverse extracellular receptors. Stimulation of Ag receptors, such as the TCR, results in rapid activation of protein tyrosine kinases such as Lck, Fyn, and Zap-70 and phosphorylation of the cytoplasmic domain of receptor accessory molecules, creating binding sites for Src homology 2 (SH2) and phosphotyrosine-binding domain-containing signaling proteins (1–6). Recruitment of cytoplasmic signaling molecules to the activated TCR couple receptor activation with multiple downstream pathways that initiate signaling events required for the transcription of lymphokine genes (2, 5–7). The critical substrates of TCR-activated protein tyrosine kinases include the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) (8), the guanine nucleotide exchange factor Vav (9), phospholipase Cγ1 (PLC-γ1) (10), c-Cbl (11), and the linker for activation of T cells (LAT) (12–15).

The three members of the Grb2 family of adaptor proteins, Grb2, Grap, and Gads, have also been implicated in the formation of protein complexes at the TCR and are important for linking specific pathways, such as Ras to TCR activation. The Grb2 family of adaptors possess amino- and carboxyl-terminal SH3 domains that flank a central SH2 domain (7, 16–21). Gads also has a 120-aa unique region between the SH2 domain and carboxy-terminal SH3 domain (20). This family of proteins couples tyrosine-phosphorylated membrane-associated proteins bound by the SH2 domain with cytoplasmic proteins associated through the SH3 domains (7, 16–22). Grb2 is ubiquitously expressed, while expression of both Grap and Gads is restricted to hemopoietic cells (18–20, 23–25). It has been proposed that Grb2 and Grap may serve overlapping functions, since their SH2 and SH3 domains appear to bind to similar proteins in T cells and Grap null mice display no apparent phenotype (26). In contrast, Gads is likely to have a unique function because it binds to distinct SH3 domain targets (20, 22).

The function of the Grb2 adaptor is best defined in the context of growth factor receptor signaling, where it couples activated receptors with downstream effector pathways, such as the evolutionarily conserved pathway involved in Ras activation (16, 26–34). The SH2 domain of Grb2 binds to activated growth factor receptors either directly or indirectly via the docking protein Shc, localizing Grb2 and the stably associated Ras guanine nucleotide exchange factor, Sos, to the membrane, resulting in the activation of Ras (28–32, 34, 35). Within T cells, Grb2 is proposed to couple TCR engagement to Ras activation through the recruitment of
Grb2-Sos complexes to tyrosine-phosphorylated LAT, a membrane-bound linker protein (14, 15, 36). By analogy, Grap may function to amplify TCR activation of Ras through recruitment of additional pools of Sos to membrane-associated LAT.

Gads also inductively associates with LAT in T cells, but unlike Grb2 and Grap, it does not form complexes with Sos (20, 22). Instead, Gads is bound to the SLP-76 adaptor protein, which plays a critical role in signaling from both pre-TCR and mature TCR (8, 37–39). Gads synergizes with SLP-76 to augment both NF-AT and IL-2 activation, and this augmentation is dependent on a functional Gads SH2 domain (22). Hence, through its association with SLP-76 and LAT, Gads appears to play a central role in signaling from the activated TCR (22, 24, 25).

To date, SLP-76 is the only Gads SH3 domain-binding protein that has been identified. In an effort to identify additional signaling effectors downstream of Gads, we used an expression screening strategy and identified hemopoietic progenitor kinase-1 (HPK1) as a Gads-binding protein. HPK1 belongs to a family of Ste20 homologous serine/threonine kinases that includes germinal center kinase (GCK), GCKR/KHS (GCK-related kinase/kinase homologous to SPS1/Ste20), and GLK (GCK-like kinase) (40). The family is defined by their N-terminally located kinase domain and the regulatory C-terminal domain, and all have been implicated as upstream effectors in activation of the stress-activated protein kinase (SAPK/c-Jun NH2-terminal kinase) pathway (40–44). Here we show that HPK1 is activated by the TCR, that Gads and HPK1 interact in vivo, and that Gads can function to link HPK1 to signaling from the activated TCR complex.

Materials and Methods

Expression library screen

A 16-day mouse embryo expression library (Novagen, Madison, WI) was plated and protein expression induced according to the manufacturer’s directions. The nitrocellulose filters were washed in TBST (1% nonfat milk powder (w/v) and 1 mM DTT in TBST) (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20) and blocked at 4°C

Materials and Methods

In vitro binding experiments

GST–Gads wild-type and mutant fusion proteins were prepared as previously described (20), and purified proteins were quantified by Coomassie staining. Wild-type or proline mutant pcDNA3-HPK1:HA-transfected COS1 cells were lysed in 1 ml of PLC lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% (v/v) glyceral, 1% (v/v) Triton X-100, 1.5 mM MgCl2, 1 mM Na3VO4, 10 mM NaPi, 100 mM NaF, 1 mM EDTA, 100 mM ZnCl2, 1% (v/v) Nonidet-P40, 10% (v/v) glycerol, and Complete protease inhibitors (Roche)), and the cellular lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Four hundred microliters of lysate was mixed with 2 μg of GST and either wild-type GST-Gads or GST-Gads mutants fusion protein conjugated to glutathione-Sepharose beads for 1 h at 4°C. The beads were washed five times with NFL lysate buffer, eluted in SDS-Laemmli sample buffer, and separated on a 10% SDS-PAGE gel, for 30 min before the addition of primary Ab for1ha troom tem-

Plasmids and Abs

The pEF-FLAG wild-type Gads and mutant Gads (N*Y*C, N*Y*C, N*Y*C, and SH2*) constructs were previously described (22). The GST–Gads fusion proteins containing a C-terminal anti-FLAG (1–22)-A) were used for in vivo binding experiments.俣

In vitro kinase assays

DO11.10 cells or lymph node-derived T cells were prewarmed at 37°C for 10 min, left unstimulated, or stimulated with soluble anti-CD3 Ab for 2 min at 37°C, followed by the addition of 700 μl of an ice-cold phosphate inhibitor mix (100 mM NaF, 10 mM NaPPi, and 1 mM Na3VO4 in PBS, pH 7.4). Cells were collected by centrifugation and lysed in 1 ml of PLC lysis buffer. The clarified lysates were incubated with the indicated Ab and 50 μl of a 20% (v/v) protein A-Sepharose bead slurry (Sigma) for 90 min at 4°C. The immune complexes were washed five times with Nonidet P-40 lysis buffer, eluted in SDS-laemmli sample buffer, and separated on a 10% SDS-PAGE gel. Proteins were electrophoretically transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were then immunoblotted with anti-HPK1 7 antisera.

Intracellular kinases

COS1 cells were maintained in DMEM supplemented with 10% (v/v) FBS, 200 mM L-glutamine, 5 U/ml penicillin C, and 5 mg/ml streptomycin sulfate. Ten micromolars of wild-type or proline-deficient HPK1 plasmids were transfected into a 100-mm dish of 50% confluent COS1 cells with lipofectin reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions, and cell lysates were prepared for in vitro binding experiments as detailed below. The murine T cell hybridoma, DO11.10, was maintained in RPMI 1640 supplemented with 10% FBS (v/v), 200 mM L-glutamine, 5 U/ml penicillin C, and 5 mg/ml streptomycin sulfate, and 55 μM 2-ME. For electroporations, 20 million cells were resuspended in 400 μl of RPMI 1640 and electroporated with a Gene Pulser (Bio-Rad, Hercules, CA) set at 250 V and 960 μF. Optimal expression of transfected constructs was obtained with 15 μg of pcDNA3-HPK1:HA and 45 μg of pEF-FLAG-Gads constructs. Primary murine T cells were isolated from dissected lymph nodes and maintained in RPMI for 4 h before stimulation with anti-CD3.

In vivo binding experiments

GST–Gads wild-type and mutant fusion proteins were prepared as previously described (20), and purified proteins were quantified by Coomassie staining. Wild-type or proline mutant pcDNA3-HPK1:HA-transfected COS1 cells were lysed in 1 ml of PLC lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% (v/v) glyceral, 1% (v/v) Triton X-100, 1.5 mM MgCl2, 1 mM Na3VO4, 10 mM NaPi, 100 mM NaF, 1 mM EDTA, 100 mM ZnCl2, 1% (v/v) Nonidet-P40, 10% (v/v) glycerol, and Complete protease inhibitors (Roche)), and the cellular lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Five hundred microliters of lysate was mixed with 2 μg of GST and either wild-type GST-Gads or GST-Gads mutants fusion protein conjugated to glutathione-Sepharose beads for 1 h at 4°C. The beads were washed five times with NFL lysate buffer, eluted in SDS-laemmli sample buffer, and separated on a 10% SDS-PAGE gel, for 30 min before the addition of primary Ab for1ha troom tem-

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Identification of HPK1 as a Gads-binding protein in activated T cells

To identify Gads-binding proteins, radiolabeled GST-Gads fusion protein was used to screen an embryonic day 16 mouse expression library. A 2.6-kb cDNA clone was isolated from this screen that encoded the full-length murine hemopoietic progenitor kinase-1 (HPK1, GenBank accession no. Y09010).

Since both Gads and HPK1 proteins are expressed in T cells, (22, 41, 45), we examined whether Gads binds to HPK1 in the DO11.10 murine T cell hybridoma. DO11.10 cells were left unstimulated or were stimulated with anti-CD3 Ab for 2 min, lysed, and immunoprecipitated with a negative control rabbit anti-mouse Ab (RoM), anti-HPK1 (HPK1), and anti-Gads Ab (Gads). Following SDS-PAGE separation and electroblotting to a PVDF membrane, phosphorylated proteins were detected in Western blot analysis using anti-phosphotyrosine Ab (α-pY; upper panel). The immunoprecipitating phosphoproteins p100, p76 (SLP-76), and p36 (LAT) are indicated with arrowheads. The blot was stripped, the top half was blotted with anti-HPK1 Ab (α-HPK1; middle panel), and the lower half with anti-Gads Ab (α-Gads; lower panel). Bands corresponding to HPK1 and Gads are denoted by an arrow.

EDTA, and once with 50 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 2 mM MnCl₂, and 1 mM DTT, and kinase reactions were performed in 20 µl of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 1 mM EDTA at 30°C for 15 min in the presence of 10 µCi of [γ-32P]ATP and 20 µg of myelin basic protein (MBP) as outlined previously (41). The kinase reactions were stopped by addition of 2X SDS-Laemmli sample buffer and boiled for 5 min, and proteins were resolved by SDS-PAGE. The proteins were electrophoretically transferred to Immobilon-P membrane, and the incorporated radiolabeled phosphate was assessed by PhosphorImager analysis using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). To confirm that equivalent levels of HPK1 were present in each kinase assay, the membrane was subsequently immunoblotted with anti-HPK1 antisera as described above.

Results

HPK1 kinase activity is up-regulated by TCR activation and requires intact proline-rich regions

It has been previously reported that activation of the TGF-β and erythropoietin receptors leads to an increase in HPK1 kinase activity (46, 47). In addition, Ling et al. have previously provided evidence that HPK1 may be involved in regulating IL-2 activation in Jurkat T cells (48). To determine whether HPK1 kinase activity is regulated by TCR engagement, in vitro kinase assays were performed on anti-HPK1 immunoprecipitates from unstimulated and anti-CD3-stimulated DO11.10 hybridoma and primary lymph node-derived T cells, using MBP as a substrate (Fig. 2A). The level of phosphorylated MBP increased by 2-fold, on the average, following TCR activation in both DO11.10 and lymph node-derived T cells, indicating that HPK1 kinase activity is up-regulated by TCR activation.

HPK1 possesses four proline-rich regions (P1, P2, P3, and P4), and three of these regions (P1, P2, and P4) have been shown to bind to SH3 domains in vitro (41, 48, 49). It has been demonstrated that SH2/SH3 domain-containing adaptor proteins can associate with HPK1, and therefore may serve to couple activated receptors with the downstream activation of HPK1 (48, 49). To examine the effect of loss of the proline-rich regions on TCR-dependent activation of HPK1 kinase activity, an HPK1 mutant was generated in which the first (P1; aa 309–311), second (P2; aa 392–401), and fourth (P4; aa 467–471) SH3 domain-binding consensus motifs were deleted in combination (ΔP1/2/4 Fig. 4A). The third (P3; aa 430–441) motif was not included in the deletion because of its poor fit to the SH3 domain-binding consensus motif, and since Anafi et al. (49) previously demonstrated that a P3 peptide was unable to compete recombinant SH3 domain binding to HPK1. HA-epitope tagged wild-type, kinase-dead, and ΔP1/2/4 HPK1 constructs were transiently expressed in DO11.10 cells, the cells were left unstimulated or were stimulated with anti-CD3 and lysed, and anti-HA immunoprecipitation was performed, followed by kinase assays using MBP as substrate. Following TCR activation, an ∼2-fold increase in HPK1 kinase activity was observed with the wild-type HPK1, while a marginal increase was observed for the kinase-dead HPK1 (Fig. 2B). The induced kinase activity of the ΔP1/2/4 HPK1 mutant was markedly reduced following TCR ligation compared with that of wild-type HPK1. The defect in activation of ΔP1/2/4 HPK1 in vivo is probably due to its inability to couple to the activated TCR rather than to a structural defect that affects the kinase domain, since the basal in vitro kinase activity of this mutant is equivalent to that of wild-type HPK1. Therefore, the proline-rich regions of HPK1 and anti-Gads immunoprecipitates from anti-CD3-stimulated DO11.10 lysates. The membranes were stripped and reprobed with anti-HPK1 Ab, identifying the 100-kDa phosphoprotein in both anti-HPK1 and anti-Gads immunoprecipitates as HPK1 and demonstrating that HPK1 is inducibly tyrosine-phosphorylated and associated with Gads in vivo following TCR ligation (Fig. 1). Following prolonged exposure of the blot to film, only a very small amount of HPK1 was detected in Gads immunoprecipitates before stimulation (data not shown). The identities of the 130- and 180-kDa phosphoproteins present in the anti-Gads immunoprecipitate from anti-CD3-stimulated lysate are unknown at this time. Gads was not detected in anti-HPK1 immunoprecipitates, suggesting that the anti-HPK1 Abs do not recognize HPK1 when complexed with Gads. HPK1 was also detected in Gads immunoprecipitates from primary lymph node-derived murine T cells following anti-CD3 stimulation (data not shown).

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A. DO11.10 T cells

IP: α-HPK1
α-CD3: - +
kinase assay

MBP

 blot: α-HPK1

Lymph Node T cells

IP: RαM HPK1
α-CD3: - + - +
kinase assay

MBP

 blot: α-HPK1

B. α-CD3:

wt KD ΔP1/2/4
- + - + - +

MBP

 IP: α-HA kinase assay

HPK1

 blot: α-HPK1

Graphs showing phosphorylation levels of MBP and HPK1 under different conditions.
FIGURE 2. A, HPK1 kinase activity is up-regulated following TCR activation. DO11.10 cells, starved overnight in 0.5% FCS containing RPMI 1640 medium, or freshly isolated lymph node-derived murine T cells were left unstimulated or were stimulated (5 × 10⁵ cells equivalents/condition) with soluble anti-CD3 Ab for 5 min. Cells were lysed and immunoprecipitated with anti-HPK1 Ab (α-HPK1), and the immune complexes were subjected to in vitro kinase assays, using MBP as a substrate (upper panel). The proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and incorporated radiolabeled phosphate was quantitated by PhosphorImager analysis; these values are depicted in chart format (middle panel), and are representative of at least three independent experiments. The membrane was immunoblotted with anti-HPK1 Ab to confirm that equivalent levels of HPK1 were present (left panel). Representative autoradiographs of the MBF phosphorylation and anti-HPK1 immunoblotting are shown. The bands corresponding to MBP and HPK1 are denoted.

B, HPK1 kinase activity up-regulation requires intact proline-rich regions (PRR). DO11.10 murine T cells (2.0 × 10⁵) were transiently transfected by electroporation with 20 μg of pcDNA3-HPK1:HA wild-type (wt), pcDNA3-KD HPK1:HA (KD) or pcDNA3-triple PRR (ΔP1/2/4):HA lacking the first, second, and fourth PRR. Twenty-four hours postelectroporation, the cells were left unstimulated or were stimulated with soluble anti-CD3 Ab for 5 min at 37°C in RPMI 1640 medium. Cellular lysates were prepared and immunoprecipitated with anti-HA Ab (IP: α-HA), and immune complexes were subjected to in vitro kinase assays, using MBP as a substrate (upper panel). The proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and incorporated radiolabeled phosphate was quantitated by PhosphorImager analysis; these values are depicted in chart format (lower panel), and are representative of at least three independent experiments. The membrane was immunoblotted with anti-HPK1 Ab to confirm that equivalent levels of HPK1 were present (middle panel). Representative autoradiographs of the MBF phosphorylation and anti-HPK1 immunoblotting are shown. The bands corresponding to MBP and HPK1 are denoted.

Since the Gads-HPK1 association was an SH3 domain-mediated interaction, we reasoned that the Gads binding site was probably within one of the four proline-rich regions (P1–P4) of HPK1. Additional HPK1 mutants were generated in which the core SH3 domain-binding consensus motifs were deleted individually or in combination (Fig. 4A). The binding of GST-Gads fusion protein to HPK1 mutants that possessed individual or combined deletions of the first, second, and fourth proline-rich regions was assayed; the third proline-rich region was not removed due to reasons described above (Fig. 4B). Deletion of the first or second proline-rich region of HPK1, either individually or together, did not eliminate the Gads-HPK1 interaction. However, all HPK1 mutants that lacked the fourth proline-rich region of HPK1 were no longer precipitated by GST-Gads, suggesting that the major Gads binding site on HPK1 is within the fourth proline-rich region (Fig. 4B). Deletion of the second proline-rich region reduced binding to HPK1, whereas loss of the fourth proline-rich region abolished the interaction. Together, these results suggest that the while the fourth proline-rich region is critical for association between Gads and HPK1, the second proline-rich region may partially contribute to the interaction. To confirm the role of the Gads SH3 domains and the HPK1 proline-rich regions in the Gads-HPK1 interaction in vivo, full-length epitope-tagged Gads and HPK1 cDNAs with mutations in the domains important for the interaction were cotransfected into DO11.10 cells. Wild-type HA-HPK1 was efficiently coimmunoprecipitated with FLAG-Gads; however, deletion of all three proline-rich regions (P1/P2/P4) or the P4 region alone resulted in loss of HPK1-Gads association (Fig. 5A, left panel). An inactivating mutation in the Gads SH2 domain (Gads-SH2*) had no effect on the ability of FLAG-Gads to coprecipitate HA-HPK1, while mutation of the SH3 domains of Gads (Gads-N*/*C*) abolished the interaction (Fig. 5A, right panel). Equivalent expression of the HA-HPK1 and FLAG-Gads proteins was confirmed by Western blots of whole cell lysates with anti-HA and anti-FLAG Abs (Fig. 5B).

Efficient HPK1 tyrosine phosphorylation requires the presence of the second or fourth proline-rich region

Recruitment of HPK1 to the proximity of activated receptors by SH2/SH3 domain-containing adaptor proteins has been shown to facilitate the subsequent tyrosine phosphorylation of HPK1 in response to activation of the epidermal growth factor and erythropoietin receptors (47, 49). To test whether the Gads-binding region, P4, is required for HPK1 tyrosine phosphorylation following TCR activation, we transiently expressed HPK1 mutants lacking probably SH3 domain-containing adaptors are important in coupling regulation of HPK1 activity to TCR activation.

The carboxyl-terminal SH3 domain of Gads mediates binding to the fourth proline-rich region of HPK1

To map the domain of Gads responsible for interacting with HPK1, recombinant full-length GST-Gads fusion proteins with inactivated SH3 or SH2 domains were assayed for their ability to precipitate HPK1 from transfected COS1 cells. Inactivation of both SH3 domains (N*/C*) or inactivation of the carboxyl-terminal SH3 domain (N*C*) eliminated the interaction between Gads and HPK1; whereas mutation of the SH2 domain (SH2*) had no effect on the ability of Gads to precipitate HPK1 in vitro (Fig. 3). Similar experiments performed in DO11.10 cells also show that the carboxyl-terminal SH3 domain is required for binding to endogenous HPK1 following activation of the TCR (data not shown).
the various proline-rich regions in DO11.10 cells and examined the tyrosine phosphorylation status of these HPK1 mutants following anti-CD3 stimulation (Fig. 6). Loss of the second or fourth proline-rich region in HPK1 led to a substantial decrease in the tyrosine-phosphorylation status of HPK1 following TCR ligation, while loss of the first proline-rich region had no effect. Together, these data suggest that the second and fourth proline-rich regions are important for the efficient tyrosine phosphorylation of HPK1.

**FIGURE 4.**

**A**, Structure of SH3 binding site mutants in HPK1. The cluster of four proline-rich motifs located in the central portion of HPK1 and sequences of the individual polyproline stretches are depicted. Three of these motifs (P1, P2, and P4) conform to the sequence consensus for SH3 binding sites in class II orientation (underlining arrow). All four motifs are schematically depicted as left-handed polyproline type II helices lining the relatively conserved hydrophobic binding pocket of an SH3 domain. P1, P2, and P4 fit the sequence consensus very well and are shown in negative orientation, P3 fits the sequence consensus poorly and is shown in positive orientation (52, 53). The binding positions were assigned as follows: motif P1, C-R(P-3) R(P-2) P(P-1) I(P-0) A(P-1) P(P-2) P(P-3) N; motif P2, C-K(P-3) P(P-2) P(P-1) I(P-0) P(P-1) P(P-2) P(P-3) N; motif P3, N-P(P-3) R(P-1) P(P-0) G(P-1) P(P-2) P(P-3) C; and motif P4, C-R(P-3) P(P-2) P(P-1) V(P-0) L(P-1) P(P-2) P(P-3) N. Positions requiring proline residues, P-1/P2 in negative orientation and P0/P3 in positive orientation are marked by asterisks. The SH3 domain binding sites P1, P2, and P4 were removed singly and in combination by introduction of the amino acid deletions depicted in the diagram at the lower part of the figure.

**B**, The fourth proline-rich region (PRR) of HPK1 is required for Gads binding. Lysates were prepared from COS1 cells transfected with 15 μg of wild-type pcDNA3-HPK1:HA (wt) or mHPK1 mutant constructs with deletions of the triple PRR (ΔP1/2/4), the first PRR (ΔP1), the second PRR (ΔP2), the fourth PRR (ΔP4), the first and second PRR (ΔP1/2), and the first and fourth PRR (ΔP1/4). Lysates were incubated with immobilized GST or GST-Gads wild-type fusion proteins. The protein complexes were separated on a SDS-PAGE gel, electroblotted onto a PVDF membrane, and immunoblotted with anti-HPK1 Ab (α-HPK1; upper panel). To confirm equivalent protein expression of the mHPK1 mutants, whole cell lysates were separated on a SDS-PAGE gel in parallel, transferred to a PVDF membrane, and Western blotted with anti-HPK1 Ab (lysates; middle panel). HPK1 is denoted with an arrow. The membrane from the top panel was stained with Coomassie brilliant blue to confirm that equal amounts of GST or GST-Gads fusion proteins were used in the in vitro binding experiments (lower panel). GST and GST-Gads fusion proteins are denoted with arrows.
Expression of mutant Gads can inhibit the tyrosine phosphorylation of HPK1

Since a Gads-HPK1 complex was detected in anti-CD3-stimulated DO11.10 cells, we examined the effect of overexpression of Gads mutant proteins on the tyrosine phosphorylation of HPK1 following TCR activation. Gads mutant proteins that possessed inactivated SH3 or SH2 domains were transiently coexpressed with HA-tagged HPK1 in DO11.10 cells, and the relative tyrosine phosphorylation level of HPK1 following anti-CD3 stimulation was assessed (Fig. 7). Expression of wild-type Gads or mutant Gads with inactivated SH3 domains (N*/C*) did not alter the tyrosine phosphorylation level of HPK1 relative to the control level. However, the expression of mutant Gads with an inactivated SH2 domain (SH2*) appreciably reduced tyrosine phosphorylation of HPK1, suggesting that Gads may be directly involved in coupling HPK1 to tyrosine kinases that are activated by the TCR.

FIGURE 5. Mutation of the Gads SH3 domains or the HPK1 P4 region disrupt Gads-HPK1 binding in vivo. A, DO11.10 murine T cells (2.0 × 10⁷) were transiently cotransfected by electroporation with 15 µg of pcDNA3-HPK1:HA (HA HPK1) or HPK1 mutants (ΔP4 and ΔP1, 2, 4) and 45 µg pEF-FLAG-Gads wild-type (wt; left panel) or with pcDNA3-HPK1:HA (HA HPK1) and pEF-FLAG-Gads wild-type (wt) or Gads mutants (SH2* and N*/C*) as indicated. Cellular lysates were prepared, and Gads proteins were immunoprecipitated with anti-FLAG Ab (IP:α-FLAG), separated on an SDS-PAGE gel, and electroblotted onto a PVDF membrane. Coimmunoprecipitating HA-tagged mHPK1 was detected by Western blot analysis with anti-HA Ab (Blot:α-HA). B, To confirm equivalent protein expression of the mHPK1 and Gads mutant constructs, whole cell lysates were separated on a SDS-PAGE gel in parallel, transferred to a PVDF membrane, and Western blotted with anti-HA Ab (upper panel) or anti-FLAG Ab (lower panel).

FIGURE 6. The second and fourth proline-rich regions of HPK1 mediate efficient tyrosine phosphorylation of HPK1 following TCR stimulation. DO11.10 murine T cells (2.0 × 10⁷) were left untransfected (mock) or were transiently transfected by electroporation with 15 µg of pcDNA3-HPK1:HA wild-type or mHPK1 mutant constructs with deletion of the first PRR (ΔP1), the second PRR (ΔP2), the fourth PRR (ΔP4), or the triple PRR (ΔP1/2/4). Twenty-four hours postelectroporation, the cells were left unstimulated or were stimulated with soluble anti-CD3 Ab for 2 min at 37°C in RPMI 1640 medium (with or without α-CD3). Cellular lysates were prepared and immunoprecipitated with anti-HA Ab (IP:α-HA), separated on an SDS-PAGE gel, and electroblotted onto a PVDF membrane. The level of tyrosine-phosphorylated HA-tagged mHPK1 was detected by Western blot analysis with antiphosphotyrosine Ab (Blot:α-pY; upper panels). To confirm that equal amounts of HPK1 were immunoprecipitated, the blots were stripped and reprobed with anti-HPK1 Ab (Blot:α-HPK1; lower panels). The bands corresponding to HPK1 are denoted with arrows. The data are representative of four independent experiments.
FIGURE 7. An inactivating mutation of the Gads SH2 domain exhibits a dominant inhibitory effect on HPK1 tyrosine phosphorylation following TCR stimulation. A. DO11.10 murine T cells (2.0 × 10^7) were transiently cotransfected by electroporation with 15 μg of pcDNA3-HPK1:HA and 45 μg of pEF-FLAG-Gads wild-type (wt) or Gads mutants with an inactivating point mutation in both SH3 domains (N*/C*) or the SH2 domain (SH2*). As a control, 15 μg of pcDNA3-HPK1:HA wild-type was transfected with 45 μg of pEF-FLAG vector. Twenty-four hours postelectroporation, the cells were left unstimulated or were stimulated with soluble anti-CD3 Ab for 2 min at 37°C in RPMI 1640 medium (with or without α-CD3). Cellular lysates were prepared and immunoprecipitated with anti-HA Ab (IP:α-HA), separated on a SDS-PAGE gel, and electroblotted onto a PVDF membrane. The level of tyrosine-phosphorylated HA-tagged mHPK1 was detected by Western blot analysis with anti-phosphotyrosine onto a PVDF membrane. The level of tyrosine-phosphorylated HA-tagged a panel and Western blotted with anti-HPK1 Ab (Blot:α-HPK1; lower panel). The band corresponding to HPK1 is denoted by an arrow. B. To compare the expression of HPK1 and Gads for each transfection condition, whole cell lysates were separated on a SDS-PAGE gel in parallel, transferred to a PVDF membrane, and Western blotted with anti-HPK1 Ab (Blot:α-HPK1; upper panel) and anti-Gads (Blot:α-Gads; lower panel). The bands corresponding to HPK1 and Gads are denoted by arrows. These data are representative of seven independent experiments.

Discussion

In this report we have identified HPK1 as a Gads-binding protein and have demonstrated that Gads inductively associates with HPK1 following the activation of the TCR. Furthermore, we have found that TCR cross-linking results in HPK1 activation in both DO11.1 murine hybridoma and primary murine T cells. Our studies also reveal that a small proportion of HPK1 becomes rapidly tyrosine-phosphorylated following TCR activation. The proline-rich motifs (P1, P2, and P4) of HPK1 are involved in facilitating both HPK1 kinase activation and tyrosine phosphorylation in response to TCR engagement. The Gads-HPK1 interaction is mediated by the carboxy-terminal SH3 domain of Gads and aa 467–471 (P4) of HPK1. Expression of a Gads SH2 mutant can inhibit the tyrosine phosphorylation of HPK1, suggesting that in T cells, Gads functions to link HPK1 to the activated TCR complex.

Substantial research focused on elucidating the pathways downstream of HPK1 has led to a common model that HPK1 initiates a kinase cascade involving several intermediates including mixed lineage kinase 3 (41), MEK1 (42), and TGF-β-activated kinase 1 (50), which can all phosphorylate and activate SAPK/ERK kinase 1 leading to the activation of SAPK. In contrast, less is known about signaling events upstream of HPK1 activation. Several groups have described the association of SH2/SH3 domain-containing adaptor proteins, including Crk, CrkL, and Grb2 with HPK1, and have implicated these adaptors in connecting HPK1 to activated receptors (45, 48, 49). Additionally, it has been previously demonstrated that expression of the HPK1 proline-rich regions in Jurkat T cells prevents association with Crk and Grb2 adaptor proteins and disrupts IL-2 activation (48). We have found that the Gads adaptor protein inductively associates with HPK1 in T cells, and that HPK1 kinase activity is regulated by TCR activation, which is dependent on the presence of its proline-rich regions for efficient activation. Taken together, this supports a model for HPK1 kinase regulation in T cells, where SH2/SH3 domain-containing adaptor proteins such as Grb2, Crk, CrkL, and Gads are able to recruit HPK1 to the proximity of the TCR, thus facilitating subsequent activation of the HPK1 kinase. These adaptor proteins probably provide a means of coupling the activated TCR complex to kinase cascades downstream of HPK1, which may ultimately regulate the induction of a downstream effector response.

HPK1 has been previously identified as a substrate for tyrosine kinases. In experiments using ectopically expressed HPK1 in COS1 cells, the activated epidermal growth factor receptor, platelet-derived growth factor receptor, and constitutively activated cytoplasmic tyrosine kinases v-Src and v-Fps induce the tyrosine phosphorylation of HPK1 (49). Our studies provide the first report that endogenous HPK1 is tyrosine phosphorylated following TCR activation. It is not known whether tyrosine phosphorylation of HPK1 directly regulates its kinase activity, although based upon the small proportion of tyrosine-phosphorylated HPK1 observed in anti-HPK1 immunoprecipitates from TCR-activated cells, this would appear unlikely. At this time, the functional significance of HPK1 tyrosine phosphorylation, as observed in response to a wide variety of activated receptors and cytoplasmic tyrosine kinases, remains elusive.

Within the proline-rich region, murine HPK1 harbors three consensus SH3 domain binding sites, and human HPK1 potentially has four. One of the motifs in mHPK1 (P2) and two in human HPK1 conform with the binding consensus for Crk proteins, PxLPxK (where x is any amino acid) (41, 45, 49). The remaining two motifs in mHPK1 (P1, P4) have arginine at the P-3 specificity position, which is usually preferred by Grb2 SH3 domains (41, 49). We have demonstrated that the Gads carboxyl-terminal SH3 domain preferentially interacts with the P4 motif. Furthermore, our studies indicate that P2 and P4 are required for the efficient tyrosine phosphorylation of HPK1, supporting a model where tyrosine phosphorylation of HPK1 is dependent on the recruitment of HPK1 to the proximity of activated tyrosine kinases via SH2/SH3 domain-containing adaptor proteins. This model is strengthened by our observations that coexpression of a Gads SH2 mutant also attenuated the tyrosine phosphorylation of HPK1. This effect might result from the ability of this mutant to effectively bind HPK1 through an intact SH3 domain, while sequestering it from the proximity of the activated TCR by preventing association with proteins such as LAT. In contrast, the Gads SH3 mutant was found...
to have only a marginal effect on HPK1 tyrosine phosphorylation. This is not entirely unexpected, since the Gads SH3 mutant can no longer interact with HPK1. Tyrosine phosphorylation of HPK1 is not completely abolished in the presence of the Gads SH2 mutant, suggesting that other SH2/SH3 domain-containing adaptor proteins such as Crk, CrkL, and Grb2, probably serve redundant roles in recruiting of HPK1 to the proximity of the activated TCR complex. This redundancy might also be perceived as a mechanism for amplifying the level of HPK1 kinase activation.

Although the interaction of Gads and HPK1 is SH3 domain mediated, we have found that in vivo the association in DO11.1 is not constitutive, but is induced following TCR activation. The inducible nature of the Gads-HPK1 interaction is analogous to the interactions reported for the Grb2 and Grap adaptor proteins with Sos and for Grap with dynamin in activated T cells (13, 19, 51). In keeping with those reports, our results suggest that certain events must be initiated by the TCR for the Gads-HPK1 interaction to occur. For example, phosphorylation of HPK1 or engagement of the Gads SH2 domain could induce a conformational change resulting in an increased accessibility of the Gads SH3 domain to the fourth proline-rich region of HPK1. In support of the latter, Ravichandran et al. have shown that binding of the Grb2 SH2 domain to a Shc-derived phosphopeptide significantly enhances the Grb2-Sos interaction (51). Alternatively, TCR activation may result in the relocalization of proteins that facilitates their interaction. The inducible association of Grb2-family members with Sos, dynamin, and HPK1 suggests that the formation of these signaling complexes is highly regulated within T cells.

We have previously described a potential role for Gads in coupling TCR activation to the distal activation of NF-AT and IL-2 through its interactions with the SLP-76 and LAT adaptor proteins (22). We now propose that Gads also functions in an additional pathway downstream of the activated TCR that regulates the kinase activity and tyrosine phosphorylation of HPK1.

Note: While this manuscript was in revision, Liou et al. (54) published similar results demonstrating that HPK1 is activated by the TCR.

Acknowledgments
We thank K. Ravichandran for the DO11.10 T cell hybridoma cells, Ian Clarke and Michelle French for assistance with isolating primary T cells, and Michelle French and Donna Berry for comments on the manuscript.

References


