Release of serine/threonine-phosphorylated adaptors from signaling microclusters down-regulates T cell activation

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Antigen recognition within immunological synapses triggers and sustains T cell activation by nucleating protein microclusters that gather T cell receptors (TCRs), kinases, and adaptors. Dissipation of these microclusters results in signal termination, but how this process is regulated is unclear. In this paper, we reveal that release of the adaptors SLP76 and GADS from signaling microclusters is induced by the serine/threonine protein kinase HPK1 and that phosphorylation of GADS plays a major role in this process. We found that HPK1 was recruited into microclusters and triggered their dissipation by inducing the phosphorylation of a threonine-containing motif of GADS, together with the previously described serine phosphorylation of SLP76. These events induced the cooperative binding of 14-3-3 proteins to SLP76–GADS complexes, leading to their uncoupling from the transmembrane adaptor LAT and consequently reducing microcluster persistence and activation-induced gene transcription. These results demonstrate that serine/threonine phosphorylation of multiple TCR-proximal effectors controls the stability of signaling microclusters, thereby determining the intensity of T cell responses.

Introduction

Adaptive immune responses are initiated upon recognition by the T cell receptor (TCR) of peptide antigen–major histocompatibility complex complexes, displayed on the surface of antigen-presenting cells. TCR engagement induces a coordinated redistribution of receptors and signaling proteins at the immunological synapse, i.e., the interface between the T cell and the antigen-presenting cells (Monks et al., 1998; Grakoui et al., 1999). These events have been analyzed with high spatial and temporal resolution by imaging T cells activated on artificial stimulatory surfaces, such as coverslips coated with anti-CD3 antibodies (Bunnell et al., 2002) or lipid bilayers containing major histocompatibility complex–peptide complexes and adhesion molecules (Campi et al., 2005; Yokosuka et al., 2005). After T cell spreading over the stimulatory surface, TCR-containing microclusters enriched in activating kinases (e.g., LCK and ZAP70) and excluding negative regulators (e.g., the phosphatase CD45) preferentially form at the periphery of the immunological synapse. They then engage in centripetal movements toward the synapse center (Yokosuka et al., 2005; Varma et al., 2006). Microcluster components undergo different fates during this travel. TCRs accumulate at submicrometer-scale protein complexes, or microclusters, containing the TCR and critical signaling molecules, including the protein kinases LCK and ZAP70, the adaptors SLP76, GADS, GRB-2, and LAT, and downstream effectors, such as VAV1, PLC-γ1, and Wiskott–Aldrich syndrome protein (Bunnell et al., 2002; Barda-Saad et al., 2004; Campi et al., 2005; Yokosuka et al., 2005; Carrizosa et al., 2009; Miletic et al., 2009). After T cell spreading over the stimulatory surface, TCR-containing microclusters enriched in activating kinases (e.g., LCK and ZAP70) and excluding negative regulators (e.g., the phosphatase CD45) preferentially form at the periphery of the immunological synapse. They then engage in centripetal movements toward the synapse center (Yokosuka et al., 2005; Varma et al., 2006). Microcluster components undergo different fates during this travel. TCRs accumulate at...
the center of the synapse forming the so-called central supra-
molecular activation cluster (cSMAC; Monks et al., 1998;
Yokosuka et al., 2005) and are eventually down-regulated
(Varma et al., 2006; Vardhana et al., 2010). On the other hand,
molecules such as SLP76 and ZAP70 segregate from TCR
microclusters before they reach the cSMAC (Bunnell et al.,
2002; Yokosuka et al., 2005). Interestingly, reducing the mo-
tility of microclusters toward the cSMAC by different means
results in increased microcluster persistence and T cell acti-
vation (Mossman et al., 2005; Nguyen et al., 2008; Lasserre et al.,
2010), suggesting that dynamic segregation of microcluster
components reflects signal termination. Although ubiquity-
lation-mediated internalization/degradation of TCR subunits
or other microcluster components has been implicated in sig-
nal inactivation (Balagopalan et al., 2007; Vardhana et al.,
2010), the mechanisms triggering protein sorting from micro-
clusters and signal termination are poorly characterized.

The cytoplasmic scaffold protein SLP76 plays a central
role in TCR signal transduction and is indispensable for both
thymocyte development and mature T cell activation (Koretzky et al.,
2006). SLP76 is recruited into microclusters by binding to the
transmembrane phosphoprotein LAT via the small adaptor
GADS (Liu et al., 1999; Bunnell et al., 2006). The LAT–
GADS–SLP76 complex is pivotal for signaling regulation and
diversification because it coordinates the recruitment and acti-
vation of effectors belonging to several downstream pathways
(Acuto et al., 2008). For instance, the association of SLP76
with the guanine nucleotide exchange factor VAV1, the adap-
tors NCK (Bubeck Wardenburg et al., 1998) and adhesion-
degranulation-promoting adaptor protein, (Peterson et al.,
2001; Wang et al., 2009), the Tyr kinase Itk (Bunnell et al.,
2001; Yokosuka et al., 2005). Interestingly, reducing the mo-
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clusters and signal termination are poorly characterized.

Recently, we have unveiled a negative feedback loop in-
volving SLP76 that modulates T cell activation. HPK1 phosphory-
lates Ser376 of SLP76 and induces its interaction with members
of the 14-3-3 protein family (Di Bartolo et al., 2007), ubiquitous
molecules that regulate multiple cellular functions (Tzivion and
Avruch, 2002). Biochemical and functional analyses suggested
that binding of 14-3-3 to SLP76 down-modulates T cell signaling
and activation (Di Bartolo et al., 2007), although the underlying
mechanism remained uncharacterized. In support of our model,
HPK1-deficient T cells showed increased signaling and respon-
siveness upon TCR stimulation (Shui et al., 2006).

Here, we demonstrate that HPK1 negatively regulates
T cell activation by reducing the persistence of signaling micro-
clusters and identify a threonine-containing motif in GADS
whose phosphorylation plays a central role in this regulatory
process. Our results indicate that HPK1-dependent phos-
phorylation of both GADS and SLP76 is required for the recruit-
ment of 14-3-3 proteins. The resulting SLP76–GADS–14-3-3 com-
plexes are released from LAT-containing microclusters and
likely inactivated, thus down-modulating T cell activation.
These data define a novel mechanistic link between Ser/Thr
phosphorylation of TCR effectors, stability of signaling protein
complexes, and down-regulation of T cell responses.

Results

HPK1 overexpression affects
SLP76 microclusters

To address whether HPK1 may alter the formation and/or the
dynamics of SLP76 signaling complexes, we analyzed SLP76-
containing microclusters induced by immobilized stimulatory
antibodies. SLP76-deficient J14 cells stably reconstituted with a
YFP-tagged SLP76 fusion protein (J14-SLP76-YFP; Lasserre et al.,
2010) were transiently transfected with a wild-type (WT) HPK1
construct fused to the red fluorescent protein mCherry
(HPK1-mCherry). These cells were dropped on anti-CD3–
coated coverslips, and protein microclusters were imaged in
fixed cells after 3-min stimulation. Microclusters containing
SLP76-YFP were detected in most untransfected cells, as ex-
pected, whereas their number was dramatically reduced in cells
expressing HPK1-mCherry (Fig. 1 A). Conversely, transfection
of an mCherry-labeled kinase-deficient mutant of HPK1
(HPK1-KD-mCherry) led to an increase in the number of SLP76
microclusters compared with control cells (Fig. 1 B), indicating
that the impairment of SLP76 microclusters by HPK1 was de-
pendent on its catalytic activity.

Interestingly, HPK1-KD-mCherry clearly accumulated in
SLP76-containing microclusters (Fig. 1 B). Thus, we asked
whether HPK1 recruitment into microclusters was a prerequi-
site for their negative regulation. Phosphorylation of Tyr379 of
HPK1 has been shown to allow binding of this kinase to the
SH2 domain of SLP76 (Sauer et al., 2001). Hence, we trans-
sected an HPK1-mCherry construct bearing a Y379F mutation
in J14-SLP76-YFP cells. As shown in Fig. 1 C, this mutant was
not incorporated into microclusters nor affected their number,
suggesting that Tyr379-dependent interaction of HPK1 with
SLP76 is a critical step in down-regulating SLP76 micro-
clusters. Thus, these data revealed that HPK1 regulates signaling
microclusters and demonstrated that this function depends on
HPK1 directly binding to SLP76 and catalytic activity.

HPK1 negatively regulates the persistence
of SLP76-containing microclusters

To understand whether HPK1 overexpression prevents the for-
mation of signaling microclusters and/or reduces their stability
after initial assembly. J14-SLP76-YFP cells transfected with
HPK1-mCherry constructs were stimulated on anti-CD3-coated
coverslips and imaged in real time. In control cells expressing
only endogenous HPK1, SLP76 microclusters appeared as soon
as cells touched the stimulatory surface and could be observed
for a few minutes after cells had completely spread (Fig. 2 A and
Video 1, bottom cell) as previously described (Bunnell et al.,
2002; Lasserre et al., 2010). Interestingly, in cells overexpress-
ing HPK1-mCherry, microclusters formed as in control cells
during spreading on the stimulatory surface, but they rapidly dis-
appeared thereafter (Fig. 2 A and Video 1, top cell). Quantifica-
tion of the lifetime of individual microclusters showed that
overexpression of HPK1 significantly reduces their persistence
compared with untransfected cells (Fig. 2 B). The rarity of micro-
clusters in HPK1-overexpressing cells rendered the incorpora-
tion of HPK1-mCherry in SLP76 microclusters hard to detect.
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(Video 1). However, when visible, it was delayed as compared with microcluster formation. For instance, Fig. 2 C demonstrates that SLP76 incorporation increased for ~40 s after microcluster detection, whereas HPK1-mCherry recruitment started to be detectable when the amount of SLP76 was at its maximum. Interestingly, the amount of SLP76 in microclusters started decreasing when HPK1 recruitment began, arguing that incorporation of HPK1 in SLP76 microclusters induces their dissipation.

Remarkably, continuous generation of new microclusters was detectable for some minutes in control cells but not in HPK1-mCherry transfectants after the extinction of the initial wave of microclusters accompanying cell spreading (Fig. 2 A and Video 1). This observation suggested that HPK1 not only promotes dissociation of SLP76 from existing complexes but also prevents its incorporation into new ones. Contrary to the WT construct, HPK1-KD-mCherry was incorporated into SLP76 microclusters and induced a significant increase in their mean lifetime (Fig. 2 B and Video 2), confirming that dissipation of SLP76 microclusters requires a catalytically active HPK1.

Figure 2. HPK1 reduces the persistence of SLP76 microclusters. (A) J14-SLP76-YFP cells transfected with HPK1-mCherry were activated on anti-CD3–coated coverslips and imaged with a spinning-disk confocal microscope at one image/20 s. Frames from Video 1 at different time points are shown. One untransfected control (CTRL) cell (closed arrowhead) and one cell expressing HPK1-mCherry (open arrowhead) are visible in each frame. (B) Lifetime of single SLP76-YFP microclusters was determined by manual tracking using time-lapse images of J14-SLP76-YFP cells, acquired as described in A. The plot shows SLP76 microcluster lifetime in untransfected cells and in cells expressing HPK1-mCherry or HPK1-KD-mCherry. (C, top) Z projection of the HPK1-mCherry–expressing cell shown in A, imaged after 80 s of activation. The boxed area highlights a microcluster containing both SLP76-YFP (green) and HPK1-mCherry (red). (bottom) Magnification of the boxed area shown in the top image at different time points. Time 0 corresponds to the first detection of this SLP76-YFP microcluster [i.e., 40 s in the time lapse displayed in A]. Similar results were obtained in three independent experiments. (D) J14-SLP76-YFP cells transfected with control (siCont) or HPK1-specific (siHPK1) siRNAs were stimulated on anti-CD3 coverslips and analyzed as described in A. Knockdown efficiency was assessed by flow cytometry (Fig. S1). (E) SLP76 microcluster lifetime assessed in cells treated as in D. The plot shows pooled data of three experiments. Horizontal lines and error bars show means and SEM. Bars: [A, C [top], and D] 5 µm; [C, bottom] 1 µm.
We then addressed the role of endogenous HPK1 by analyzing the effect of HPK1 silencing on the persistence of SLP76 microclusters in live cells. SLP76-YFP–expressing cells were transfected with control or HPK1-specific siRNA oligonucleotides, typically yielding a knockdown efficiency of >70% (Fig. S1). Reduced HPK1 expression correlated with an increased lifetime of SLP76 microclusters (Fig. 2, D and E), similar to the effect of overexpressing HPK1-KD-mCherry (see previous paragraph and Fig. 2 B). It is noteworthy that changes in SLP76 expression were not detected upon overexpression or silencing of HPK1 (Fig. S1, B and C); hence, they cannot explain the observed effect on SLP76 microclusters.

Persistence of SLP76 microclusters is partially dependent on HPK1-induced binding of 14-3-3 proteins to SLP76

Because we previously showed that HPK1 phosphorylates Ser376 of SLP76 and induces its binding to 14-3-3 proteins (Di Bartolo et al., 2007), we probed the involvement of these events in the dissipation of SLP76 microclusters. First, we compared the distribution of SLP76 microclusters and SLP76–14-3-3 complexes in SLP76-YFP–expressing cells. SLP76–14-3-3 complexes were specifically detected in activated cells by using an in situ proximity ligation assay (PLA; Fig. S4, A and B; see Materials and methods and Söderberg et al., 2006) but appeared associated with target cells (Fig. 4 A, top). Accordingly, only SLP76 microclusters, which are localized in close proximity of the plasma membrane, could be detected by total internal reflection fluorescence (TIRF) microscopy, whereas SLP76–14-3-3 complexes were visible only by epifluorescence (Fig. S4 C). These data strongly suggest that 14-3-3 binding to SLP76 and retention of SLP76 in microclusters are mutually exclusive.

Additionally, the number of microclusters was significantly increased in cells expressing a YFP-labeled SLP76-S376A mutant, which cannot bind 14-3-3 proteins (Fig. 4 B). This result, implicating the SLP76–14-3-3 interaction in the regulation of SLP76 microclusters, was reminiscent of the effect of HPK1 knockdown (Fig. 3). However, the latter produced larger effects than SLP76-S376A-YFP expression (~63 vs. 29% increase in microclusters compared with control cells, respectively, at 3 min of stimulation; compare Figs. 3 C and 4 B), suggesting that HPK1 may exert additional effects on these signaling complexes.

Based on the propensity of 14-3-3 proteins to form homo- or heterodimers simultaneously binding two phosphorylated motifs (Tzivion and Avruch, 2002), we hypothesized that HPK1 might generate a second 14-3-3 binding site in SLP76 or in an associated protein, stabilizing the interaction with Ser376. Hence, we probed SLP76–associated proteins, isolated from clustering of SLP76, although it may exert some delayed effect on phospho-LAT microclusters.
Figure 3. **HPK1 uncouples SLP76 from LAT microclusters.** (A) J14-SLP76-YFP cells transfected with control (siCont) or HPK1-specific (siHPK1) siRNAs were activated for 3 min on anti-CD3–coated coverslips, fixed, stained with antiphospho-LAT (Y191) mAbs, and imaged by confocal microscopy. Results are representative of three independent experiments. (B and C) J14 cells expressing SLP76-YFP were transfected as in A and stimulated for the indicated time.
identified GADS as a novel 14-3-3–interacting protein and suggested its potential involvement in mediating HPK1 regulation of signaling microclusters.

**HPK1 phosphorylates GADS and regulates its interaction with 14-3-3 proteins**

The aforementioned results prompted us to ask whether HPK1 may regulate 14-3-3 binding to GADS. First, we used an in vitro approach to assess the interaction between HPK1 and GADS. We transfected J14-SLP76-WT cells with constructs encoding HPK1-MYC and FLAG-SLP76. After stimulation with anti-CD3 antibodies, we performed immunoprecipitation and analyzed the samples by immunoblotting. The results showed that HPK1 knockdown reduced the interaction between HPK1 and GADS, indicating that HPK1 promotes the interaction between GADS and 14-3-3 proteins.

**HPK1 regulates T cell microcluster stability**

J14-SLP76-WT cells, with a GST–14-3-3ζ fusion protein in an overlay assay. In addition to SLP76, a protein of ~40 kD was bound by GST–14-3-3ζ but not by GST (Fig. 4 C, middle and left). This interaction was detectable in unstimulated cells and was increased after stimulation. Immunoblotting identified this protein as GADS (Fig. 4 C, right). Similar results were obtained when the GST–14-3-3ζ overlay assay was performed on SLP76 immunoprecipitates from human CD4+ T cells (Fig. 4 D). These results identified GADS as a novel 14-3-3–interacting protein and suggested its potential involvement in mediating HPK1 regulation of signaling microclusters.
Figure 5. **HPK1-dependent binding of 14-3-3ζ to GADS.** (A) HPK1-HA constructs, wild type (WT) or kinase deficient (KD), were transfected in COS7 cells and immunoprecipitated (IP) with anti-HA mAbs. Recombinant MBP or MBP-GADS was added to either sample and incubated with [32P]ATP. Supernatants were then blotted onto Whatman filters, and incorporated radioactivity was measured. Comparable results have been obtained in three independent experiments. Error bars indicate SDs of triplicates. (B) J14-SLP76-WT cells expressing FLAG-SLP76 and transfected with control (siCont) or HPK1-specific (siHPK1) siRNAs were stimulated by anti-CD3 mAbs for the indicated time points, lysed, and immunoprecipitated with anti-FLAG mAbs. Samples were analyzed by GST (top) and GST–14-3-3ζ (middle) overlay assay followed by Western blotting with anti-SLP76 and anti-GADS antibodies (bottom).
kinase assay to test whether GADS is a substrate of HPK1. Immunoprecipitated WT or kinase-deficient HPK1 was incubated with maltose-binding protein (MBP) or an MBP-GADS fusion protein in the presence of \([^{32}P]ATP\). A significant incorporation of \(^{32}\)P over background was observed when MBP-GADS was incubated with WT HPK1 but not with the kinase-deficient construct (Fig. 5 A).

We then asked whether HPK1 regulates the interaction of 14-3-3\(\zeta\) with GADS. We transfected J14-SLP76-WT cells with control or HPK1-specific siRNAs and immunoprecipitated FLAG-SLP76 and associated proteins. Efficient HPK1 knockdown (Fig. S3 C) almost completely inhibited of GST–14-3-3\(\zeta\) binding to FLAG-SLP76 in an overlay assay (Fig. 5, B [middle] and C [right]). Maximum binding of GST–14-3-3\(\zeta\) to GADS was reduced by \(\sim 50\%\) in HPK1 knockdown cells compared with control cells (Fig. 5, B [middle] and C [right]). Although the inhibition of GST–14-3-3\(\zeta\) binding to GADS in this assay was not complete, this result indicated a role of HPK1 in inducing 14-3-3 binding to GADS.

Finally, we probed the role of HPK1 in regulating the interaction of 14-3-3\(\zeta\) with GADS in intact cells. Jurkat cells, transfected with control or HPK1-specific siRNAs, were stimulated on anti-CD3–coated coverslips and analyzed by in situ PLA with different antibody combinations. Labeling with anti-SLP76 and anti-14-3-3\(\zeta\) antibodies yielded a positive PLA signal that was abolished upon HPK1 silencing (Fig. 5, D [top] and E), confirming that SLP76 binding to 14-3-3\(\zeta\) is induced by HPK1 (Di Bartolo et al., 2007). Staining with anti-GADS and anti-14-3-3\(\zeta\) antibodies also yielded a strong PLA signal in control cells, validating the interaction between these proteins, which was impaired by HPK1 silencing (Fig. 5, D [middle] and E). Conversely, no detectable PLA signal was observed after staining with anti-LAT and anti-14-3-3\(\zeta\) antibodies (Fig. 5, D [bottom] and E). Collectively, these data demonstrated that HPK1 controls the interaction of 14-3-3 proteins with GADS.

### 14-3-3\(\zeta\) binds a phosphothreonine-containing motif of GADS

Because 14-3-3 protein binding to their target is usually induced by Ser/Thr phosphorylation of conserved motifs (Tzivion and Avruch, 2002), we inspected the human GADS sequence in search of potential 14-3-3 binding sites. We identified a conserved RRRHTDP sequence, resembling a type I 14-3-3 binding motif (RXXp(S/T)XP; Johnson et al., 2010), encompassing Thr262 (Thr254 in murine GADS). Importantly, neither mutation altered the constitutive SLP76–GADS interaction (Fig. 6 B, top and middle). These data identified Thr254 as a 14-3-3 binding site and highlighted its prominent role in regulating the association of 14-3-3 proteins to SLP76–GADS complexes.

14-3-3 binding to SLP76 and GADS cooperatively affects microcluster stability and T cell activation

Based on the aforementioned observations, we aimed to correlate 14-3-3 binding to SLP76 and GADS with microcluster stability and T cell activation. First, we analyzed the persistence of SLP76 microclusters in cells expressing various SLP76-YFP and CFP-GADS constructs stimulated on anti-CD3–coated coverslips for 5 min. The number of SLP76 microclusters was significantly increased in cells expressing the CFP-GADS-T254A compared with those transfected with CFP-GADS (Fig. 7 A). Expression of YFP-SLP76-S376A also increased the number of microclusters compared with YFP-SLP76–expressing cells, although it was less effective than CFP-GADS-T254A (Fig. 7 A). Simultaneous expression of both mutants further increased the number of microclusters as compared with either mutant alone (Fig. 7 A). Of note, phospho-LAT microclusters

Similar results have been obtained in three independent experiments. Open arrowheads indicate heavy chains of the precipitating antibodies. HPK1 knockdown efficiency was assessed by immunoblotting (Fig. S1 C). Mobility of molecular mass markers is shown on the right in kilodaltons. (C) Quantification of GST–14-3-3\(\zeta\) binding to SLP76 [left] and GADS [right] in overlay assays shown in B. Bands in B [middle and bottom] were acquired and quantified as outlined in Materials and methods. Intensity of bound GST–14-3-3\(\zeta\) bands was normalized by the relative amount of SLP76 [left] or GADS [right] in the same lane. (D) Jurkat cells transfected with control or HPK1-specific siRNAs were activated for 10 min on anti-CD3–coated coverslips, fixed, and stained with the antibody pairs indicated on the left and by DAPI for visualizing nuclei (blue). Protein complexes [red] were detected by in situ PLA. A z-stack projection is shown in each image. Bars, 5 pm. (E) Images obtained by in situ PLA experiments as shown in D were analyzed using the BlobFinder software to automatically count spots generated by protein–protein interactions. Histograms show means and SEM of the number of spots per cell from two independent experiments. The number of cells analyzed is indicated beside each bar.
Discussion

This work shows for the first time that HPK1 modulates TCR-proximal signaling and T cell activation by regulating the stability of critical protein complexes at the immunological synapse. We found that the persistence of SLP76 microclusters induced by TCR stimulation is dependent on HPK1 activity. HPK1 incorporation into SLP76 microclusters coincides with their rapid dissipation caused by a 14-3-3–mediated uncoupling of SLP76–GADS complexes from LAT. Persistence of phospho-LAT microclusters appears also affected by HPK1 but with some delay compared with SLP76 microclusters. These kinetic differences suggest that the effect on phospho-LAT clustering is consequent to the release of the SLP76–GADS complex, possibly because of the loss of stabilizing protein–protein interactions dependent on SLP76 and/or GADS. In support of the hypothesis that HPK1 does not target LAT directly, phospho-LAT microclusters are increased when 14-3-3 binding to SLP76 and GADS is impaired (Fig. S5 A). Collectively, these results show that a major function of HPK1 is to uncouple the SLP76–GADS from phosphorylated LAT, leading to negative regulation of T cell activation.

Finally, we evaluated the functional response of T cells expressing SLP76 and GADS mutants by measuring the activation of the nuclear factor of activated T cell (NFAT) transcription factor. J14-SLP76-WT and -S376A cell lines expressing FLAG-tagged WT SLP76 or the S376A mutant (Di Bartolo et al., 2007) were transfected with CFP-GADS or CFP-GADS-T254A expression vector, together with an NFAT-luciferase reporter plasmid. Measurement of luciferase activity in cells stimulated with anti-CD3 and anti-CD28 antibodies showed that overexpressing CFP-GADS-T254A in J14-SLP76-WT cells led to higher NFAT activation than CFP-GADS transfection (Fig. 7 B). J14-SLP76-S376A cells expressing CFP-GADS also showed higher NFAT activity compared with J14-SLP76-WT transfected with this construct. Moreover, expression of CFP-GADS-T254A in J14-SLP76-S376A further increased NFAT activity (Fig. 7 B), indicating that simultaneous mutation of both 14-3-3 binding sites resulted in the highest T cell activation. Comparable expression of all constructs was verified by immunoblotting (Fig. S5 B). Altogether, these results indicated that the simultaneous interaction of 14-3-3 to SLP76 and GADS negatively regulates the stability of signaling complexes and functional activation of T cells.
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of SLP76 (Di Bartolo et al., 2007). Thr254 in murine GADS (corresponding to Thr262 in the human protein) was necessary for 14-3-3 binding and for negative regulation of microcluster stability and T cell activation. Moreover, disruption of this 14-3-3 binding site had a stronger impact on SLP76 microcluster persistence than mutation of Ser376, possibly because binding of 14-3-3 to GADS disturbs the interaction of the SH2 domain of GADS with phospho-LAT, either by steric hindrance or by inducing a conformational change. However, simultaneous mutation of both Ser376 and Thr254 had additive effects on both persistence of SLP76 microclusters and NFAT transcriptional activity upon T cell stimulation, suggesting a cooperative stabilization of the interaction of 14-3-3 with the SLP76–GADS complex. This finding is consistent with 14-3-3 proteins forming homo- and heterodimers that usually require interaction with two phosphorylation sites for stable binding (Tzivion and Avruch, 2002). Based on these results, we propose a model whereby recruitment of HPK1 in SLP76-containing microclusters leads to phosphorylation of both SLP76 and GADS on Ser376 and Thr262, respectively. These posttranslational modifications enable recruitment of a 14-3-3 protein dimer, which in turn enforces dissociation of the SLP76–GADS–14-3-3 complex. This finding is consistent with 14-3-3 proteins forming homo- and heterodimers that usually require interaction with two phosphorylation sites for stable binding (Tzivion and Avruch, 2002). Based on these results, we propose a model whereby recruitment of HPK1 in SLP76-containing microclusters leads to phosphorylation of both SLP76 and GADS on Ser376 and Thr262, respectively. These posttranslational modifications enable recruitment of a 14-3-3 protein dimer, which in turn enforces dissociation of the SLP76–GADS–14-3-3 complex from phospho-LAT and consequently down-regulates TCR-induced signal transduction (Fig. 8).

The subsequent fate of the pool of SLP76 (and GADS) released from microclusters is unknown yet. Although a fraction of it could still be signaling competent because of its interaction with other effectors (Bunnell et al., 2006), the correlation between removal from microclusters and reduced T cell activation reported here rather hints at an inactivation process. Hence, 14-3-3–bound SLP76 might be sorted for degradation or recycling. We could not detect significant changes in the total amount of SLP76 during the stimulation time analyzed (10 min), but we cannot exclude that degradation occurs later. Reincorporation of SLP76 into new microclusters has been previously described by others (Barr et al., 2006), but according to our model, it would imply release of 14-3-3 and dephosphorylation of Ser376. However, we found that both Ser376 phosphorylation and 14-3-3 binding to SLP76 were long lasting (i.e., 45–60 min; Di Bartolo et al., 2007) and that no additional microclusters were generated after the extinction of the first wave accompanying cell spreading on anti-CD3 coverslips in cells overexpressing HPK1 (Fig. 2 A and Video 1). Both observations argue against a recycling of released SLP76; hence, further studies are required to address these issues.

Intriguingly, HPK1 knockdown more potently impaired GST–14-3-3ζ binding to SLP76 than to GADS in the overlay and in situ PLA assays, whereas direct mutation of the 14-3-3ζ binding site in GADS had stronger effects on GST–14-3-3ζ coprecipitation and on microcluster stability than mutation in SLP76. The reason for this difference is unknown and may depend, in part, on the different assay used. However, although HPK1 phosphorylates GADS in vitro, they do not allow us to exclude the existence of redundant kinases that may replace HPK1 in phosphorylating GADS in vivo.

The effect of HPK1 on microcluster persistence is reminiscent of that previously described for the ubiquitin E3
Interestingly, anti-CD3–stimulated binding of GST–14-3-3 to GADS in vitro is reinforced by pre-treating Jurkat cells with PGE2 (unpublished data). Hence, it will be interesting to address the involvement of HPK1-dependent control of microclusters in altering T cell responsiveness under pathological conditions.

In conclusion, our data demonstrate that negative feedback triggered by HPK1 plays an important role in regulating the stability of critical signaling complexes at immunological synapses. This mechanism may represent a flexible device adapting T cell responsiveness according to cell differentiation and/or external cues.

**Materials and methods**

**Plasmids and antibodies**

The pEF-FLAG-SLP76-WT expression vector was provided by G. Koretzky (University of Pennsylvania, Philadelphia, PA); pMT2-HPK1-HA and pMT2-HPK1-KD-HA plasmids (Arnold et al., 2005) were provided by F. Kiefer (Max Planck Institute for Molecular Biomedicine, Münster, Germany). HPK1-MYC and -mCherry constructs were generated by subcloning mouse HPK1 cDNA from pMT2-HPK1-HA plasmids into the pmCherry-N1 plasmid (Takara Bio Inc.) using a PCR cloning system (In-Fusion; Takara Bio Inc.). SLP76-YFP and CFP-GADS (Bunnell et al., 2006) were provided by L.E. Samelson (National Cancer Institute–Center for Cancer Research, Bethesda, MD). All point mutations were generated using the mutagenesis kit (QuickChange II; Agilent Technologies) and verified by sequencing of the entire insert cDNA. The following antibodies were used: 14-3-3ζ (C-16; Santa Cruz Biotechnology, Inc.), SLP76 (clone SLP76/03 mAb or goat polyclonal obtained from AbD Serotec and rabbit polyclonal obtained from Cell Signaling Technology), HPK1 (Cell Signaling Technology), FLAG (M2; Sigma-Aldrich), CD3 (clone UCHT1 obtained from BioLegend, MEM92 obtained from Dr. R. A. Flavell, University of British Columbia, Vancouver).

Figure 8. **Model of regulation of microcluster persistence and signaling by HPK1.** (A) SLP76–GADS complexes (only one is depicted for clarity) are recruited to the phosphorylated transmembrane adaptor LAT to form signaling-competent microclusters. (B and C) When phosphorylated on Tyr379, HPK1 is incorporated into microclusters by interacting with the SH2 domain of SLP76 (B) and phosphorylates Ser376 of SLP76 and Thr262 of GADS (C). (D and E) 14-3-3 proteins then bind to SLP76–GADS complexes through these phosphorylated residues (D), thus leading to dissociation of these complexes from phospho-LAT (p-LAT) and terminating signaling (E).
from Exbio, or HiT-3a obtained from biobolab, Inc.) and eluted with maltose according to the manufacturer’s instructions. Plates were performed using the Cell Culture Lysis Reagent and the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Plates were read using a microplate luminometer (Tropix TX717; Applied Biosystems). The percentage of luciferase activity for anti-CD3/CD28-stimulated samples was calculated as follows: percent $= [\text{RLU - MIN}]/(\text{MAX - MIN}) \times 100$, in which RLU is the measured luciferase activity of the sample, MIN is the measured RLU for the unstimulated sample, and MAX is the mean RLU for a sample stimulated with PMA plus Ca$^{2+}$ ionophore.

**PLA**

This assay allows detection of protein-protein interactions when two antibodies coupled to complementary oligonucleotides are in sufficient proximity, by annealing and priming of a rolling circle amplification reaction. Amplification products are then visualized by incubation with fluorescent probes. Interactions between 14-3-3 and potential targets were analyzed in situ by intracellular labeling with anti-14-3-3 rabbit polyclonal antibody combined with anti-SLP76, anti-GADS, or anti-LAT mouse mAbs followed by detection using the PLA kit (Duolink; Olink) according to the manufacturer’s instructions. Fluorescence spots generated were automatically counted, and the mean number of spots per cell was calculated from nuclei counting using BlobFinder v3.2 software (Centre for Image Analysis, Uppsala University, Uppsala, Sweden).

**Transient transfection and RNA interference**

For transient protein expression, cells were electroporated with 5–15 µg plasmid using an electroporation device (260 V, 950 µF; Gene Pulser Xcell; Bio-Rad Laboratories), a Neon transfection system, or a Nucleofector system [Lonza]. HPK1 knockdown in cell lines was obtained by electroporating 500 nM of control or HPK1-specific siRNAs (Thermo Fisher Scientific) as previously described (Lasserre et al., 2010). CD4$^+$ T cells were transfected with 1 µM siRNA using a Nucleofector system and the Human T Cell Nucleofector kit (Lonza). Cells were analyzed 72–96 h after transfection.

**Cell stimulation and lysis, immunoprecipitation, and immunoblotting**

Cells were stimulated by incubation at 37°C with 15 µg/ml of soluble anti-CD3 mAb (IgM, clone MEM-92). Activation was stopped by adding ice-cold PBS containing 2 mM sodium orthovanadate and 0.05% sodium azide. Cells were lysed for 10 min in ice-cold buffer containing 0.25% lauryl-m-maltoside and a cocktail of protease and phosphate inhibitors, and insoluble material was removed by centrifugation at 20,800 g for 10 min at 4°C. Immunoprecipitations were performed by incubating lysates for 1.5–2 h at 4°C with either anti-FLAG M2 affinity gel (Sigma-Aldrich) or protein G-coupled Sepharose beads previously coated with relevant antibodies. Beads were then washed, and bound proteins were eluted by incubating beads with a 0.2 mg/ml FLAG peptide (Sigma-Aldrich) solution or by heating them at 70°C in gel sample loading buffer. Protein electrophoresis was performed on standard SDS-PAGE or NuPAGE gels (Invitrogen). After protein electrotransfer on nitrocellulose (LICOR Biosciences), immunoblots were saturated with blocking buffer for near-infrared fluorescence blotting (Rockland Immunocchemicals) and incubated with primary antibodies. After incubation with secondary antibodies Alexa Fluor 680 (Invitrogen), IRDye 800 (Rockland Immunocchemicals), or DyLight 800 (Thermo Fisher Scientific), near-infrared fluorescence was detected and quantified by using an Odyssey scanner and the Odyssey v3.0 software (LICOR Biosciences).

**Overlay assay**

Immunoprecipitated proteins were transferred onto nitrocellulose membranes that were sequentially incubated in renaturation buffer (20 mM Hepes, 50 mM NaCl, 1 mM EDTA, and 10% glycerol) containing decreasing amounts of guanidine hydrochloride (6–0.19 M). After extensive washes in renaturation buffer, membranes were saturated as described for immunoblots and incubated overnight with 10 µg/ml GST or GST–14-3-3[Δ13] (provided by Y.-C. Liu, La Jolla Institute for Allergy and Immunology, La Jolla, CA; Di Bartolo et al., 2007) followed by mouse anti-GST antibody and Alexa Fluor 680– or IRDye 800–coupled anti–mouse antibodies (see previous paragraph).

**In vitro phosphorylation assays**

WT or kinase-dead HA-tagged HPK1 was expressed in COS7 cells and purified by immunoprecipitation with an anti-HA mAb. Human GADS was subcloned into pMal C8X (New England Biolabs, Inc.), and the MPB fusion protein or MBP alone was bound on an amylose column (New England Biolabs, Inc.) and eluted with maltose according to the manufacturer’s instructions.
stuctions. Equal amounts of bead-bound HPK1 constructs were mixed with 3 μg of the indicated substrate in kinase buffer (25 mM Tris, pH 7.5, 4 mM MgCl₂, and 1 mM MnCl₂) containing 5 μCi γ[32P]ATP and 20 μM of unlabeled ATP. After 30-min incubation at 30°C, the reaction was stopped by adding EDTA to a final concentration of 80 mM. Beads were then spun down, and 60% of the supernatant was spotted onto Whatman filters, which were then washed extensively with 10% TCA containing 10 mM sodium pyrophosphate and rinsed once with ethanol and then with acetone. Finally, filters were dried and radioactivity counted.

Online supplemental material

Fig. S1 shows quantification of HPK1 knockdown efficiency and effect of HPK1 knockdown or overexpression on SLPI76 levels (Figs. 1 and 2). Fig. S2 shows that HPK1 knockdown or overexpression affects SLPI76- but not phosphorylated LAT-containing microclusters at early stimulation time points. Fig. S3 shows that HPK1 expression and SLPI76 Ser376 phosphorylation is not affected by HPK1 knockdown or overexpression on SLP76 levels (Figs. 1 and 2).

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References


