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Sequential phosphorylation of SLP-76 at tyrosine 173 is required for activation of T and mast cells

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Cooperatively assembled signalling complexes, nucleated by adaptor proteins, integrate information from surface receptors to determine cellular outcomes. In T and mast cells, antigen receptor signalling is nucleated by three adaptors: SLP-76, Gads and LAT. Three well-characterized SLP-76 tyrosine phosphorylation sites recruit key components, including a Tec-family tyrosine kinase, Itk. We identified a fourth, evolutionarily conserved SLP-76 phosphorylation site, Y173, which was phosphorylated upon T-cell receptor stimulation in primary murine and Jurkat T cells. Y173 was required for antigen receptor-induced phosphorylation of phospholipase C-γ1 (PLC-γ1) in both T and mast cells, and for consequent downstream events, including activation of the IL-2 promoter in T cells, and degranulation and IL-6 production in mast cells. In intact cells, Y173 phosphorylation depended on three, ZAP-70-targeted tyrosines at the N-terminus of SLP-76 that recruit and activate Itk, a kinase that selectively phosphorylated Y173 *in vitro*. These data suggest a sequential mechanism whereby ZAP-70-dependent priming of SLP-76 at three N-terminal sites triggers reciprocal regulatory interactions between Itk and SLP-76, which are ultimately required to couple active Itk to its substrate, PLC-γ1.

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Introduction

The adaptive immune system responds to antigens through a variety of receptor types, including the T-cell receptor (TCR), B-cell receptor (BCR) and Fc receptors. The latter are indirect antigen receptors whose specificity is determined by the bound antibody. An important example is the FcεRI of mast cells, which mediates immediate type hypersensitivity responses upon exposure to the cognate antigen of the bound IgE molecule.

Antigen receptors signal through broadly similar pathways, in which Src-, Syk- and Tec-family tyrosine kinases form a cascade that results in tyrosine phosphorylation and activation of phospholipase C-γ isoforms (PLC-γ1 or PLC-γ2) (Carpenter and Ji, 1999). In addition to the kinases, cell type specific adaptor proteins are absolutely required for PLC-γ phosphorylation. In T cells and mast cells, this function is carried out by a heterotrimeric complex of adaptor proteins consisting of LAT, Gads and SLP-76 (reviewed in Koretzky *et al*, 2006; Alvarez-Errico *et al*, 2009; Kambayashi *et al*, 2009). LAT is a transmembrane adaptor that is heavily phosphorylated on tyrosine residues upon TCR or FcεRI stimulation. PLC-γ1 binds directly to LAT, whereas SLP-76 is indirectly recruited to LAT by Gads (Liu *et al*, 1999; Ishiai *et al*, 2000). Within this complex, SLP-76 binds and activates Itk, a Tec-family kinase that can phosphorylate PLC-γ1 at the sites required for its activation (Liu *et al*, 1998; Houtman *et al*, 2005; Bogin *et al*, 2007). In addition, SLP-76 binds to other proteins that regulate PLC-γ1 activation by incompletely understood mechanisms; the most prominent among these is Vav (Reynolds *et al*, 2002).

Once activated, PLC-γ1 produces second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), that trigger calcium flux and Ras activation, respectively. In mast cells, increased intracellular calcium triggers rapid release of preformed mediators, through a process of vesicle exocytosis, known as degranulation. These mediators produce most of the symptoms of immediate type hypersensitivity. Over a longer time course, both T and mast cells transcribe and secrete cytokines, through processes that involve calcium–calcineurin-mediated dephosphorylation and activation of the NFAT transcription factor, and Ras-dependent activation of AP-1. As an essential regulator of PLC-γ1 activation, the SLP-76 adaptor protein is required for all of the above signalling events (Yablonski *et al*, 1998; Pivniouk *et al*, 1999).

SLP-76 is expressed in all haematopoietic cells except B cells, where an analogous protein, SLP-65/BLNK is expressed (Fu *et al*, 1998; Wienands *et al*, 1998). SLP-76-deficient mice fail to develop mature T cells due to a block in pre-TCR

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signalling (Clements *et al*, 1998; Pivniouk *et al*, 1998). SLP-76-deficient mast cells develop normally, but exhibit defective responses to FcεRI activation (Pivniouk *et al*, 1999). In particular, FcεRI-induced PLC-γ1 activation is defective, as are the ensuing steps of degranulation and cytokine production. In addition, a SLP-76-deficient derivative of the Jurkat T cell line, known as J14, is useful for mechanistic studies of SLP-76. J14 cells fail to activate PLC-γ1 or to transcribe IL-2 in response to TCR stimulation, but signalling is restored upon reconstitution with wild-type SLP-76 (Yablonski *et al*, 1998). Based on these genetic models, SLP-76 has become an important paradigm for understanding adaptor protein function.

SLP-76 contains three regions that mediate interactions with other signalling proteins: an N-terminal acidic domain that includes three well-characterized tyrosine phosphorylation sites, a central proline-rich domain and a C-terminal SH2 domain (Koretzky *et al*, 2006). An N-terminal SAM domain is also required for full functionality (Shen *et al*, 2009).

Upon TCR stimulation, the three N-terminal tyrosines are phosphorylated by ZAP-70 (Wardenburg *et al*, 1996; Raab *et al*, 1997), and bind to three proteins, Nck, Vav and Itk (Tuosto *et al*, 1996; Wu *et al*, 1996; Raab *et al*, 1997; Bubeck Wardenburg *et al*, 1998; Su *et al*, 1999; Wunderlich *et al*, 1999; Bunnell *et al*, 2000). Mutation of all three tyrosines eliminates SLP-76 tyrosine phosphorylation (Fang *et al*, 1996; Wardenburg *et al*, 1996) and nearly abrogates its function (Myung *et al*, 2001; Yablonski *et al*, 2001; Kettner *et al*, 2003).

The central proline-rich domain contains two additional regions that are required for SLP-76 function: a short Gads-binding motif (Musci *et al*, 1997; Berry *et al*, 2002), and the P-I region, which is found between the N-terminal tyrosine phosphorylation sites and the Gads-binding motif (Yablonski *et al*, 2001). The P-I region can bind to the SH3 domains of PLC-γ1, Itk and Lck (Sanzenbacher *et al*, 1999; Bunnell *et al*, 2000; Yablonski *et al*, 2001; Grasis *et al*, 2010); but its role in T-cell activation has been subject to multiple, often conflicting interpretations (Singer *et al*, 2004; Gonen *et al*, 2005; Kumar *et al*, 2005; Grasis *et al*, 2010).

Of the proteins that bind to SLP-76, Itk is the most directly connected to PLC-γ1 activation, since it can phosphorylate PLC-γ1 at the sites that are required for its activation (Bogin *et al*, 2007). Catalytic activation of Itk depends on the inducible interaction of its SH2 domain with the N-terminal tyrosines of SLP-76 (Bogin *et al*, 2007). The additional interaction of its SH3 domain with the P-I region of SLP-76 appears to facilitate recruitment of Itk to the immune synapse (Bunnell *et al*, 2000; Grasis *et al*, 2010). An ongoing interaction of SLP-76 with Itk is required to maintain its catalytic activity (Bogin *et al*, 2007). This close interaction raises the possibility of reciprocal regulation; whereby SLP-76-activated Itk could feed back onto SLP-76 by phosphorylating it at other sites. Although SLP-76 is considered to have only three tyrosine phosphorylation sites, we suspected that these sites, by recruiting and activating Itk, might prime SLP-76 for phosphorylation at other sites.

In this study, we identified a new tyrosine phosphorylation site on SLP-76 and characterized its importance for antigen receptor signalling in both T cells and mast cells. This new site, Y173, is located in the P-I region of SLP-76, a region that is critical for SLP-76-mediated signalling (Yablonski *et al*, 2001; Singer *et al*, 2004), but whose mechanistic role has been difficult to dissect (Gonen *et al*, 2005). By revealing an additional layer of regulation in the antigen receptor signalling pathways, this observation brings us closer to understanding the reciprocal interactions between enzymes and adaptor proteins that mediate the rapid and exquisite responsiveness of the immune system.

Results

SLP-76 tyrosine Y173 is selectively phosphorylated by Itk

TCR stimulation triggers a cascade of kinases, each with a distinct role and substrate specificity. The N-terminus of SLP-76 is efficiently phosphorylated by ZAP-70, but not by Src-family kinases (Wardenburg *et al*, 1996; Raab *et al*, 1997). Phosphorylated SLP-76 recruits and activates Itk (Bunnell *et al*, 2000; Bogin *et al*, 2007); in turn, the PLC-γ1 sites required for its activation are efficiently phosphorylated by Itk, but not by ZAP-70 (Bogin *et al*, 2007). Despite their different substrate specificity, Itk and ZAP-70 exhibited comparable ability to phosphorylate a recombinant SLP-76 substrate *in vitro* (Lin *et al*, 2004; Bogin *et al*, 2007). This observation prompted us to search for tyrosine phosphorylation sites on SLP-76 that may be selectively phosphorylated by Itk.

To map the sites targeted by each kinase, we immunopurified ZAP-70 and Itk from TCR-stimulated Jurkat cells and tested their ability to phosphorylate recombinant fragments of SLP-76 *in vitro*. An N-terminal fragment of SLP-76 encompassing residues 2–163 was efficiently phosphorylated by ZAP-70 but not by Itk (Figure 1A). Using phosphospecific antisera to SLP-76 Y113, 128 and 145, we detected ZAP-70-mediated phosphorylation of each of these sites (data not shown). This fragment was not phosphorylated by Itk; however, we noted that SLP-76 contains a fourth, evolutionarily conserved tyrosine, located at position 173 (Supplementary Figure S1). A substrate encompassing this residue was efficiently phosphorylated by Itk, but not by ZAP-70, and phosphorylation was abolished by mutation of Y173 to phenylalanine (Figure 1B). These experiments identify Y173 as a potential Itk-targeted phosphorylation site on SLP-76.

TCR-inducible phosphorylation of SLP-76 at Y173 proceeds by a sequential mechanism

We postulated that the efficient phosphorylation of SLP-76 by Itk *in vitro* may recapitulate what happens upon TCR stimulation of intact cells. To test this idea, we performed mass spectrometric analysis of SLP-76, purified from TCR-stimulated cells. SLP-76 protein was digested with the endoprotease, Asp-N, chosen for its ability to cleave the acidic region of SLP-76, where the known tyrosine phosphorylation sites are found. Phosphorylated peptides were enriched by titanium oxide chromatography followed by MS and MSMS analysis. The expected Asp-N cleavage product, encompassing phosphorylated Y173, was unambiguously detected in this analysis (Figure 2A). In addition, we detected one of the previously known phosphorylation sites, Y145 (Supplementary Figure S2).

For routine detection of Y173 phosphorylation, we prepared an affinity-purified, polyclonal, phospho-Y173-specific antiserum. Using this reagent, we observed rapid and transient phosphorylation of Y173 in primary murine thymocytes

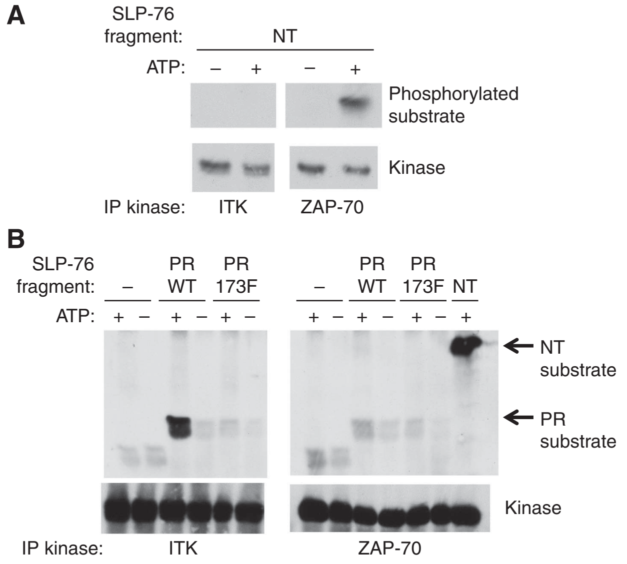

Figure 1 Selective phosphorylation of SLP-76 sites by Itk and ZAP-70. (**A**, **B**) Itk (left panels) or ZAP-70 (right panels) immune complexes were prepared from the lysates of 8×10^6 TCR-stimulated cells, and their catalytic activity was assayed, using the indicated GST-SLP-76 recombinant fusion proteins as *in vitro* substrates, in the presence or absence of 100 μM ATP. Kinase reaction products were separated by SDS/PAGE, and substrate phosphorylation was detected by probing with anti-phosphotyrosine (4G10) antibody (top panels). The immune complex beads were probed by western blotting with anti-Itk and anti-ZAP-70 antibodies, as indicated (bottom panels). Recombinant substrates were GST alone (–), GST fused to the N-terminal region of SLP-76 (NT; residues 2–163), or GST fused to a fragment of the proline-rich region of SLP-76 (PR; residues 150–196, either wild-type or mutated at Y173). Migration of the NT and PR substrates is indicated with arrows to the right of (**B**). Results are representative of three independent experiments. See also Supplementary Figure S1.

upon co-crosslinking of CD3 and CD4, whereas CD3 crosslinking was sufficient to induce phosphorylation of Y173 in primary murine splenic T cells (Figure 2B).

To confirm the specificity of this reagent, we probed lysates from TCR-stimulated J14 cells, stably reconstituted with FLAG-tagged wild-type or Y173-mutated SLP-76. Wild-type SLP-76 was inducibly phosphorylated at Y173, with a time course roughly parallel to that of the three previously known phosphorylation sites (Figure 2C, left four lanes). Mutation of Y173 to phenylalanine abolished the signal detected with the phosphoY173 reagent, but did not affect phosphorylation of the three previously known phosphorylation sites (Figure 2C). This result provides strong evidence for TCR-inducible phosphorylation of Y173. Incidentally, this result also shows that phosphorylation of the three N-terminal sites proceeds independently of Y173.

Phosphorylation of many proteins proceeds according to a stepwise mechanism, whereby one site primes the protein for subsequent phosphorylation at additional sites. In the case of SLP-76, the three N-terminal sites are required for recruitment and activation of Itk (Bogin *et al*, 2007), suggesting that they may be required for phosphorylation of Y173. Consistent with this idea, Y173 was not phosphorylated in J14 cells that stably express the Y3F mutant of SLP-76, in which tyrosines 113, 128 and 145 are mutated to phenylalanine (Figure 3A).

The N-terminal phosphorylation sites of SLP-76 have been divided into two groups according to the sequence immediately surrounding the phosphorylated tyrosine. Y113 and 128 are embedded in the sequence DYESP, whereas Y145 occurs in the sequence DYEPPP (Fang *et al*, 1996). To address their contribution to Y173 phosphorylation, J14 cells were transiently transfected with SLP-76 that was either wild-type or mutated at one (Y145F), two (Y2F; Y113,128F) or three (Y3F; Y113,128,145F) tyrosines. As previously reported (Jordan *et al*, 2006), TCR-induced phosphorylation of PLC-γ1 was markedly reduced by the single and double mutations of SLP-76 and abrogated by the triple mutation. Phosphorylation of Y173 followed a similar pattern (Figure 3B), suggesting that both the DYESP and the DYEPPP motifs contribute to Y173 phosphorylation. Broadly similar results were obtained upon stimulation of thymocytes (Figure 3C) or splenic T cells (Figure 3D) from gene-targeted mice that bear genomic Y145F or Y112,128F point mutations on SLP-76 (Jordan *et al*, 2008). Whereas the Y145F mutation produced a substantial reduction in Y173 phosphorylation, it was virtually eliminated in mice bearing the Y112,128F allele of SLP-76 (Figure 3C and D). Taken together, these results strongly support TCR-induced sequential phosphorylation of SLP-76 on at least four tyrosines.

Activation of PLC-γ1 by the TCR depends on tyrosine 173 of SLP-76

To explore the role of Y173 in TCR signalling, we stably reconstituted J14 cells with wild-type or mutant FLAG-tagged SLP-76, by infection with an IRES-GFP-containing retroviral vector, followed by FACS-based cell sorting to remove non-infected cells. Y173 was disrupted by mutation to phenylalanine (Y173F), to alanine (Y173A) or by a short deletion (Δ158–180). GFP and TCR expression in each of the cell lines is presented in Supplementary Figure S3.

We first tested whether Y173 is required for recruitment of proteins to the SLP-76- and LAT-nucleated signalling complex. Upon TCR stimulation, wild-type SLP-76 associated with a number of proteins, including PLC-γ1, Vav, Itk, Lck and Nck. None of these interactions was disrupted by the Y173F mutation; however, we reproducibly observed a somewhat extended association of the Y173F mutant with PLC-γ1 and Vav (Figure 4A). Taken together with Figure 2C, these experiments demonstrate that the Y173F mutation does not affect phosphorylation of SLP-76 at its N-terminal tyrosine phosphorylation sites, nor does it affect the recruitment of Nck, Vav and Itk to these sites. Even the indirect association of SLP-76 with PLC-γ1 was not reduced by the Y173F mutation, suggesting that the SLP-76- and LAT-nucleated signalling complex is largely intact.

The overall pattern of TCR-induced tyrosine-phosphorylated proteins was not grossly affected by mutation of Y173; however, phosphorylation of the 150-kDa band corresponding to PLC-γ1 was abrogated (Figure 4B). The marked dependence of PLC-γ1 phosphorylation on tyrosine 173 was more convincingly shown by using a phosphospecific antibody for PLC-γ1 Y783, one of the sites required for its activation (Serrano *et al*, 2005) (Figure 4C, top two panels). A similar impairment of PLC-γ1 phosphorylation was observed upon mutation of Y173 to alanine, or upon deletion of the region of SLP-76 surrounding Y173 (Δ158–180) (Supplementary Figure S4). This impairment was quite profound; indeed, PLC-γ1 phosphorylation in the Y173F mutant cells was only slightly higher than in the absence of SLP-76 (Figure 4C).

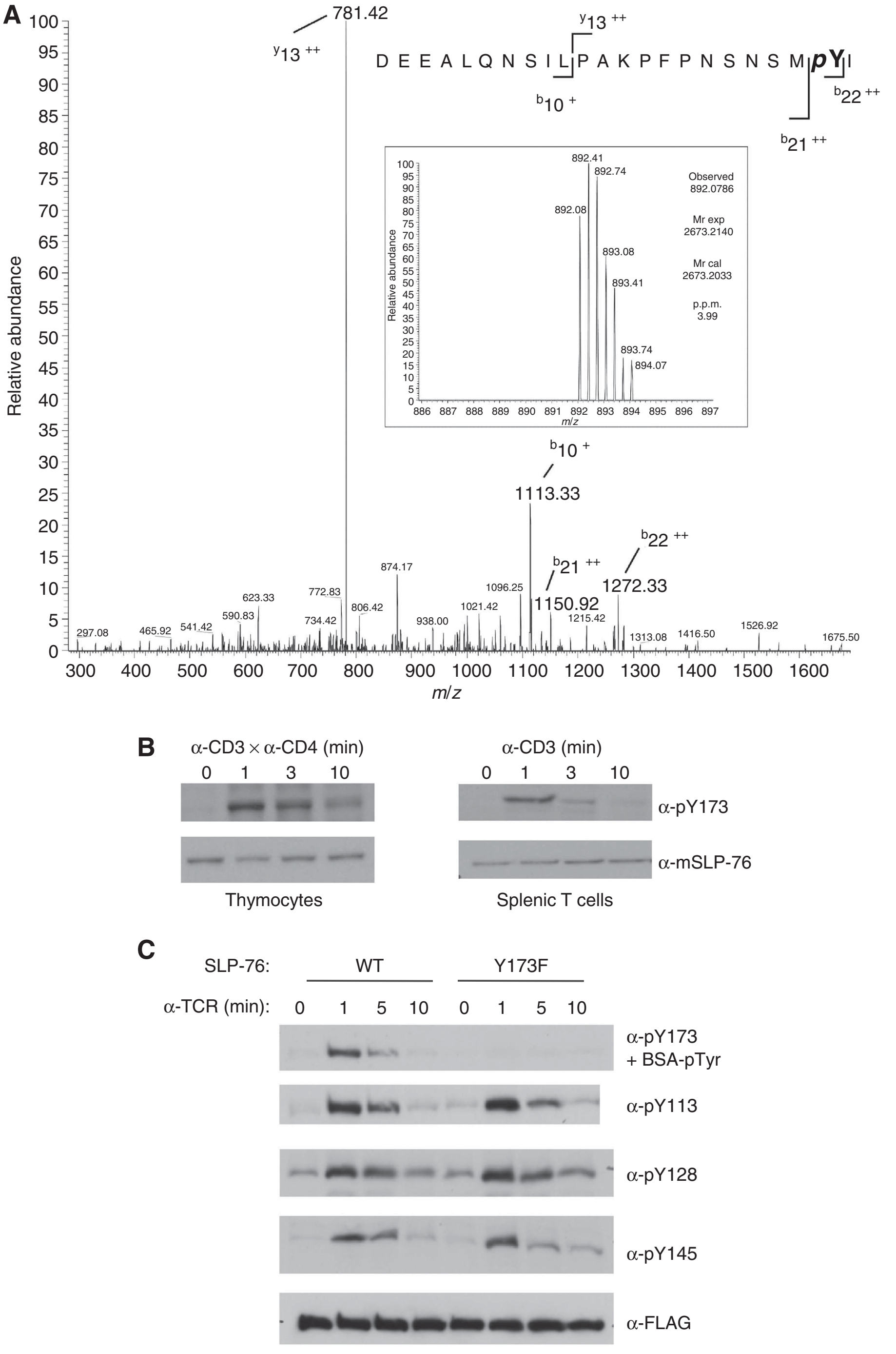

Figure 2 TCR-inducible phosphorylation of Y173 in intact T cells. (**A**) Mass spectrometry analysis of a peptide derived from SLP-76 with Y173 being phosphorylated. FLAG-tagged SLP-76 was immunopurified from TCR-stimulated J14-76-11 cells and digested with Asp-N endoprotease, followed by enrichment of phosphopeptides with titanium oxide chromatography and LC-coupled MSMS analysis. Shown is the MSMS analysis of the peptide DEEALQNSILPAKPFPNSNSM*p*YI derived from SLP-76. The insert shows the intact mass-to-charge ratios of the particular phosphopeptides selected for analysis by MSMS, the sequence of the peptide, and the corresponding b-type ions that unambiguously identified Tyr173 to be phosphorylated. The mass of the intact peptide (MS) and the mass deviation between calculated and experimental mass are shown. See also Supplementary Figure S2. (**B**) TCR-inducible phosphorylation of Y173 in primary T cells. Murine thymocytes (left panels) and negatively purified splenic T cells (right panels) were stimulated for the indicated time with avidin-crosslinked anti-CD3 and anti-CD4 (thymocytes) or avidin-crosslinked anti-CD3 (splenic T cells) and lysed. Lysates were probed with anti-SLP-76 phospho-Y173, then stripped and reprobed with anti-murine SLP-76. Results are representative of three independent experiments. (**C**) Y173-independent phosphorylation of the N-terminal tyrosines. J14 cells, retrovirally reconstituted wild-type or Y173F-mutated, FLAG-tagged SLP-76, were stimulated with anti-TCR for the indicated time and lysed. Anti-FLAG immunoprecipitates from 15 million cells (top two panels) or lysates from 0.5 million cells (third and fourth panels) were probed with the indicated anti-SLP-76 phosphospecific antibodies. Subsequent stripping and reprobing of membranes with anti-FLAG indicated equivalent loading of SLP-76 in all lanes (bottom panel and data not shown). Results are representative of two independent experiments.

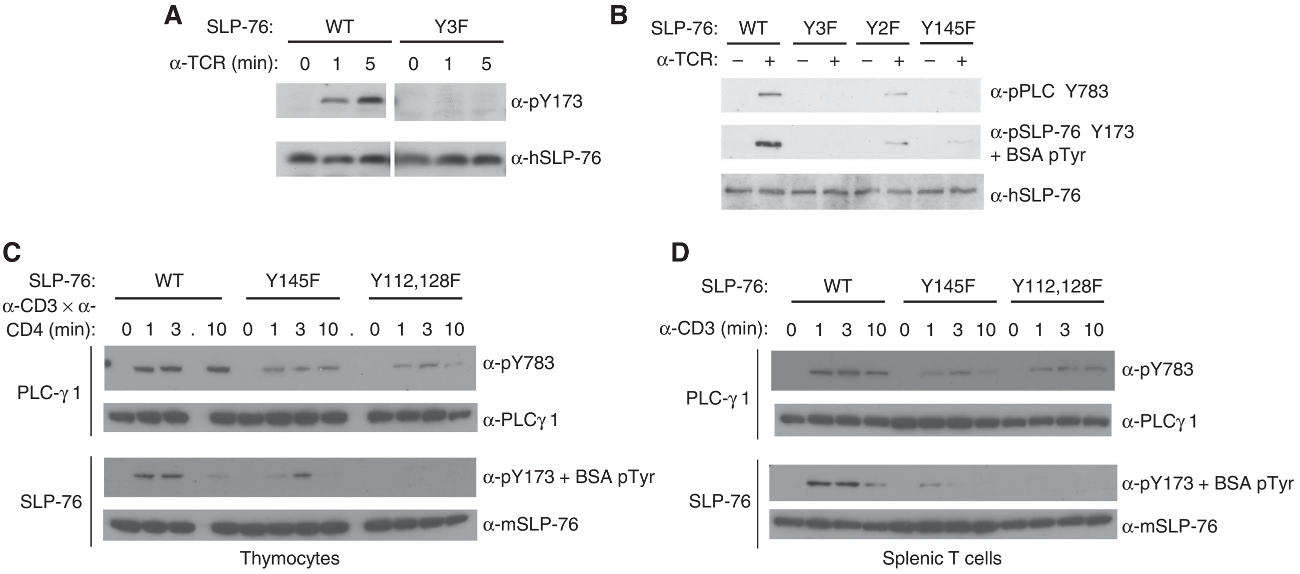

Figure 3 Phosphorylation of Y173 is primed by three N-terminal tyrosines. The indicated cell types were stimulated and lysed. Western blots of the lysates were probed with the indicated phosphospecific antibodies, then stripped and reprobed for the total protein, as indicated. All results shown are representative of at least three independent experiments. The cell types used in these experiments were as follows: (**A**) J14 cells, stably reconstituted with wild-type or Y3F-mutated (Y113,128,145F) human SLP-76. (**B**) One day before stimulation and lysis, J14 cells were transiently transfected with the indicated alleles of FLAG-tagged human SLP-76: wild-type; Y3F (Y113,128,145F) Y2F (Y113, 128F) or Y145F. (**C**) Thymocytes were isolated from gene-targeted 'knockin' mice bearing the indicated point mutations in SLP-76, and stimulated with avidin-crosslinked anti-CD3 and anti-CD4. (**D**) CD90.2$^+$ purified splenic T cells were isolated from the strains of mice shown in (**C**), and stimulated with crosslinked anti-CD3.

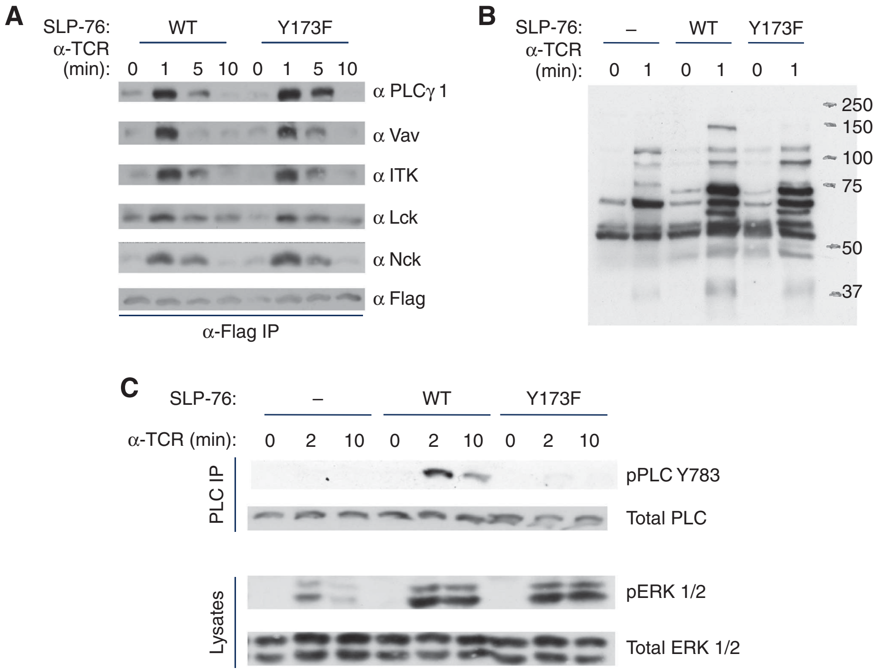

Figure 4 Y173 is required for TCR-induced phosphorylation of PLC-γ1. J14 cells were retrovirally reconstituted with wild-type or Y173F-mutated, FLAG-tagged SLP-76 (see Supplementary Figure S3). Cells were stimulated for the indicated time with anti-TCR and lysed. All results shown are representative of at least three independent experiments. (**A**) TCR-inducible recruitment of signalling proteins to the SLP-76-nucleated complex. Anti-FLAG immunoprecipitates prepared from the lysates of 20 million cells were separated by SDS–PAGE on a 9–12% gradient gel, and were analysed by probing the western blot with the indicated antibodies. (**B**) TCR-induced tyrosine phosphorylation. Lysates were probed with anti-phosphotyrosine (4G10). (**C**) TCR-induced phosphorylation of PLC-γ1 and Erk1/2. Anti-PLC-γ1 immunoprecipitates (top two panels) or lysates (bottom two panels) were probed with the indicated phosphospecific antibodies, then stripped and reprobed to detect total PLC-γ1or Erk1/2. See also Supplementary Figure S4.

Unlike the profound decrease in PLC-γ1 phosphorylation, TCR-induced tyrosine phosphorylation of Itk did not depend on Y173 of SLP-76 (Figure 5A). This result was somewhat surprising since Itk is thought to directly phosphorylate PLC-γ1, and we expected that reduced phosphorylation of PLC-γ1 might correlate with reduced activation of Itk.

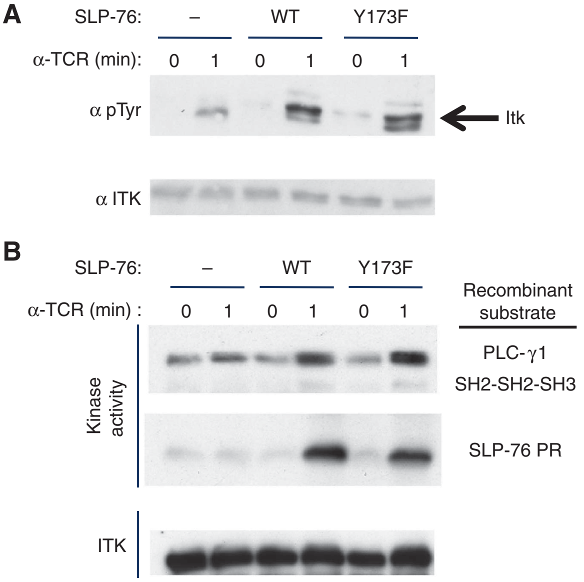

Figure 5 Phosphorylation and activation of Itk do not depend on Y173. The indicated, retrovirally reconstituted J14-derived cell lines were stimulated and lysed as in Figure 4. (**A**) TCR-induced phosphorylation of Itk. Anti-Itk immunoprecipitates, prepared from the lysates of 19×10^6 cells, were probed by western blotting with anti-phosphotyrosine (4G10, top) then stripped and reprobed with anti-Itk (bottom). The band corresponding to Itk in the upper blot is indicated with an arrow. The faint tyrosine-phosphorylated band seen just above Itk represents co-immunoprecipitating SLP-76. Results are representative of three independent experiments. (**B**) TCR-induced Itk kinase activity. Itk immune complexes were prepared from the lysates of 15×10^6 cells, and their catalytic activity was assayed. Assay conditions were similar to those used in Figure 1 except that the concentration of ATP was 10 μM, and two different recombinant GST fusion proteins were included in each kinase reaction: GST-PLC-$\gamma1^{\mathrm{SH2-SH2-SH3}}$ (Bogin *et al*, 2007), and GST fused to residues 150–196 of SLP-76 (SLP-76 PR). Reaction products were separated by SDS–PAGE and probed with anti-phospho-Y783, to detect phosphorylation of GST-PLC-$\gamma1^{\mathrm{SH2-SH2-SH3}}$ (top panel), 4G10, to detect phosphorylation of the recombinant SLP-76 substrate (middle panel) and anti-Itk, to verify comparable amounts of the kinase in each reaction (bottom panel). Figure is representative of four independent experiments.

To more directly measure Itk kinase activity, we performed an immune complex kinase assay to measure the catalytic activity of Itk, isolated from TCR-stimulated cells that express either wild-type or Y173F SLP-76. This assay was performed using two different recombinant substrates: glutathione S-transferase (GST) fused to a fragment of PLC-γ1 that can be phosphorylated by Itk at Y783 (Bogin *et al*, 2007), and GST fused to a fragment from the proline-rich region of SLP-76, which can be phosphorylated by Itk at Y173 (see Figure 1B). Mutation of SLP-76 at Y173 did not reduce activation of Itk, as measured by its ability to phosphorylate either of the exogenous substrates in this assay (Figure 5B). Taken together, our results suggest that residue Y173 of SLP-76 is required in the context of intact cells, to couple active Itk to its substrate, PLC-γ1.

SLP-76 Y173 is required for signalling events downstream of PLC-γ1

PLC-γ1 produces two second messengers, IP_3 and DAG, which trigger calcium flux and Ras activation, respectively. TCR-induced calcium flux was reduced in cells expressing SLP-76 Y713F, as compared with cells expressing wild-type SLP-76 (Figure 6A). Although the reduction in calcium flux was moderate, it was sustained for at least 1.5 h after TCR stimulation (Supplementary Figure S5). This ongoing reduction in TCR-induced calcium levels could have a cumulative effect on the ability to mount downstream effector responses.

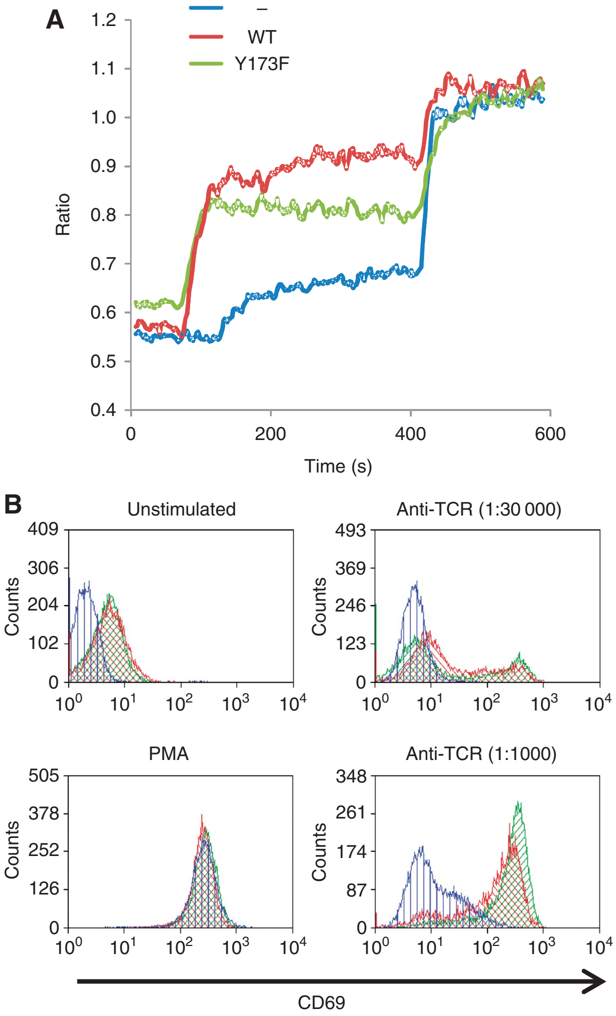

Figure 6 Y173 is required for TCR-induced calcium flux. (**A**) TCR-induced calcium flux. J14 cells (blue) or cells reconstituted with wild-type (red) or Y173F-mutated (green) SLP-76 were loaded with indo1-AM and intracellular calcium was measured using a plate fluorimeter as described (Gonen *et al*, 2005). Anti-TCR (C305, 1:10 000) was added at 50 s and thapsigargen (1 μM) was added at 410 s. The ratio of emission at 405 nm (calcium-bound indo1) to 486 nm (calcium-free indo1) is presented as a moving average calculated over overlapping 10 s intervals. Results are representative of at least five experiments. See also Supplementary Figure S5. (**B**) TCR-induced expression of CD69. J14 cells (blue) or cells reconstituted with wild-type SLP-76 (red) or Y173F-mutated SLP-76 (green) were mock stimulated, or stimulated overnight with plate-bound anti-TCR, or with 25 ng/ml of PMA. Cells were surface stained with PE-Cy5-conjugated anti-CD69, and analysed by FACS. Results are representative of three independent experiments.

The partial impairment of TCR-induced calcium flux suggests that second messenger production is not completely abrogated by the Y173F mutation. Whereas IP_3 directly triggers calcium release, DAG-mediated activation of the Ras-MAPK pathway proceeds via a positive feedback loop that involves both DAG-dependent activation of Ras-GRP and subsequent activation of a second Ras exchange factor, SOS;

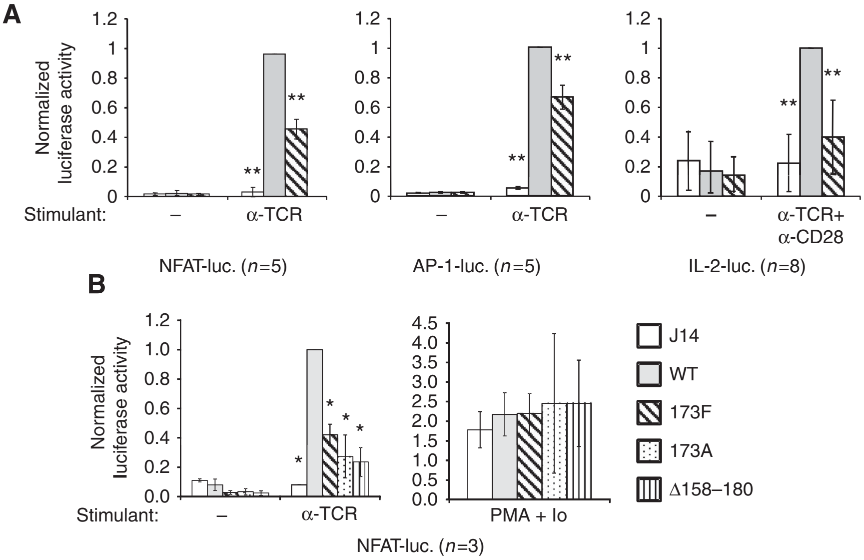

Figure 7 Role of Y173 in transcriptional responses to TCR stimulation. (**A**) J14 cells (white bars), or cells stably infected with wild-type SLP-76 (grey bars) or Y173F-mutated SLP-76 (striped bars) were transiently transfected with the indicated luciferase reporter plasmids along with a renilla luciferase plasmid for normalization purposes. After 16 h, the cells were mock stimulated (−) or stimulated for 6 h with plate-bound anti-TCR alone, or in combination with 10 μg/ml soluble α-CD28, as indicated in the graphs. Firefly luciferase activity was normalized to the renilla luciferase activity measured in the same well. Normalized values were expressed relative to the activity measured in TCR-stimulated wild-type cells from the same experiment. '*n*' indicates the number of independent experiments averaged in each panel; error bars indicate the s.d. between independent experiments. The two-tailed Student's *t*-test was used to evaluate the statistical significance of differences in TCR-induced luciferase activity; '**' indicates a *P*-value of <0.005 for the indicated cell type as compared with wild-type cells. (**B**) J14 cells stably infected with the indicated alleles of SLP-76 were transiently transfected with an NFAT-luciferase reporter plasmid and renilla luciferase control plasmid. Luciferase activity was measured following 6 h of stimulation with plate-bound anti-TCR, or with PMA (50 ng/ml) and ionomycin (1 μM). Shown is the average normalized luciferase activity from three independent experiments, error bars indicating the s.d. '*' indicates a *P*-value of <0.02 for the indicated cell type as compared with wild-type cells.

this loop mediates robust activation of the Ras-MAPK pathway in response to even weak signals (Das *et al*, 2009). Consistent with this notion, TCR-induced phosphorylation of Erk1 and Erk2 was not reduced by the Y173F mutation (Figure 4C, bottom two panels); and TCR-induced surface expression of the Ras-dependent activation marker, CD69 was not impaired (Figure 6B).

PLC-γ1 activation is required for downstream activation of the NFAT and AP-1 transcription factors that participate in transcriptional activation of IL-2. Consistent with reduced PLC-γ1 phosphorylation and calcium flux, cells expressing SLP-76 Y173F exhibited markedly reduced activation of an NFAT-luciferase reporter plasmid in response to TCR stimulation (Figure 7A, left panel). TCR-induced NFAT activation was impaired to a similar extent by three different mutations that disrupt Y173 (Figure 7B, left panel), but as expected, none of the mutations disrupted NFAT activation in response to PMA and ionomycin, pharmacologic stimuli that bypass SLP-76 (Figure 7B, right panel). These mutations disrupt tyrosine 173 in different ways: increasing hydrophobicity while preserving a similar structure (Y173F), not affecting hydrophobicity (Y173A), or deleting the entire region (Δ158–180). The similar effect of structurally different mutations suggests that impaired signalling results from the loss of phosphorylation at tyrosine 173, rather than from any regional effect on hydrophobicity or local structure. Activation of an AP-1 luciferase reporter plasmid was also reduced (Figure 7A, middle panel). Most importantly, activation of a luciferase reporter construct driven by the IL-2 promoter was markedly reduced upon mutation of tyrosine 173 (Figure 7A, right panel). Together these results support the functional importance of this evolutionarily conserved tyrosine phosphorylation site.

Mast cell activation depends on tyrosine 173 of SLP-76

Functional studies in the J14 background are quite informative, but may not fully reflect the role of Y173 in untransformed, primary haematopoietic cells. Primary bone marrow-derived mast cells (BMMCs) are a complementary system for addressing the signalling functions of SLP-76, since SLP-76 is required for their activation through the FcεRI, but is not required for their development (Pivniouk *et al*, 1999). To this end, we retrovirally reconstituted SLP-76-deficient or wild-type bone marrow with different alleles of SLP-76, followed by *in vitro* differentiation to the mast cell lineage and sorting for infected cells. We chose to compare the Y173F mutant of SLP-76 to the Y145F mutant, which exhibits dramatically reduced SLP-76 functionality in both T cells and mast cells (Jordan *et al*, 2006, 2008; Lenox *et al*, 2009).

On a biochemical level, both the Y173F and Y145F mutations markedly impaired FcεRI-induced phosphorylation of PLC-γ1 and p38 Map kinase (Figure 8A). As a measure of upstream processes, we looked at phosphorylation of SLP-76 itself at tyrosine 128, which was not diminished by the Y173 mutation (Figure 8A, middle panels). Thus, it appears that in mast cells, as in the J14 model, Y173 is required to couple upstream events to the activation of PLC-γ1.

In line with the reduction in PLC-γ1 phosphorylation, FcεRI-induced calcium flux was consistently reduced by the Y173F mutation, but was somewhat above the baseline level exhibited by SLP-76-deficient mast cells and cells expressing the Y145F mutation (Figure 8B, left panel). Thapsigargin-

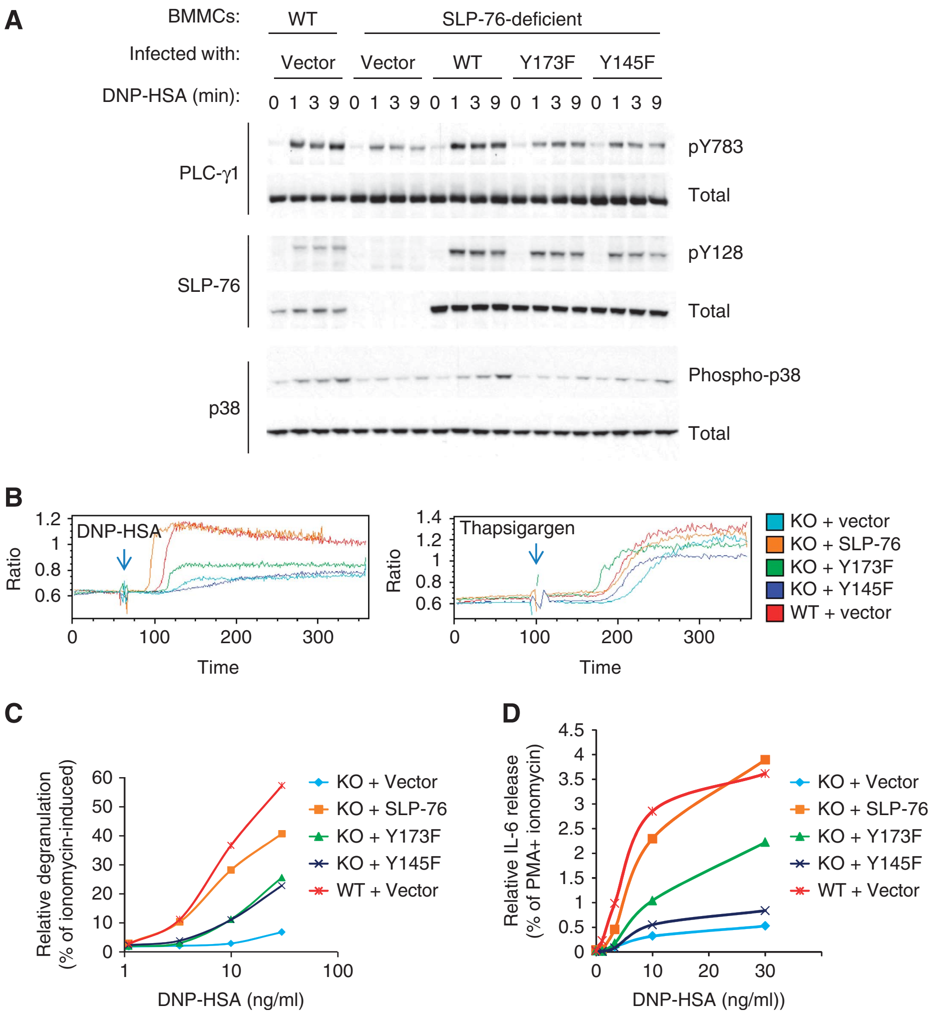

Figure 8 Role of Y173 in mast cell activation. Fully differentiated, retrovirally reconstituted BMMCs from wild-type or SLP-76-deficient mice, expressing the indicated alleles of SLP-76, were sensitized with a monoclonal IgE type antibody specific for DNP and activation was triggered with DNP conjugated to HSA (DNP-HSA). Four different FcεRI-induced responses were measured. (**A**) Phosphorylation of PLC-γ1 and p38. Cells were stimulated with DNP-HSA for the indicated time and lysates were probed with the indicated antibodies. (**B**) Calcium flux. Cells were labelled with indo1 and ratiometric detection of intracellular calcium was performed by FACS, with stimulant added at the time indicated with the arrow. Increased intracellular calcium results in an increased blue/violet ratio. Shown is the average ratio exhibited by each of the cell types upon stimulation with 10 ng/ml DNP-BSA (left), or 1 μM thapsigargen (right). (**C**) Degranulation. Cells were stimulated for 45 min with the indicated concentration of DNP-HSA or with 1 μM ionomycin. Degranulation is expressed as the β-hexosaminidase activity released into the medium, relative to the activity released upon stimulation with ionomycin. Results are representative of six experiments. (**D**) IL-6 secretion. IgE-sensitized cells were stimulated for 6 h with the indicated concentration of DNP-HSA, or with 50 ng/ml PMA and 1 μM ionomycin. IL-6 released into the medium was measured by ELISA. Results are representative of six experiments and are expressed relative to the amount of IL-6 produced by the same cells upon stimulation with PMA and ionomycin.

induced calcium flux was also measured, to rule out differences in cell viability or calcium stores, and did not differ between the cell types (Figure 8B, right panel). The effect on downstream calcium-dependent effector responses was consistent with the reduction in calcium flux. FcεRI-induced degranulation was consistently reduced to a similar extent by the 173F and 145F mutations (Figure 8C). On a longer time scale, FcεRI-induced secretion of IL-6 was markedly reduced by the Y173F mutation, although not to the same extent as the Y145F mutation (Figure 8D). Taken together, these experiments support the idea that Y173 of SLP-76 has an important regulatory role in antigen receptor signalling pathways, by coupling the early events of the pathway to activation of PLC-γ1, thereby impacting PLC-γ1-dependent downstream events.

Discussion

Current models depict two aspects of SLP-76 activation: its phosphorylation by ZAP-70 at three N-terminal sites, and its recruitment to LAT via Gads (Zou *et al*, 2010). In this study, we uncover an additional layer of regulation, via tyrosine phosphorylation of SLP-76 at a fourth, evolutionarily conserved site, Y173. This site is inducibly phosphorylated upon TCR stimulation of primary murine or Jurkat T cells. Y173 phosphorylation occurs by a sequential mechanism that depends on prior phosphorylation of the three N-terminal sites. This is most easily understood in terms of the requirement of the N-terminal sites for TCR-induced recruitment and activation of Itk (Bogin *et al*, 2007), along with the ability of Itk, but not ZAP-70 to phosphorylate Y173 *in vitro*.

Most importantly, Y173 is required for antigen receptor-induced phosphorylation of PLC-γ1, and for consequent, PLC-γ1-dependent responses to antigen receptor stimulation. Our mast cell reconstitution experiments establish the importance of Y173 in the context of nontransformed, primary haematopoietic cells, and demonstrate its role in both short- and long-term responses to antigen, exemplified by degranulation and cytokine production, respectively. Surprisingly, mutation of Y173 produced a signalling defect comparable to mutation of Y145, a site of well-established importance in both T and mast cells (Jordan *et al*, 2006, 2008; Lenox *et al*, 2009).

SLP-76 exemplifies the reciprocal regulatory interactions that can occur between kinases and adaptor proteins. Upon TCR stimulation, SLP-76 binds and activates two protein kinases, Itk and HPK1 (Sauer *et al*, 2001; Bogin *et al*, 2007). By virtue of their close interaction with SLP-76, these kinases are ideally situated to provide feedback by phosphorylating SLP-76 at one or more sites. Indeed, HPK1 phosphorylates SLP-76 at a negative regulatory site (Di Bartolo *et al*, 2007; Shui *et al*, 2007), and we now show that Itk can exert positive feedback on SLP-76 by phosphorylating it at tyrosine 173. The sequential phosphorylation of SLP-76 by ZAP-70 and Itk may allow diverse regulatory inputs to modulate T-cell responsiveness. We speculate that at some developmental stages, decreased phosphorylation of Y173 may uncouple antigen receptor signalling from calcium-dependent effector functions, whereas at other developmental stages, increased Y173 phosphorylation could recouple these events. While entirely speculative, this hypothesis suggests potential implications of studying the multiple layers of regulation of SLP-76.

In intact cells, Y173 phosphorylation is not necessarily limited to Itk, but may be carried out by other Tec-family kinases, such as Rlk and Btk. Rlk is partially redundant with Itk for T-cell development and function (Schaeffer *et al*, 1999), and was previously reported to phosphorylate SLP-76 (Schneider *et al*, 2000), although the precise sites and their physiologic relevance were not mapped. Mast cell activation is regulated by both Itk and Btk (Iyer *et al*, 2011), and it remains to be seen which kinase may phosphorylate Y173 in this cell type, and with what time course. The strong mast cell phenotype observed upon mutation of Y173 is consistent with the notion that it is phosphorylated upon FcεRI stimulation; nonetheless, we have not yet observed its phosphorylation in this cell type. Btk function is better understood in B cells, where it binds to SLP-65 (Hashimoto *et al*, 1999; Su *et al*, 1999), a B-cell adaptor analogous to SLP-76 (Fu *et al*, 1998; Wienands *et al*, 1998). We note a conserved motif Y(V/I/A)DNR (Y138 in chicken SLP-65) that is inducibly phosphorylated upon BCR stimulation (Oellerich *et al*, 2009), and roughly aligns by location and sequence with SLP-76 Y173. It will be interesting to see whether this motif has a regulatory role resembling Y173 in SLP-76.

Phosphorylation of Y173 was profoundly dependent on the three, previously characterized tyrosine phosphorylation sites, both in a J14-based model and in primary mouse thymoctes and splenocytes. Past studies have ascribed discrete functions to these sites, suggesting that Y112 and 128 bind to Nck and Vav, whereas Y145 binds to Itk (Raab *et al*, 1997; Fang and Koretzky, 1999; Wunderlich *et al*, 1999; Bunnell *et al*, 2000). More recent studies suggest that Itk, Vav and Nck bind to SLP-76 in a cooperative, interdependent manner, probably due to the direct interaction of Vav with both Itk and Nck (Dombroski *et al*, 2005; Barda-Saad *et al*, 2010). It therefore becomes difficult to clearly differentiate between Vav- and Itk-mediated signalling events; and indeed, analyses of the SLP-76 N-terminal tyrosine mutants in a mouse model revealed graded defects, where mutating SLP-76 Y112 and Y128 was somewhat less severe than mutating Y145 (Jordan *et al*, 2006, 2008; Lenox *et al*, 2009). Consistent with this notion, mutation of Y112 and 128 (Y2F) profoundly disrupted Y173 phosphorylation, as did mutation of Y145 alone. We therefore suggest that cooperative interactions at the N-terminus of SLP-76 create a signalling complex that is competent to phosphorylate the adjacent Y173.

The most prominent biochemical phenotype of T and mast cells observed upon mutation of SLP-76 at Y173 was reduced phosphorylation of PLC-γ1 at Y783. In mast cells, reduced PLC-γ1 phosphorylation was associated with dramatically reduced calcium flux and downstream effector functions. In contrast, calcium flux was moderately but persistently reduced in the J14 background, while DAG-dependent events such as Erk phosphorylation and CD69 expression were not appreciable reduced, suggesting that PLC-γ1 activity was not completely abrogated. To some extent, calcium flux in this model may occur independently of PLC-γ1 phosphorylation; indeed, tyrosine phosphorylation-independent mechanisms of PLC-γ activation have been described (Sekiya *et al*, 1999a, b; Piechulek *et al*, 2005), and PLC-γ-independent mechanisms of TCR-induced calcium flux have been proposed (Matza *et al*, 2008). One important aspect of PLC-γ1 activation is its recruitment to the membrane, where its substrate is located. Constitutive recruitment of PLC-γ1 to the membrane partially activates the enzyme (Veri *et al*, 2001), although it can be further activated upon its phosphorylation at tyrosine 783 (Beach *et al*, 2006). Vav and Cbl also contribute to PLC-γ1 activation through incompletely understood mechanisms (Reynolds *et al*, 2002; Rellahan *et al*, 2003; Chiang *et al*, 2009; Saveliev *et al*, 2009). Finally, PI-3-kinase activity, which is constitutively high in Jurkat-derived cell lines due to the absence of PTEN (Shan *et al*, 2000), contributes to PLC-γ1 membrane recruitment and activation. Most likely, constitutive activation of the PI-3-kinase pathway, together with intact recruitment of PLC-γ1 and Vav to the SLP-76-nucleated complex may permit partial PLC-γ1 activation and facilitate its interaction with membrane-bound substrates, despite a low level of PLC-γ1 phosphorylation.

Our observations highlight the importance of subtle regulatory interactions that occur within the SLP-76–Gads–LAT-nucleated complex. Itk-mediated phosphorylation of PLC-γ1 depends on a docking interaction between the kinase domain of Itk, and a basic surface on the back of the C-terminal SH2 domain of PLC-γ1 (PLC-SH2C); in brief, Itk binds to PLC-SH2C, and this allows it to phosphorylate PLC-γ1 at the adjacent Y783 (Joseph *et al*, 2007; Min *et al*, 2009). The marked reduction in PLC-γ1 phosphorylation upon mutation of SLP-76 Y173 suggests that it may participate in this docking event. This contribution cannot be viewed in terms of recruitment of signalling molecules to the complex, since mutation of Y173 did not reduce recruitment of Itk, Vav or PLC-γ1 to the SLP-76-nucleated complex. The contribution of Y173 also does not involve activation of Itk, since its tyrosine phosphorylation and catalytic activity were not appreciably reduced upon mutation of SLP-76 Y173. We therefore suggest that phosphorylation of Y173 triggers conformational changes within the SLP-76-nucleated complex, that facilitate the interaction of SLP-76-bound, active Itk with its substrate, PLC-γ1.

Y173 phosphorylation may trigger a conformational change in SLP-76 itself that brings the catalytic domain of Itk closer to its target sites in PLC-γ1. This type of phosphorylation-induced conformation change occurs in ZAP-70, where phosphorylation of interdomain B tyrosines disrupts hydrophobic interactions and promotes a switch to the active conformation (Deindl *et al*, 2007). Consistent with their structural role, mutation of interdomain B tyrosines to phenylalaine stabilizes the inactive conformation, whereas deletion or mutation to alanine reduces hyrophobicity and stabilizes the active conformation (Brdicka *et al*, 2005). In contrast, SLP-76 function was disrupted to an equal extent by a Y173F mutation, Y173A mutation or deletion of the region surrounding Y173. These data are most consistent with a model in which phosphotyrosine 173 promotes SLP-76-mediated signalling by binding to an SH2 domain within a target protein.

Since mutation of Y173 profoundly affects PLC-γ1 phosphorylation, we speculate that the target protein that binds to phospho-Y173 may be PLC-γ1 itself. PLC-γ1 has two SH2 domains followed by one SH3 domain. Whereas the N-terminal SH2 domain recruits PLC-γ1 to LAT (Stoica *et al*, 1998), we suggest that PLC-SH2C may bind to SLP-76 phospho-Y173. Consistent with this notion, mutation of the PLC-SH2C profoundly impairs TCR-induced phosphorylation of PLC-γ1 at Y783, and abrogates its interaction with SLP-76 (Braiman *et al*, 2006). In further support of this idea, we note that SLP-76 Y173 is found within a motif (MYIDR) that is somewhat homologous to PLC-γ1 Y783 (FYVEA); this similarity may underlie the selective phosphorylation of both motifs by Itk (Bogin *et al*, 2007 and this study). Although neither motif corresponds precisely to the optimal binding motif of PLC-SH2C (Songyang *et al*, 1993; Huang *et al*, 2008), PLC-SH2C is known to undergo an intramolecular interaction with phosphoY783 (Poulin *et al*, 2005), and we speculate that the similar motif at SLP-76 Y173 may likewise have affinity for this SH2 domain. Binding of PLC-SH2C and Itk to adjacent sites on SLP-76 (Y173 and Y145, respectively) may facilitate docking of Itk onto PLC-SH2C. The subsequent phosphorylation of PLC-γ1 is associated with the above-mentioned intramolecular rearrangement, whereby PLC-SH2C binds to the adjacent phospho-Y783, thereby promoting a catalytically active conformation (Poulin *et al*, 2005). This rearrangement would detach PLC-γ1 from SLP-76, perhaps allowing for successive binding and activation of multiple PLC-γ1 molecules by each SLP-76-nucleated complex. This model shows how sequential phosphorylation of SLP-76 at Y173 may trigger conformational changes within the SLP-76-nucleated complex that modify the interaction of Itk with its substrate and promote PLC-γ1 activation.

Materials and methods

Antibodies

The monoclonal antibody C305 was used for anti-TCR stimulations of Jurkat-derived cell lines (Weiss and Stobo, 1984). Monoclonal anti-FLAG epitope (M2) and monoclonal anti-dinitrophenol (anti-DNP, IgE isotype) were from Sigma. Polyclonal anti-PLC-γ1 and anti-Vav were from Santa Cruz Biotechnology. Anti-phosphotyrosine (4G10) and anti-Nck were from Upstate Cell Signaling Solutions. Phosphospecific anti-PLC-γ1 pY783 was from MBL International or Cell Signaling Technology. Polyclonal anti-Itk (BL12) (Tomlinson *et al*, 1999) and monoclonal anti-Lck (clone 1F6) were provided by Michael G Tomlinson and Joseph Bolen. Polyclonal anti-ZAP-70 (Qian *et al*, 1997) was provided by Dapeng Qian and Arthur Weiss. Polyclonal anti-human SLP-76 was previously described (Gonen *et al*, 2005). Polyclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), anti-p38 and rabbit monoclonal anti-phospho-p38 (Thr180/Tyr182) were from Cell Signaling Technology. Monoclonal anti-mouse SLP-76, anti-mouse CD3ε-biotin (clone 145-2C11), anti-mouse CD4-biotin (clone GK1.5) and anti-human CD3-APC were from eBioscience. Polyclonal phosphospecific antibodies to human SLP-76 Y113 and Y145 were from Novus Biologicals. Mouse monoclonal antibody to human SLP-76 Y128 was from BD Pharmingen. PE-Cy5-conjugated anti-CD69 was from Serotec.

A polyclonal, affinity-purified antibody against SLP-76 phosphoY173 was prepared by Eurogentec. Briefly, a phospho-peptide NSNSMpYIDRPPSG, corresponding to amino acids 168–180 of human SLP-76 was conjugated to KLH, and used to immunize rabbits, followed by two steps of affinity chromatography, to remove antibodies that recognize the nonphosphorylated peptide and enrich for those that recognize the phosphorylated peptide. In some experiments, we supplemented the diluted antiserum with 5 μg/ml phosphotyrosine-conjugated BSA; this additive decreased background without blocking the sequence-specific recognition of phospho-Y173.

Plasmids

Transient transfections were performed by electroporation using the Gene Pulser (Bio-Rad Laboratories, Hercules, CA), at a setting of 234 V and 1000 μF, using a 0.4-cm cuvette. EcoVR-Blast, the ecotropic viral receptor cloned into pEF6/Myc-His A, was provided by Jeroen Roose. pEF-BOS-based plasmids encoding wild-type or Y to F mutated, FLAG-tagged SLP-76, were used for transient transfection (Fang *et al*, 1996). Retroviral infections were performed using the pMIGR1 vector (Pear *et al*, 1998) into which N-terminally FLAG-tagged human SLP-76 alleles were subcloned upstream of an IRES-GFP cassette. pMIGR1-based plasmids encoding untagged wild-type or Y145F-mutated mouse SLP-76 were previously described (Jordan *et al*, 2006). Point mutations of human and mouse SLP-76 at codon 173 were made using the QuikChange site-directed mutagenesis kit (Stratagene). All mutant constructs were verified by sequencing the entire insert.

Cell lines and retroviral infections

The Jurkat-derived SLP-76-deficient cell line, J14, and J14-derived cell lines stably transfected with FLAG-tagged wild-type SLP-76 (J14-76-11) or with FLAG-tagged SLP-76 bearing tyrosine to phenylalanine mutations at tyrosines 113, 128 and 145 (J14-Y3F) were previously described (Yablonski *et al*, 1998, 2001). J14Eco.1 was created by stable transfection of J14 with EcoVR-Blast, followed by limiting dilution in 10 μg/ml blasticidin, and screening colonies for efficient infection with ecotropic retroviruses. Retroviral packaging was performed in 293T cells by calcium–phosphate-mediated cotransfection of pMIGR1-based retroviral plasmids along with the ecotropic packaging vectors, pVPack-GP and pVPack-Eco (Stratagene). Undiluted (high titer) or diluted (low titer) cell supernatants were used for infection of J14Eco.1 cells, followed by FACS sorting for GFP expression at 2 weeks following infection.

In vitro phosphorylation of recombinant proteins

All GST fusion proteins were expressed in *Escherichia coli* BL21 bacteria, using the pGEX-2TK bacterial expression vector (Amersham Pharmacia Biotech) and purified on glutathione–agarose beads (Sigma), followed by elution with free glutatione (Sambrook and Russel, 2001). *In vitro* phosphorylation reactions using anti-Itk or anti-ZAP-70 immune complexes as the source of kinase activity were performed as described (Bogin *et al*, 2007).

Cell stimulation and lysis

J14 derivatives were stimulated with C305 and lysed as described (Bogin *et al*, 2007).

Murine splenic T cells were purified by negative or positive selection using a Pan T-cell isolation kit or CD90.2 microbeads, respectively (Miltenyi Biotec). Washed, purified splenic T cells or total thymocytes were resuspended in Dulbecco's PBS and were stimulated at 37°C with biotinylated anti-CD3, with or without biotinylated anti-CD4, that was pre-crosslinked with avidin, at a final concentration of 10 μg/ml of each biotinylated antibody. Lysis buffer contained 50 mM Tris pH 8, 1% nonidet P40, 100 mM NaCl, 10% glycerol, 50 mM NaF, 2 mM Na_3VO_4, 50 mM β-glycerol phosphate, 0.5 mM $CaCl_2$, 20 mM sodium pyrophosphate, 5 mM EDTA and 1 mM DTT, 2 mM PMSF and a 1:100 dilution of the Sigma protease inhibitor cocktail (P8340).

Luciferase assays

Luciferase assays were performed as described (Yablonski *et al*, 1998), except that each luciferase reporter plasmid (20 μg of NFAT luciferase, 10 μg of AP-1 luciferase or 20 μg of IL-2 luciferase) was cotransfected with 5 μg of pRL-null, which drives constitutive expression of renilla luciferase (Promega). Following 6 h of stimulation, cells were lysed with passive lysis buffer (Promega) and activity was measured with the Dual Luciferase Kit (Promega).

Mass spectrometry

TCR-stimulated J14-76-11 cells were lysed and SLP-76 was purified by immunoprecipitation with anti-FLAG, followed by elution with a triple FLAG peptide (Sigma). Phosphorylated peptides derived from SLP-76 were obtained after in-gel digestion with endoproteinase Asp-N and subsequent enrichment of the phosphopeptides with TiO2 exactly as described (Oellerich *et al*, 2009). Enriched phosphopeptides were analysed by LC-coupled MSMS and searched against a database as described (Oellerich *et al*, 2009).

Retroviral transduction of BMMCs

Primary BMMCs expressing different alleles of SLP-76 were generated by infection of bone marrow from SLP-76-deficient (Clements *et al*, 1998) or wild-type mice with an IRES-GFP-marked retroviral vector (pMIGR) that was either empty or encoded for different alleles of murine SLP-76. Infection, *in vitro* differentiation into the mast cell lineage under the influence of IL-3 and SCF, and sorting for GFP-expressing cells were performed as previously described (Kambayashi *et al*, 2010). Experiments were begun when the cells were >95% $cKit^+$, $FcERI^+$, as shown by FACS staining. The entire process was repeated three times with comparable results in all functional assays. Prior to functional assays, cells were washed in cytokine-free medium and incubated overnight in medium containing 10 ng/ml IL-3, but lacking SCF. Cells were sensitized by incubation with 0.5–1 μg/ml IgE (anti-DNP) for 2–12 h and were then washed and resuspended in Tyrode's buffer (Kambayashi *et al*, 2010) containing 0.5 mg/ml BSA. Stimulation was initiated by the addition of DNP-conjugated human serum albumin (DNP-HSA). FcεRI-induced phosphorylation events, calcium flux, degranulation and IL-6 production were measured exactly as described (Kambayashi *et al*, 2010).

Multiple Sequence Alignments

Multiple Sequence Alignments were prepared using ClustalW2 (Larkin *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: The study was conceived by DY, YB and MS, with important contributions from all other authors. MS developed the cell lines used in this study, performed most of the experiments and analysed the data. YB developed reagents and performed the experiments depicted in Figure 1. DB prepared samples for mass spectrometry and performed the experiments depicted in Figure 3A, 3B, 4B and 5B. TO and JL performed the mass spectrometry analysis, which was conceived by JW and HU. JES-G performed the experiments depicted in Figure 3C and D, which were conceived by GK. ES and EL developed reagents and protocols for this study. TK conceived and planned the mast cell experiments, which were performed and analysed by MO (Figure 8A) and DY (Figure 8B–D), with the assistance of RK and JC. DY wrote the manuscript with the assistance of all other authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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