The Src family kinase Hck regulates mast cell activation by suppressing an inhibitory Src family kinase Lyn

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IgE/antigen-dependent mast cell activation plays a central role in immediate hypersensitivity and other allergic reactions. The Src family tyrosine kinase (SFK) Lyn is activated by the cross-linking of high-affinity IgE receptors (FcεRI). Activated Lyn phosphorylates the FcεRI subunits, β and γ, leading to subsequent activation of various signaling pathways. Lyn also plays a negative regulatory function by activating negative regulatory molecules. Another SFK, Fyn, also contributes to mast cell degranulation by inducing Gab2-dependent microtubule formation. Here we show that a third SFK, Hck, plays a critical role in mast cell activation. Degranulation and cytokine production are reduced in FcεRI-stimulated *hck*$^{-/-}$ mast cells. The reduced degranulation can be accounted for by defects in Gab2 phosphorylation and microtubule formation. Importantly, Lyn activity is elevated in *hck*$^{-/-}$ cells, leading to increased phosphorylation of several negative regulators. However, positive regulatory events, such as activation of Syk, Btk, JNK, p38, Akt, and NF-κB, are substantially reduced in *hck*$^{-/-}$ mast cells. Analysis of *lyn*$^{-/-}$*hck*$^{-/-}$, *lyn*$^{-/-}$*FcεRI*β$^{-/-}$, and *hck*$^{-/-}$*FcεRI*β$^{-/-}$ cells shows that Hck exerts these functions via both Lyn-dependent and Lyn-independent mechanisms. Thus, this study has revealed a hierarchical regulation among SFK members to fine-tune mast cell activation. (Blood. 2007;110: 2511-2519)

Introduction

Mast cells are key effector cells for IgE-dependent immediate hypersensitivity and other allergic reactions. These reactions are triggered by cross-linking of the high-affinity IgE receptor, FcεRI, with IgE and multivalent antigen. FcεRI consists of an IgE-binding α subunit, a signal-amplifying tetramembrane-spanning β subunit, and 2 signal-generating γ subunits.[1] According to the widely accepted model,[2] the following events occur on receptor aggregation: FcεRIβ-associated Lyn, a Src family protein-tyrosine kinase (PTK), phosphorylates tyrosine residues of the immunoreceptor tyrosine-based activation motifs (ITAMs) in β and γ subunits.[3,4] The phosphorylated ITAMs in the β and γ subunits recruit Lyn and Syk molecules, respectively.[4] These ITAM-bound PTKs phosphorylate a multitude of signaling proteins, leading to the activation of several signaling pathways including phosphatidylinositol 3-kinase, phospholipase C/Ca^{2+}, and mitogen-activated protein kinases.[2,5-7] In addition to Lyn, recent studies suggested the presence of another pathway required for degranulation: Fyn, another FcεRI-associated Src family tyrosine kinase (SFK), mediates phosphorylation of the adaptor protein Gab2, leading to phosphatidylinositol 3-kinase activation,[8] as well as Ca^{2+}-independent microtubule formation.[9] Concerted action of these pathways leads to degranulation (release of preformed vasoactive amines and other proinflammatory mediators), synthesis and release of leukotrienes and their derivatives, and production and secretion of cytokines.

In addition to its signal-initiating activity through phosphorylation of tyrosine residues in the γ-ITAM, Lyn also plays a negative regulatory role in aspects of mast cell activation[10-14]: IgE/mast cell-dependent in vivo anaphylactic reactions are enhanced in young *lyn*$^{-/-}$ mice.[12] FcεRI stimulation induces greater production of cytokines in *lyn*$^{-/-}$ than in wild-type (WT) mast cells,[10] whereas the same stimulation induces reduced degranulation in mast cells expressing a constitutively active Lyn.[14] The β subunit also plays both positive and negative regulatory roles in mast cell activation.[15-20] In mouse mast cells, the β subunit is required for stable surface expression of FcεRI. The β-ITAM is unique in 2 ways: the spacing between the 2 canonical tyrosines (Tyr-219 and Tyr-229) harbors a third tyrosine (Tyr-225), and it is one amino acid shorter than canonical ITAMs, making it unfit to bind the tandem SH2 domains of Syk, a PTK essential for most, if not all, activation outcomes.[21,22] Phosphorylation of Tyr-219 contributes to the enhancement of mast-cell activation, whereas that of Tyr-225 opposes it.[19,20] Lyn can phosphorylate both Tyr-219 and Tyr-225 residues, and therefore its roles in positive and negative regulation of mast cell activation are exerted at least in part through phosphorylation of β-ITAM residues. Our recent study showed that Lyn plays a positive regulatory role in survival, degranulation, and cytokine production when mast cells were

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stimulated with "low-intensity" stimuli such as IgE^+ low-concentration antigen (Ag) and IgE^+ anti-IgE, whereas these activation events were negatively regulated by Lyn on "high-intensity" stimulation with IgE^+ high-concentration Ag.[23] Lyn appears to use its associated protein, FcεRIβ, as a pivotal molecule to negatively regulate downstream events on the latter stimulation, because "low-intensity" stimuli leads to a dissociation of Lyn with FcεRIβ, but "high-intensity" stimuli leads to an increased association of Lyn with FcεRIβ.[23] "High-intensity" or supraoptimal antigen stimulation results in the downward phase of bell-shaped antigen dose-response curves. Suppression of mast-cell activation in this phase depends on Lyn,[11] Src homology-2-containing inositol 5′-phosphatase (SHIP),[24] protein kinase C-δ,[25] and the actin cytoskeleton.[26-28]

Here we show that Hck plays a positive regulatory role in mast-cell activation induced under "high-intensity" FcεRI stimulation, in part by suppressing the negative regulatory Lyn kinase activity. In contrast, Lyn-mediated inhibitory signaling does not work under "low-intensity" stimulation.

Materials and methods

Sources of antibodies and some procedures are given in Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Mice, cell culture, and FcεRI stimulation

Bone marrow cells from WT and mutant mice were cultured in IL-3 for 4 to 6 weeks to generate bone marrow mast cells (BMMC) with more than 95% purity (c-Kit^+ FcεRI^+). *Lyn*$^{-/-}$,[29] *hck*$^{-/-}$,[30] and *FcεRI*β$^{-/-}$[31] mice were used. These mice were backcrossed to C57BL/6 mice for at least 8 generations. *lyn*$^{-/-}$*hck*$^{-/-}$, *lyn*$^{-/-}$*FcεRI*β$^{-/-}$, and *hck*$^{-/-}$*FcεRI*β$^{-/-}$ double-knockout mice were generated by crossing single-knockout mice. For FcεRI stimulation, BMMCs were first sensitized by a 24-hour incubation with 0.5 μg/mL of H1 DNP-ε-206 IgE. BMMCs were washed and stimulated with the indicated concentrations of antigen, DNP_{23}-HSA.

Measurements of histamine and cytokines

Amounts of histamine secreted from BMMCs were measured as described.[32] Supernatants of BMMCs were measured by enzyme-linked immunosorbent assay for IL-6 and tumor necrosis factor-α (BD Biosciences Pharmingen, San Diego, CA).

Ca^{2+} measurement

IgE-sensitized BMMCs were loaded with Indo 1-AM (Calbiochem, San Diego, CA) and stimulated with various concentrations of antigen. Fluorescence ratio (525:405 nm) was continuously measured using flow cytometer BD-LSR, as described previously.[33]

Immunoblotting and in vitro kinase assays

Immunoblotting with or without immunoprecipitation and kinase assays for Lyn, Fyn, Syk and JNK molecules were performed as described previously.[33]

Retroviral transduction

Retroviral transduction of *lyn*$^{-/-}$*hck*$^{-/-}$, *lyn*$^{-/-}$*FcεRI*β$^{-/-}$, and *hck*$^{-/-}$*FcεRI*β$^{-/-}$ mast cells was performed as described previously.[34] Briefly, pMX-puro plasmids harboring WT or kinase-dead mouse *hck* cDNA, or WT (YYY) or mutant FcεRI β cDNAs[20] were transfected into packaging cells to generate recombinant retroviruses. BMMCs in culture media containing IL-3 and stem-cell factor (SCF) were infected with the viruses. Mass populations of puromycin-resistant cells were used for FcεRI stimulation.

Microscopy

Slides were viewed with a Zeiss Axiovert Zoom inverted microscope (Carl Zeiss MicroImaging, Gottingen, Germany) using a Zeiss W-Pi Lens at 10×/23 and Zeiss Plan-Neofluar lens at 40×/1.3 and ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, OR). Images were acquired using a Photometrics Cool Snap HQ2 camera (Intelligent Imaging Innovations, Denver, CO), and were processed with Slidebook version 4.1 (Intelligent Imaging Innovations), and Adobe Illustrator version CS2 software (Adobe Systems, San Jose, CA).

Results

Hck protein is 30- to 50-fold less abundant than Lyn protein in mast cells

We determined the amount of 3 SFKs, Lyn, Fyn, and Hck, expressed in BMMCs by immunoblot analysis, using as a reference predetermined amounts of recombinant glutathione-S-transferase (GST)-tagged fusion proteins that contain the antigenic sequences of N-terminal unique regions of SFKs. As expected, Lyn was the most abundant SFK, with its $p53^{lyn}$ isoform present at approximately 500 ng/mg total cellular protein, whereas $p56^{lyn}$ was present at approximately 200 ng/mg (Figure 1C). The amount of $p59^{fyn}$ was estimated as 30 ng/mg. The amounts of $p59^{hck}$ and $p56^{hck}$ isoforms were estimated as low as 10 and 15 ng/mg, respectively (Figure 1B,C). Expression of Hck proteins was comparable in WT and *lyn*$^{-/-}$ BMMCs.

Hck positively regulates the proliferation of mast cells

To investigate the role of Hck in mast cells, bone marrow cells from WT and *hck*$^{-/-}$ mice were cultured in the presence of IL-3. Four

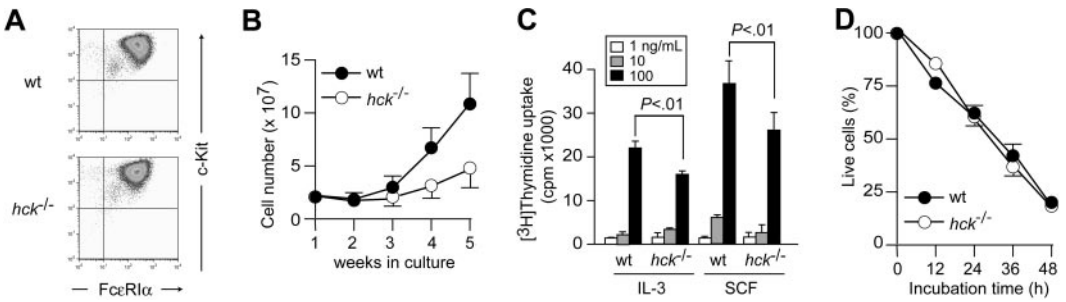

Figure 1. Hck deficiency results in reduced mast cell proliferation. (A) Flow cytometric analysis of FcεRI and c-Kit expression on the surface of WT and *hck*$^{-/-}$ BMMCs. (B) Growth curves of bone marrow cells cultured in IL-3–containing medium. (C) Proliferation of WT and *hck*$^{-/-}$ BMMCs in response to the indicated concentrations of IL-3 or SCF were measured by thymidine uptake. Error bars represent standard deviation (SD) unless otherwise mentioned. (D) Growth factor-deprivation–induced apoptosis in WT and *hck*$^{-/-}$ BMMCs. Percentages of annexin V^-/$7AAD^-$ live cells are plotted as a function of incubation time. Representative results from at least 3 independent experiments are shown.

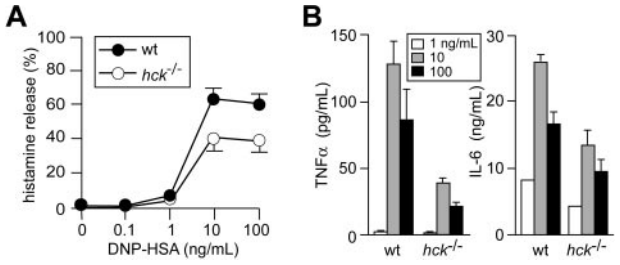

Figure 2. Hck deficiency results in reduced histamine release and cytokine production when mast cells are stimulated with high concentrations of antigen. IgE-sensitized WT and *hck*$^{-/-}$ BMMCs were stimulated with the indicated concentrations of antigen for 45 minutes (A) or 20 hours (B). Histamine, tumor necrosis factor-α, and IL-6 secreted into culture media were measured. Representative results from 3 experiments are shown. Error bars represent SD.

weeks later, more than 95% pure populations of mast cells were generated as determined by flow cytometry for cell-surface expression of c-Kit and FcεRI (Figure 1A), showing no significant differences between WT and *hck*$^{-/-}$ mice. Microscopic analysis of toluidine blue–stained cells revealed an indistinguishable metachromatic cell morphology (data not shown). Therefore, Hck deficiency does not seem to affect the mast-cell differentiation program. This notion was further supported by our observation showing that mast-cell numbers in the ear, back skin, stomach, and small intestine were not different between WT and *hck*$^{-/-}$ mice (data not shown).

However, culturing bone marrow cells from *hck*$^{-/-}$ mice in IL-3–containing medium yielded only approximately one-third of the number of mast cells derived from WT mice (Figure 1B). Thymidine uptake experiments indicated that proliferation of *hck*$^{-/-}$ mast cells in response to either IL-3 or SCF was significantly reduced compared with WT cells (Figure 1C). In contrast, growth factor deprivation induced comparable levels of apoptosis in both WT and *hck*$^{-/-}$ mast cells (Figure 1D). Taken together, these results demonstrate that Hck positively regulates proliferation, but not development or survival, of mast cells.

Hck deficiency leads to impaired FcεRI-mediated degranulation and cytokine production

Degranulation and secretion of inflammatory mediators including various cytokines are cardinal features of FcεRI-induced mast-cell activation. Histamine release (as a surrogate marker for degranulation) was significantly reduced in *hck*$^{-/-}$ mast cells stimulated with 10 or 100 ng/mL DNP_{23}-HSA, compared with WT cells (Figure 2A). Hck deficiency drastically affected FcεRI-induced tumor necrosis factor-α production: a 3- to 4-fold reduction was observed when anti-DNP IgE-sensitized mast cells were stimulated with 10 or 100 ng/mL DNP_{23}-HSA (Figure 2B). IL-6 production was also reduced by Hck deficiency, albeit to a lesser extent. Therefore, these results show that Hck impacts on FcεRI-mediated activation by playing a positive regulatory role, particularly when cells are stimulated with high concentrations of antigen.

Microtubule formation is defective in *hck*$^{-/-}$ mast cells

Ca^{2+} mobilization is required for maximal degranulation in FcεRI-stimulated cells.[35] However, Ca^{2+} mobilization induced by stimulation of IgE-sensitized cells with various antigen concentrations was comparable between WT and *hck*$^{-/-}$ cells (Figure 3A), suggesting that the Ca^{2+}-dependent pathway is not affected in *hck*$^{-/-}$ cells. A recent study revealed 2 steps of FcεRI-induced degranulation, that is, a Ca^{2+}-independent microtubule-dependent translocation of granules to the plasma membrane and Ca^{2+}-dependent membrane fusion and exocytosis. Consistent with this notion, microtubule formation was defective in *hck*$^{-/-}$ mast cells, as detected by immunofluorescence staining (Figure 3B-1,B-2). The amount of polymeric tubulin was also reduced in *hck*$^{-/-}$ cells, as measured by immunoblotting (Figure 3C). Consistent with the role for Gab2 in microtubule formation, Gab2 phosphorylation on Tyr452 was reduced in *hck*$^{-/-}$ cells (Figure 3D-1,D-2). Therefore, these results indicate that Hck controls degranulation by promoting microtubule formation via Gab2 phosphorylation.

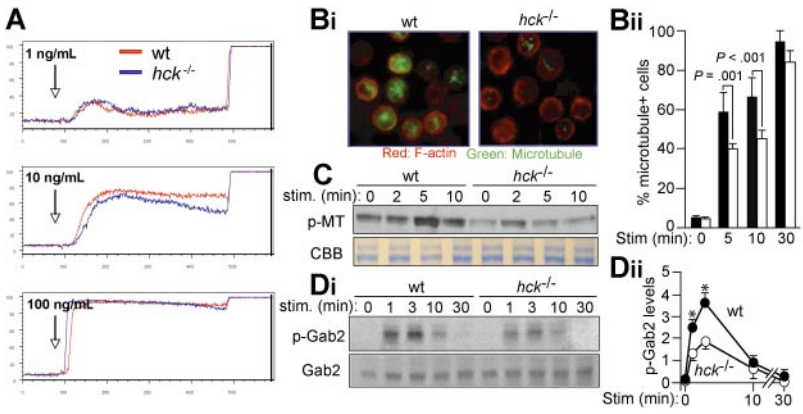

Figure 3. Hck deficiency results in impaired microtubule formation associated with reduced Gab2 phosphorylation. (A) IgE-sensitized WT and *hck*$^{-/-}$ BMMCs were stimulated with the indicated concentrations of antigen at the indicated points and with 2.5 μg/mL ionomycin 400 seconds later. Ca^{2+} flux was measured by flow cytometry. Representative results from 3 experiments are shown. (B) IgE-sensitized cells were stimulated with 100 ng/mL DNP_{23}-HSA for 5, 10, and 30 minutes. Immunofluorescence analysis for F-actin (stained by rhodamine-phalloidin) and microtubules (stained by anti-α-tubulin) was performed. Images shown are taken from cells stimulated for 10 minutes (Bi). The percentage of microtubule^{+} cells is shown in panel Bii. See "Microscopy" for image acquisition information. (C) IgE-sensitized cells were stimulated with 100 ng/mL DNP_{23}-HSA for the indicated periods (minutes). Polymeric tubulin (p-MT) in Triton-insoluble fractions was measured as described in Document S1 (top). An SDS-PAGE gel containing Triton-soluble proteins was stained with Coomassie Brilliant Blue to show that comparable amounts of lysates were used for this assay. (Di) Immunoblot analysis of phospho-Gab2 (Tyr-452) in IgE/antigen-stimulated BMMCs (top panel). The same blot was reprobed with anti-Gab2 (bottom panel). Densitometric analysis was performed (Dii). Values shown in panel Dii represent means from at least 3 independent experiments at each time point. Error bars represent SEM. *Statistically significant differences between WT and *hck*$^{-/-}$ cells ($P < .05$ by Student *t* test).

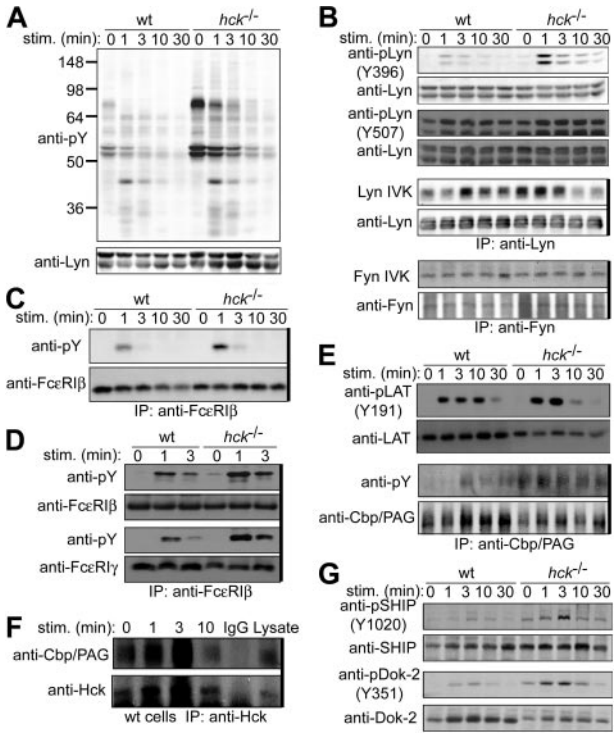

Figure 4. Hck deficiency leads to increased Lyn activity and increased phosphorylation of Lyn phosphorylation targets. IgE-sensitized WT and *hck*$^{-/-}$ cells were stimulated with 100 ng/mL DNP_{23}-HSA for the indicated periods. Cell lysates were either directly analyzed by SDS-PAGE and immunoblotting with the indicated antibodies (A,B,E,G) or first immunoprecipitated (indicated by thick vertical lines on the right of gels) with anti-FcεRIβ mAb (C) or anti-Cbp/PAG (E,F), and followed by immunoblotting with antiphosphotyrosine mAb (C,E) or anti-Hck antibody (F). (B) Immunoprecipitated SFKs were subjected to in vitro kinase assays. (D) Cell lysates were fractionated into lipid raft and soluble compartments by sucrose density gradient ultracentrifugation. Lipid raft compartments were immunoprecipitated with anti-FcεRIβ mAb, and followed by immunoblotting with antiphosphotyrosine mAb. Immunoprecipitated antigens were detected by reprobing the blots. Representative results from 2 experiments are shown, except for Lyn and Fyn kinase assays (B), which represent 3 experiments, and phosphotyrosine probing (A), which represent at least 4 experiments.

Lyn kinase activity is increased in *hck*$^{-/-}$ mast cells

To investigate the molecular mechanism by which Hck positively regulates FcεRI-induced activation, we analyzed signaling events in more detail. Immunoblot analysis revealed increased tyrosine phosphorylation of several proteins, including those of 53 and 56 kDa, in *hck*$^{-/-}$ cells before and after FcεRI stimulation with IgE plus 100 ng/mL of antigen, compared with WT cells (Figure 4A). Consistent with the possibility that these 53- and 56-kDa proteins contain $p53^{lyn}$ and $p56^{lyn}$, levels of phosphorylation at Tyr396 in the activation loop were increased in *hck*$^{-/-}$ cells, whereas phosphorylation at Tyr507 in the C-terminal region of Lyn was not significantly increased. Importantly, the kinase activity of Lyn was increased before, and at early time points of (up to 3 minutes), FcεRI stimulation (Figures 4B,S2). In contrast, Fyn kinase activity was similar in WT and *hck*$^{-/-}$ cells (Figure 4B). Of note, Hck deficiency did not affect expression of Lyn and Fyn proteins. Therefore, it seems that Hck negatively regulates Lyn kinase activity constitutively as well as under "high-intensity" FcεRI stimulation conditions.

Because Lyn kinase activity was increased before and right after FcεRI stimulation in *hck*$^{-/-}$ cells, we predicted that tyrosine phosphorylation of Lyn targets might be increased in *hck*$^{-/-}$ cells. Lyn phosphorylation targets include FcεRI β and γ subunits, LAT (linker for activation of T cells),[36] Cbp/PAG (a lipid raft-resident protein important for Csk recruitment[37,38]), and NTAL (non–T-cell activation linker)/LAB (linker for activation of B cells).[39] Indeed, tyrosine phosphorylation of β and γ subunits was increased in whole cell lysates and lipid raft fractions of *hck*$^{-/-}$ cells (Figure 4C,D). Tyrosine phosphorylation of LAT (Tyr-191), Cbp/PAG and NTAL/LAB was also increased in *hck*$^{-/-}$ cells with kinetics similar to those of Lyn kinase activity (Figure 4E and data not shown). Therefore, the increased tyrosine phosphorylation of Lyn substrates in *hck*$^{-/-}$ cells at early times correlates well with increased Lyn kinase activity.

Lyn deficiency results in increased Fyn activity.[11,12] This can be accounted for by Lyn's role in the phosphorylation of Cbp/PAG, which results in the recruitment of Csk to the plasma membrane,[37,38] where Fyn is phosphorylated by Csk on its C-terminal negative regulatory residue.[40] We next tested whether a similar mechanism, ie, physical association of Hck with Cbp/PAG, might operate for Hck-mediated inhibition of Lyn activity. As shown in Figure 4F, Cbp/PAG associated constitutively with Hck, and this association was increased on FcεRI stimulation. These results suggest that Hck may phosphorylate Cbp/PAG, leading to inhibition of Lyn activity.

Phosphorylation of SHIP and Dok-2 is upregulated in *hck*$^{-/-}$ mast cells

Previous studies showed that phosphorylation of SHIP, an important negative regulator in mast-cell activation,[41] is Lyn- and β-ITAM-dependent.[11,23] Consistent with increased Lyn kinase activity and FcεRIβ phosphorylation, tyrosine phosphorylation of SHIP was also increased in *hck*$^{-/-}$ cells on "high-intensity" FcεRI stimulation, compared with WT cells (Figure 4G). $p56^{dok-2}$, a $p62^{dok}$ homolog, inhibits IL-2–induced and endothelial growth factor receptor–induced mitogen-activated protein kinase activation.[42,43] Not surprisingly, phosphorylation of $p56^{dok-2}$ was also increased in *hck*$^{-/-}$ cells (Figure 4G). These results suggest that Hck inhibits negative regulators by downregulating Lyn kinase activity.

Activities of Syk, Btk, p38, JNK, and Akt and IκBα degradation are positively regulated by Hck

Syk is an essential PTK required for mast-cell activation.[21,22] Btk, a Tec family PTK, is also required for FcεRI-induced cytokine production.[44,45] Stimulation with 100 ng/mL of antigen induced strong enzymatic activation of Syk in WT cells, as measured by in vitro kinase assays using immunoprecipitated Syk. As shown previously,[10] Syk activity was drastically reduced in *lyn*$^{-/-}$ cells (Figure 5A). Importantly, Syk activity was more modestly reduced in *hck*$^{-/-}$ and *fyn*$^{-/-}$ cells. Phosphorylation of Btk on Tyr223, an autophosphorylation site whose phosphorylation reflects its kinase activity,[46] was also reduced in SFK-deficient cells with their rank order of impairment being *lyn*$^{-/-}$ more than *hck*$^{-/-}$ more than *fyn*$^{-/-}$ cells (Figure 5A), consistent with our previous observation that Btk activity is dependent on Syk.[47]

Downstream of these receptor-proximal PTKs, mitogen-activated protein kinases, Akt, and NF-κB function as intermediary positive regulators for FcεRI-induced mast-cell activation.[45,48-51] Stimulation with 100 ng/mL of antigen-induced robust phosphorylation and thus activation of mitogen-activated protein kinases (ERK1, ERK2, and p38) and Akt in WT cells (Figure 5B). In contrast, there was considerably reduced activation of p38 and Akt in *hck*$^{-/-}$ cells, although phosphorylation of ERK1 and ERK2 was not affected by Hck deficiency. JNK activity was also decreased in *hck*$^{-/-}$ cells (Figure 5C). Furthermore, FcεRI stimulation induces

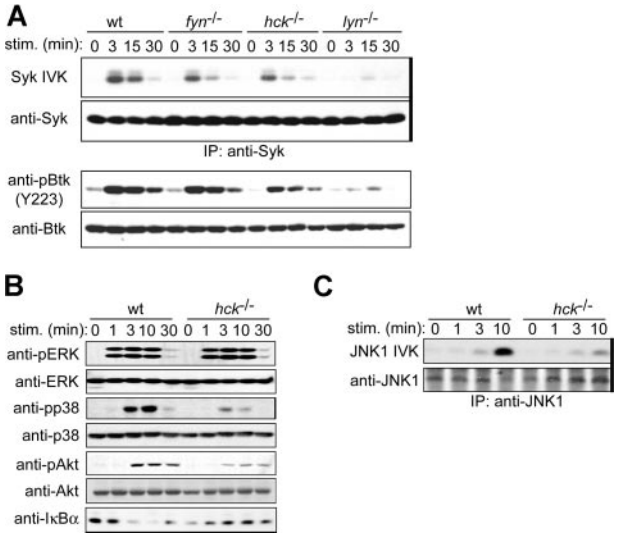

Figure 5. Hck deficiency results in reduced activities of positive regulatory molecules. IgE-sensitized WT and *hck*$^{-/-}$ cells were stimulated with 100 ng/mL DNP_{23}-HSA for the indicated periods. (A) Syk was immunoprecipitated (indicated by thick vertical line on the right of gel) from cleared cell lysates and immune complexes subjected to in vitro kinase assays using GST-HS1 as a substrate. Portion of the autoradiogram including GST-HS1 phosphorylation is shown. Cell lysates were directly analyzed by SDS-PAGE and immunoblotting with anti-Syk or anti-phospho-Btk (Tyr223). The pBtk blot was reprobed with anti-Btk antibody. (B) Cell lysates were directly analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. The same blots were reprobed with antibodies that detect antigens irrespective of their phosphorylation status. (C) Immunoprecipitated JNK1 (indicated by thick vertical line on the right of gel) was subjected to in vitro kinase assays. Representative results from 2 experiments are shown.

IκB kinase-mediated phosphorylation and rapid degradation of IκBα, which binds and masks the NF-κB nuclear localization signal and thus sequesters NF-κB in the cytoplasm.[52,53] IκBα degradation was abolished in *hck*$^{-/-}$ cells, indicating that the NF-κB pathway is positively regulated by Hck (Figure 5B). This result is consistent with our observation that SHIP phosphorylation is increased in *hck*$^{-/-}$ cells (Figure 4G), combined with observations that SHIP negatively regulates NF-κB and IL-6 production in FcεRI-stimulated mast cells.[51] Taken together, these results indicate that the activities of Syk, Btk, p38, JNK, Akt, and NF-κB are under the control of Hck, which is consistent with reduced cytokine production in *hck*$^{-/-}$ cells.

"Low-intensity" stimuli uncouple the increased Lyn activity from its negative regulatory function

These signaling studies were performed in the cells stimulated with 100 ng/mL of antigen in "high-intensity" stimulation conditions.[23] Compared with these conditions, differences in histamine release and cytokine production induced by stimulation with 1 ng/mL DNP_{23}-HSA ("low-intensity" stimulus) were smaller between WT and *hck*$^{-/-}$ cells (Figure 2). To examine whether Hck plays any significant roles under "low-intensity" conditions, IgE-sensitized cells were stimulated with 1 ng/mL DNP_{23}-HSA. Tyrosine phosphorylation of cellular proteins including Lyn was generally higher in *hck*$^{-/-}$ cells unstimulated or stimulated with 1 ng/mL DNP_{23}-HSA compared with WT cells (data not shown), similar to that in *hck*$^{-/-}$ cells stimulated with 100 ng/mL DNP_{23}-HSA. Importantly, despite the increased Lyn phosphorylation on Tyr396 and kinase activity, phosphorylation of FcεRIβ and SHIP (ie, Lyn substrates important for negative regulation of mast-cell activation) was not increased in *hck*$^{-/-}$ cells stimulated with 1 ng/mL DNP_{23}-HSA (Figure 6A). Indeed, FcεRIβ was not significantly tyrosine-phosphorylated under these conditions in WT or *hck*$^{-/-}$ cells. Phosphorylation of ERK1/2 and Akt is generally lower and more transient under "low-intensity" stimulation conditions than under "high-intensity" stimulation conditions (Figure S3). Interestingly, LAT-Tyr191, ERK1/2, and p38 were phosphorylated at slightly higher levels and Akt phosphorylation was slightly lower in *hck*$^{-/-}$ cells than in WT cells (Figure 6B), although it is not clear whether these minor differences in weak signaling translated into biologic consequences. IκBα degradation was not seen in either WT or *hck*$^{-/-}$ cells.

We evaluated the effect of another "low-intensity" stimulus, IgE^{+}anti-IgE, on activation of mast cells. IgE-sensitized WT and *hck*$^{-/-}$ cells were stimulated with 2 or 20 μg/mL anti-IgE mAb E1B3.[23] Similar to cells stimulated with 1 ng/mL DNP_{23}-HSA, cytokine production was similar between WT and *hck*$^{-/-}$ cells (Figure S4A). Tyrosine phosphorylation of cellular proteins including Lyn was higher in *hck*$^{-/-}$ cells unstimulated or stimulated with anti-IgE (Figure S4B), similar to that in *hck*$^{-/-}$ cells stimulated with 1 or 100 ng/mL DNP_{23}-HSA. Despite the increased Lyn phosphorylation on Tyr396, FcεRIβ was not tyrosine-phosphorylated[23] and SHIP phosphorylation was not increased in *hck*$^{-/-}$ cells (Figure S4B). Furthermore, phosphorylation of ERK1/2, p38, and Akt was either reduced in *hck*$^{-/-}$ cells or comparable in WT and *hck*$^{-/-}$ cells (Figure S4C). These results indicate that "low-intensity" FcεRI stimuli in *hck*$^{-/-}$ cells uncouple the increased Lyn activity from its ability to exert negative regulation on downstream signaling events (such as FcεRIβ and SHIP phosphorylation), indicating that Lyn's increased kinase activity per se is not sufficient for its negative regulatory function, but that "high-intensity" stimulus is required.

Positive regulatory roles of Hck can be exerted in Lyn-dependent and Lyn-independent pathways

These data suggest that positive signaling roles for Hck are exerted through negative regulation of Lyn activity. To test whether the positive regulatory roles for Hck in mast cells are absolutely Lyn-dependent, mast-cell activation phenotypes were compared between WT, *lyn*$^{-/-}$, *hck*$^{-/-}$, and *lyn*$^{-/-}$*hck*$^{-/-}$ mice.

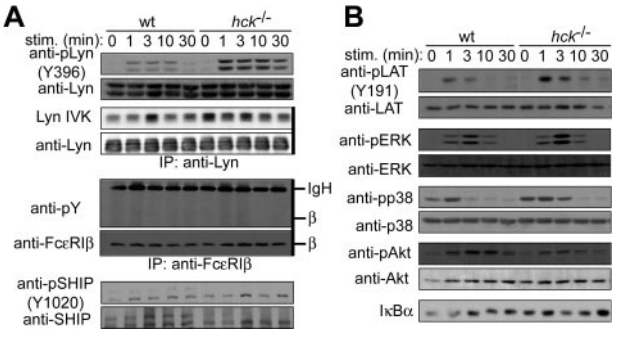

Figure 6. Stimulation with a low concentration of antigen does not induce phosphorylation of FcεRIβ or increase SHIP phosphorylation in *hck*$^{-/-}$ cells despite increased Lyn activity. IgE-sensitized WT and *hck*$^{-/-}$ cells were stimulated with 1 ng/mL DNP_{23}-HSA for the indicated periods. Cell lysates were directly analyzed by SDS-PAGE and immunoblotting with the indicated phospho-specific antibodies. The same blots were reprobed with antibodies that detect antigens irrespective of their phosphorylation status. (A, third and fourth rows) Immunoprecipitated Lyn was subjected to autophosphorylation assays. Comparable immunoprecipitations were confirmed by immunoblotting. (A, middle) Immunoprecipitated FcεRIβ was analyzed by immunoblotting with anti-phosphotyrosine mAb and then reprobed with anti-FcεRIβ mAb. Immunoprecipitations are indicated by thick vertical lines on the right of gels. Representative results from 2 experiments are shown.

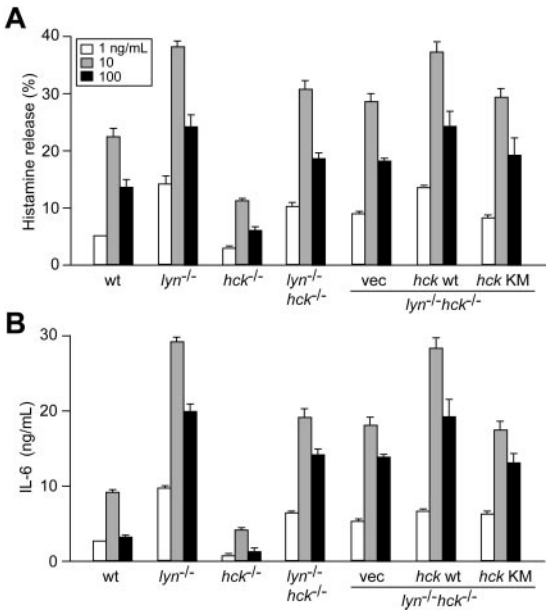

Figure 7. Hck/Lyn doubly deficient mast cells exhibit an intermediate activation phenotype between Hck- or Lyn-deficient cells, and positive and negative regulation via FcεRI β subunit is exerted by Lyn-mediated phosphorylation of the canonical and noncanonical tyrosine residues. IgE-sensitized mast cells of the indicated genotypes and FcεRIβ-transduced cells were stimulated with 1 (□), 10 (■), or 100 (■) ng/mL DNP_{23}-HSA for 45 minutes (A) or 20 hours (B). Histamine and IL-6 secreted into culture media were measured. ND indicates not detected. Representative results from 2 independent transduction experiments are shown. Error bars represent SD.

Comparable expression of FcεRI and c-Kit on the surface of these mast cells was confirmed by flow cytometry (data not shown). As shown previously,[8] histamine release and cytokine production induced by FcεRI stimulation were increased in $lyn^{-/-}$ cells, but decreased in $hck^{-/-}$ cells, compared with WT cells (Figure 7A,B). Importantly, an intermediate phenotype was noticed in $lyn^{-/-}hck^{-/-}$ cells: both histamine release and cytokine production were higher in $lyn^{-/-}hck^{-/-}$ than in $hck^{-/-}$ cells (and WT cells), but lower than in $lyn^{-/-}$ cells, suggesting that the suppressing activities in $hck^{-/-}$ cells are not totally dependent on Lyn and that Hck positively functions partly independent of Lyn. Moreover, restoration of WT, but not kinase-dead, Hck in $lyn^{-/-}hck^{-/-}$ cells could upregulate histamine release and cytokine production to the levels in $lyn^{-/-}$ cells (Figure 7A,B), demonstrating that the Lyn-independent positive regulatory function of Hck is exerted through its kinase activity. In these *hck*-transduced cells, Hck expression was very high (approximately 100-fold over its expression level in WT cells, as measured by immunoblotting using predetermined amounts of GST-Hck as a reference). However, Hck overexpression in $lyn^{-/-}hck^{-/-}$ cells simply reversed the defective degranulation/cytokine phenotype to that of $lyn^{-/-}$ cells, indicating that mast cells tolerate overexpression of Hck without their FcεRI-mediated activation phenotype being affected.

Because a fraction of Lyn and Fyn interacts with the FcεRI β subunit,[3,8] we examined the possibility that Hck might also interact with the FcεRI β subunit and exert its Lyn-independent function. Robust interactions between Lyn and FcεRI β subunit were seen by coimmunoprecipitation from 0.2 mg of WT BMMC lysates. However, consistent with a previous report,[8] no interaction could be detected between Hck and FcεRI β subunit using even 100 times more (20 mg of) lysates (data not shown).

The canonical and noncanonical tyrosine residues of FcεRI β-ITAM, respectively, mediate positive and negative regulatory functions of Lyn and, to a lesser extent, Hck

Previous studies including our own indicate that the negative regulatory role for Lyn is β-ITAM–dependent, and mediated by phosphorylation of the noncanonical β-ITAM tyrosine residue, Y-225.[19,20,23] To further dissect the functional relationship between Lyn, Hck, and FcεRI β subunit, different β-ITAM mutants were introduced retrovirally into $lyn^{-/-}$*FcεRI*$\beta^{-/-}$ and $hck^{-/-}$*FcεRI*$\beta^{-/-}$ mast cells. Comparable expression of FcεRI on mast cells expressing WT and mutant FcεRIβ was confirmed by flow cytometry (data not shown). The $lyn^{-/-}$*FcεRI*$\beta^{-/-}$ cells reconstituted with WT FcεRIβ (designated $lyn^{-/-}$-YYY cells) largely restored IgE/antigen-induced IL-6 production (Figure 7C), as previously shown.[23] The $lyn^{-/-}$-FFF cells showed a lower, but still substantial, amount of IL-6 production than $lyn^{-/-}$-YYY cells, indicating that some cytokine production requires neither intact β-ITAM nor Lyn. The $lyn^{-/-}$-YFY cells induced levels of IL-6 production similar to that in $lyn^{-/-}$-YYY cells, consistent with the notion that Y-225 is phosphorylated mainly by Lyn. However, lower IL-6 production induced by the $lyn^{-/-}$-FYF mutant relative to the $lyn^{-/-}$-FFF mutant suggests that a PTK other than Lyn may also be able to phosphorylate the noncanonical tyrosine residue, Y-225. The $lyn^{-/-}$-YYF and $lyn^{-/-}$-FYY mutants induced intermediate levels of IL-6 production, between those induced by $lyn^{-/-}$-FYF and $lyn^{-/-}$-FFF, suggesting that both Y-219 and Y-229 residues are important for positive regulation and can be phosphorylated by a PTK other than Lyn. However, YYY expression in $hck^{-/-}$*FcεRI*$\beta^{-/-}$ cells restored IL-6 production to a level similar to $hck^{-/-}$ cells (Figure 7D). Expression of YFY induced more IL-6 production than that of YYY, consistent with the role for Lyn in phosphorylation of the negative regulatory noncanonical tyrosine residue. This interpretation was also supported by our observation that IL-6 production in $hck^{-/-}$-FYF cells was lower than that in $hck^{-/-}$-FFF cells. IL-6 production was even lower in $hck^{-/-}$-YYF and $hck^{-/-}$-FYY cells than in $hck^{-/-}$-FYF cells, consistent with the notion that phophorylation of Y-219 and Y-229 residues by Hck is important for positive regulation. Therefore, Hck-dependent, Lyn-independent positive regulation also seems to be at least partly FcεRIβ-dependent. To confirm the ability of Hck to phosphorylate the FcεRIβ ITAM, we performed in vitro kinase assays using WT and mutant FcεRIβ peptides. Lyn and Hck immunoprecipitated from BMMC lysates showed a very similar phosphorylating activity toward FcεRIβ peptides, with the rank order of preference being YYY more than YFF more than FYF more than FFY (Figure S5). These results are in agreement with the preferential phosphorylation of Y-219 of FcεRIβ molecules expressed in transfected cells.[19]

Discussion

This study demonstrates positive regulatory functions of Hck in FcεRI-induced mast-cell activation. These functions are exerted by both Lyn-dependent and Lyn-independent mechanisms. Both mechanisms appear to at least partly involve phosphorylation of the tyrosine residues in the β-ITAM. The Lyn-dependent mechanism is exerted by inhibition of the phosphorylation and catalytic activity of Lyn. These results, together with previous observations that Fyn activity is enhanced in $lyn^{-/-}$ mast cells,[11,12] indicate a hierarchical relationship among these SFKs: Hck negatively regulates Lyn and Lyn negatively regulates Fyn.

Here we estimated cellular concentrations of these SFKs in mast cells for the first time. Our measurements confirmed a broadly held assumption that Lyn is the most abundant SFK in mast cells.[2,5]

Fyn, which is expressed at an approximately 17-fold lower level than $p53^{lyn}$, was also shown to play a unique role by inducing Gab2 phosphorylation, and thus contributing to degranulation.[8] It may appear surprising that $p56^{hck}$ and $p59^{hck}$, expressed at 30- and 50-fold lower expression levels than $p53^{lyn}$, play a significant role in mast-cell activation. However, the combined amount of $p59^{hck}$ and $p56^{hck}$ is similar to the amount of $p59^{fyn}$. Therefore, it may not be so surprising that $hck^{-/-}$ mast cells exhibited defective activation phenotypes, but the results indicate that these SFKs have unique roles in mast cells. This argument is also supported by our observation that 100-fold expression of WT Hck over endogenous levels did not affect activation levels of degranulation or cytokine production. Although concentrations of these kinases at the subcellular locations where they exert their function should be more important than their average cellular concentrations, low expression of Hck hampered further detailed analysis of its subcellular concentrations.

The present study showed that Hck is required for optimal in vitro proliferation of mast cells in response to IL-3 and SCF. However, mast cell numbers in several tissues are comparable between WT and $hck^{-/-}$ mice. In a recent study, $lyn^{-/-}$ mice were shown to have more peritoneal and dermal mast cells than WT mice, and $lyn^{-/-}$ mast cells expand faster in response to IL-3 and SCF.[12,54] These contrasting phenotypes might be accounted for by the increased Lyn activity in $hck^{-/-}$ mast cells. However, in another study, bone marrow cells from $lyn^{-/-}$ mice generated similar numbers of mast cells as cells from WT mice did.[10] The 2 studies also differed with respect to growth factor withdrawal-induced apoptosis: Hernandez-Hansen et al[54] showed less apoptosis in $lyn^{-/-}$ mast cells and the latter showed comparable apoptosis in WT and $lyn^{-/-}$ cells. These differences could be attributable to differences in the genetic background of the mice studied. In this study, $hck^{-/-}$ cells died as fast as WT cells.

The hierarchical relationship among SFKs suggests exquisite mechanisms that mast cells use to fine-tune their activation. Lyn kinase activity is increased in $hck^{-/-}$ cells (this study) and Fyn kinase activity is increased in $lyn^{-/-}$ cells.[11,12] c-Src activity is reduced in $lyn^{-/-}$ cells.[12] However, Fyn activity is not altered by Hck deficiency and Lyn activity is not altered by Fyn deficiency. Thus, Hck specifically inhibits Lyn activity and Lyn specifically inhibits Fyn activity in mast cells. SFK activity is positively regulated by phosphorylation of the tyrosine residue (Tyr396 in Lyn) in the activation loop,[55,56] whereas phosphorylation of the C-terminal tyrosine residue (Tyr507 in Lyn) by Csk inhibits its kinase activity.[40] Csk is recruited to the plasma membrane by tyrosine-phosphorylated Cbp/PAG via interactions between Csk's SH2 domain and phosphorylated Tyr-314 of Cbp/PAG.[37,38] Consistent with previous studies that Cbp/PAG is phosphorylated by Lyn,[12] tyrosine phosphorylation of Cbp/PAG is increased in $hck^{-/-}$ mast cells in which Lyn activity is increased. Lyn-mediated Cbp/PAG phosphorylation can account for Lyn-dependent Fyn inhibition. A similar mechanism might operate for Hck-mediated Lyn inhibition, because Hck is physically associated with Cbp/PAG (Figure 4F). However, this scenario cannot explain why Fyn activity is not increased in $hck^{-/-}$ cells and c-Src activity is not increased in $lyn^{-/-}$ cells. It is not clear whether Cbp/PAG phosphorylation affects each SFK with equal potency, although localization of each SFK relative to that of Cbp/PAG may be important for their activity. Another potential, nonmutually exclusive mechanism for hierarchical regulation among SFKs can be through regulation of protein-tyrosine phosphatases that dephosphorylate critical tyrosine residues of SFKs. This counteracting response is induced in response to an activating mutation in Hck, Hck^{Y499F}.[57] This response might be dampened in $hck^{-/-}$ cells, leading to the enhanced Lyn activity. Phosphorylation of Lyn on Tyr396 is increased in $hck^{-/-}$ cells. Future investigation into these regulations of Hck versus other SFKs will be necessary for our better understanding of the initial activation mechanisms of mast cells.

Transduction of FcεRIβ mutants in $lyn^{-/-}Fc\epsilon RI\beta^{-/-}$ cells confirmed that the canonical tyrosine residues of β-ITAM are involved in Lyn-dependent positive regulation of mast-cell activation, whereas the noncanonical tyrosine residue is involved in Lyn-dependent negative regulation. Similar experiments with $hck^{-/-}Fc\epsilon RI\beta^{-/-}$ cells not only supported Lyn's roles in positive and negative regulation through phosphorylation of β-ITAM tyrosine residues but also suggested Hck's role in positive regulation by phosphorylating the canonical tyrosine residues. Therefore, the Lyn-independent positive regulatory function of Hck also appears to involve, at least in part, β-ITAM phosphorylation (Figure S6). Interestingly, the noncanonical tyrosine Tyr-225 can be phosphorylated by a PTK other than Lyn (albeit to a lesser extent), because IL-6 production was lower in $lyn^{-/-}$-FYF than in $lyn^{-/-}$-FFF cells. To gain a better understanding of these key regulatory mechanisms in mast-cell activation, further study on other SFKs is warranted.

Parravicini et al[8] suggested that FcεRI can use an alternative activation pathway for mast-cell degranulation that involves Fyn-mediated Gab2 phosphorylation and subsequent phosphatidylinositol 3-kinase activation. Recently, this Fyn/Gab2 pathway was shown to be required for microtubule formation and consequent translocation of granules to the plasma membrane.[9] Unlike Parravicini et al, Yu et al[58] suggested that Syk is the kinase that phosphorylates Gab2. Importantly, Hck deficiency results in defective Gab2-Tyr452 phosphorylation and microtubule formation, leading to reduced degranulation, despite normal levels of Fyn kinase activity. Our study also showed that Syk activity was reduced in $hck^{-/-}$ cells. Irrespective of which PTK is responsible for Gab2 phosphorylation, these results indicate that both Fyn and Hck are required for Gab2-dependent degranulation.

Topographical studies point to the critical importance of locations of FcεRI and signaling molecules for their proper functioning.[59-63] They support the notion that there are functional and nonfunctional pools of signaling molecules: it is tempting to speculate that a pool of Lyn molecules, probably those prebound to FcεRIβ, can phosphorylate the canonical β-ITAM residues and γ-ITAM to initiate FcεRI signaling; another pool of Lyn molecules, which have a configuration relative to FcεRIβ different from the activating Lyn pool, phosphorylates the noncanonical tyrosine residue to trigger the negative regulatory signal. Similar to FcεRIβ-bound Lyn, FcεRIβ-bound Fyn as well as receptor-proximal Hck molecules might belong to an activating pool that can phosphorylate the canonical β-ITAM residues (and γ-ITAM). However, it is not clear whether Hck plays a negative regulatory role, whereas Fyn deficiency results in increased IL-13 production.[64]

In conclusion, Hck plays a positive regulatory role in FcεRI-stimulated mast-cell activation probably by phosphorylating the canonical tyrosine residues in β-ITAM and suppressing Lyn kinase activity. Together with previous studies showing Lyn-mediated Fyn inhibition, these 3 SFKs exhibit a hierarchical relationship, ie, Hck inhibits Lyn and Lyn inhibits Fyn. This hierarchical relationship seems critical in fine-tuning mast-cell activation.

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Authorship

Contribution: H.H., J.K., and W.X. performed experiments. V.H., C.R., and C.A.L. provided crucial reagents. Y.K., W.X., and T.K. designed experiments. T.K. wrote the paper.

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