

Molecular mechanisms in allergy and clinical immunology

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Molecular regulation of mast cell activation

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The mast cell is a central player in allergy and asthma. Activation of these cells induces the release of preformed inflammatory mediators localized in specialized granules and the *de novo* synthesis and secretion of cytokines, chemokines, and eicosanoids. The balance of engaging inhibitory and activatory cell-surface receptors on mast cells determines whether the cell becomes active on encountering a challenge. However, recent evidence suggests that, once activated, a mast cell's response is further regulated by the balance of both positive and negative intracellular molecular events that extend well beyond the traditional role of kinases and phosphatases. These functional responses are also carefully governed by other protein and lipid mediators that determine the rate and extent of the response. Molecules that have adaptor functions, modulate lipids, and provide synergistic signals add to the regulatory complexity. Considerable information has been obtained from the study of the high-affinity receptor for IgE (FcεRI), and thus it is the major focus of this review. The unifying theme is that the regulatory steps mentioned herein are required for promoting effective responses while protecting against unwanted inflammatory responses. (*J Allergy Clin Immunol* 2006;117:1214-25.)

Key words: FcεRI, Lyn, Fyn, IgE, mast cell, degranulation

In recent years, there has been a growing realization that the mast cell is a key component of innate immunity and can serve to amplify adaptive immunity.^{1,2} Mast cells can respond to pathogens, such as bacteria and viruses, through multiple Toll-like receptors expressed on their cell surface. These receptors, for the most part, fail to elicit the hallmark response of release of preformed

Abbreviations used

Akt/PKB:	Protein kinase B
BMMC:	Bone marrow–derived mast cell
Btk:	Bruton's tyrosine kinase
Csk:	COOH-terminal Src kinase
DAG:	Diacylglycerol
ERK:	Extracellular signal-regulated kinase
Gab2:	Grb2-associated binding protein 2
ITAM:	Immunoreceptor tyrosine–based activation motif
ITIM:	Immunoreceptor tyrosine–based inhibitory motif
LAT:	Linker for activation of T cells
MAP:	Mitogen-activated protein
MIST/Clnk:	Mast cell immunoreceptor signal transducer or cytokine-dependent hemopoietic cell linker
NTAL:	Non-T-cell activation linker
PA:	Phosphatidic acid
PH:	Pleckstrin homology
PIP ₃ :	Phosphatidylinositol 3, 4, 5-trisphosphate
PI3K:	Phosphatidylinositol 3-OH kinase
PKC:	Protein kinase C
PLA ₂ :	Phospholipase A ₂
PLD:	Phospholipase D
PLCγ:	Phospholipase Cγ
PTEN:	Phosphatase and tensin homologue deleted on chromosome 10
SHIP:	SH2 domain–containing 5'-inositol phosphatase
SHP-1/2:	SH2 domain–containing phosphatase 1/2
SphK:	Sphingosine kinase
S1P:	Sphingosine-1-phosphate
Src PTK:	Src family protein tyrosine kinase

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inflammatory mediators stored in granules but instead cause the induction of *de novo* cytokine production.² Mast cells also can mediate immunity through the binding of antigen-specific Igs.³⁻⁵ This plays a significant role in the ability to combat certain parasitic infections, an important role of mast cells in adaptive immunity. This dual role suggests that dysregulation of mast cell function could have untoward physiologic consequences. A clue for the necessity of controlling mast cell activation is provided by the evolution of multiple cell-surface receptors that can activate, as well as inhibit, mast cell activation.⁶

Additional evidence for this concept is provided by studies conducted in murine models, as well as clinical studies, that demonstrate that mast cell dysregulation can cause disorders ranging from mastocytosis to autoimmunity and allergy.¹ Although the role of the mast cell in a given disease might differ, the underlying mechanism is that aberrant regulation or persistent stimuli cause unwanted mast cell activation and responses.

Appropriate activation of mast cells is mediated by a number of factors, including the cells' ability to distinguish activating or inhibitory stimuli¹ and the strength and duration of the stimulus.^{7,8} Multiple cell-surface receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) provide inhibitory signals to counter stimuli that can activate mast cells.⁶ Such receptors include the low-affinity IgG receptor (FcγRIIb),⁹ CD200R,¹⁰ the 49-kd surface glycoprotein,¹¹ the Ig-like receptor p60,¹² the myeloid-associated Ig-like receptor,¹³ mast cell function-associated antigen,¹⁴ and the paired Ig-like receptor B.¹⁵ Ligation or coligation of these receptors with FcεRI results in downregulation of FcεRI-mediated mast cell activation.⁶ The common feature of these inhibitory receptors is their ITIM-dependent association with tyrosine or lipid phosphatases, which mediate their inhibitory function. However, the ITIMs expressed within specific receptors appear to display selectivity for the individual classes of phosphatases. For example, whereas the 49-kd surface glycoprotein appears to exert its inhibitory effect on mast cell activation by recruiting the tyrosine phosphatases, SH2 domain-containing phosphatase 1/2 (SHP) 1 and SHP-2,¹⁶ FcγRIIb, and mast cell function-associated antigen receptors appear to do so by recruiting the inositol phosphatases SH2 domain-containing 5'-inositol phosphatase (SHIP) 1 and SHIP-2.^{17,18} Mention should also be made that mast cell function can be downregulated by receptors that do not possess ITIMs. In this respect β₂-adrenergic receptors effectively inhibit antigen-dependent mast cell activation after engagement of a cyclic AMP-regulated inhibitory pathway.¹⁹

Further complexity is introduced by the varied functions of diverse signaling proteins that associate with or are influenced by immunoreceptor tyrosine-based activation motif (ITAM) signaling. In general, activation of kinases and phosphatases, adaptor proteins, lipid kinases and phosphatases, and lipases by ITAM-bearing receptors results in a combination of both positive and negative signals that are essential in fine tuning the cell response. Much of what is known results from studies focused on IgE/allergen-mediated stimulation through the high-affinity receptor for IgE (FcεRI) on mast cells. Here we concentrate on events most proximal to stimulation of this receptor.

FcεRI STRUCTURE AND FUNCTION: ITAMs AS POSITIVE AND NEGATIVE REGULATORY MOTIFS

The FcεRI expressed on mast cells and basophils is a tetrameric receptor⁴ comprised of the IgE-binding α chain,

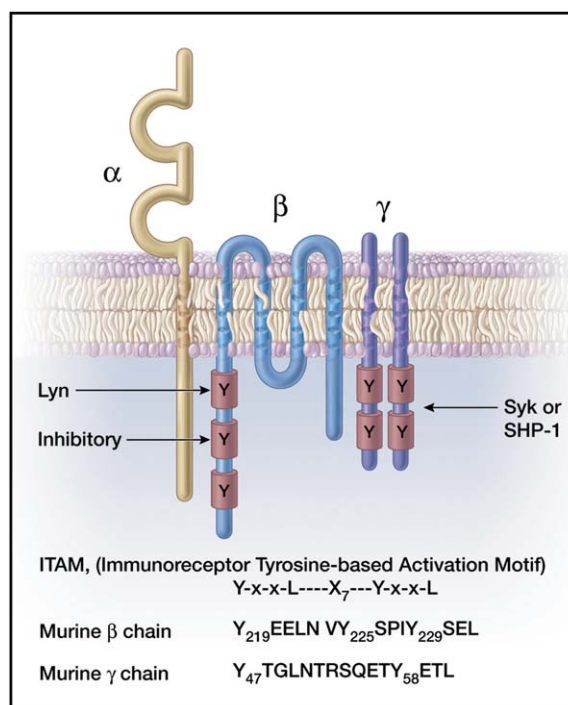


FIG 1. Structure of FcεRI and newly defined inhibitory motifs. FcεRI on mast cells is comprised of an IgE-binding α chain, a 4-transmembrane-spanning β chain, and a homodimer of γ chains. Both the β and γ chains contain the ITAM. The β chain contains a noncanonical tyrosine at position 225, which is involved in the negative regulation of cytokine production in mast cells. A recent study shows that the γ chain ITAM can associate with both positive (ie, Syk) and negative (ie, SHP-1) effector molecules and in this manner mediate both positive and negative function.

the membrane-tetraspanning β chain, and a disulfide-linked homodimer of the γ chains (Fig 1). The β chain functions as an amplifying module²⁰ for this receptor, and in its absence the receptor initiates weak signals. The γ chain homodimer imparts signaling competence to this receptor.⁴ Both the β and γ chains contain the ITAM motifs demonstrated to be essential for the amplifying and signaling competence of these receptor chains (Fig 1). The FcεRIβ ITAM possesses a noncanonical tyrosine residue that is situated between the 2 canonical tyrosines found in conventional ITAMs. Phosphorylation of the tyrosine residues in these motifs occurs through transphosphorylation by the Src family protein tyrosine kinase (Src PTK) Lyn, and normal phosphorylation requires the liquid-ordered phase of membranes (lipid rafts) where this kinase is concentrated.^{21,22} Once phosphorylated, novel binding sites are created where other signaling proteins can bind and propagate signals required for mast cell effector responses.⁴ It has been demonstrated that both the β and γ chains function to generate positive signals that are key in initiating and amplifying the mast cells' effector responses. However, recent evidence suggests that these 2 chains can also function to negatively regulate cell activation and effector responses.

New insights on FcεRIβ function

Polymorphisms in the coding region of the amplifying FcεRIβ have been associated with allergy and asthma.²³⁻²⁵ How these amino acid changes affect FcεRI function and mast cell physiology is not well understood. One might envision that the change in amino acid composition might augment mast cell functional responses. However, this has not been supported by studies in which FcεRIβ-null cells or mice were reconstituted with polymorphic FcεRIβ and a limited analysis of mast cell responses were performed.^{26,27} Mutational analysis of the tyrosine residues in the ITAM of this subunit, however, has revealed several unexpected features. First, the loss of phosphorylation of Y219 alone caused a marked reduction of receptor-associated Lyn (Fig 1),²⁸ suggesting that the other canonical tyrosine residue (Y229) is not required for FcεRIβ interaction with Lyn. Second, loss of phosphorylation of Y219 caused a markedly delayed and diminished calcium response associated with partially inhibited mast cell degranulation and cytokine production.^{28,29} Third, unexpectedly, mutation of the noncanonical tyrosine (Y225) caused a marked increase in mast cell cytokine production (IL-6 and IL-13) without affecting mast cell degranulation or leukotriene C₄ production.²⁹ This was associated with decreased tyrosine phosphorylation of SHIP-1. Importantly, activation of nuclear factor κB was found to be augmented by mutation of Y225, indicating that this residue plays an important role in negative regulation of this transcription factor. Interestingly, mutation of both canonical tyrosines (Y219/Y229) resulted in reduced, but not ablated, binding of SHIP-1 and the p85 regulatory subunit of phosphatidylinositol 3-OH kinase (PI3K), suggesting that Y225 might also be important for SHIP-1 and PI3K interactions.²⁹ Thus it appears that the FcεRIβ ITAM can promote negative signals that affect mast cell responses. Interestingly, a recent study by Xiao et al³⁰ promotes the view that the strength of the stimulus determines whether FcεRIβ serves as a positive or negative regulator. This study demonstrates that under a low-intensity stimulus, the FcεRIβ functions to amplify responses, whereas at a high intensity of stimulus, it might exert a negative regulatory role.

A new role for FcεRIγ

In addition to FcεRIβ, recent studies also extend a negative regulatory role to FcεRIγ.³¹ Engagement of FcαRI, a receptor that uses FcεRIγ for cell activation, was similarly found to inhibit FcεRI-dependent mast cell degranulation.³¹ This response, which was mediated through the association of FcεRIγ with FcαRI and required an intact phospho-ITAM, was seemingly fostered by association with the phosphatase SHP-1 (Fig 1). Collectively, these studies alter the view of ITAMs as solely positive effectors in immune cell activation and indicate that the context in which ITAMs are engaged might determine whether they function to activate or inhibit responses.

REGULATION BY RECEPTOR-PROXIMAL TYROSINE KINASES

FcεRI-mediated activation of mast cells requires both Lyn and the related Src PTK Fyn (Fig 2) as receptor-proximal kinases.³² As discussed above, Lyn is essential for the phosphorylation of FcεRIβ and FcεRIγ ITAMs, but because residual FcεRI phosphorylation was observed in Lyn-null mast cells,³³⁻³⁵ other kinases are likely required or can substitute. In addition to Lyn, Fyn was found to be activated by FcεRI engagement, and this initiates signals that complement Lyn-mediated responses required for normal mast cell activation.^{32,35} However, in the absence of Lyn, Fyn and the related Hck and Fgr (Yamashita et al, unpublished observation) are not responsible for the phosphorylation of FcεRI.³⁵ Thus for the moment it is unclear what other kinase can phosphorylate FcεRI, but Src family kinase activity is clearly essential for Lyn-independent FcεRI-mediated mast cell activation because ITAM-null FcεRI and Lyn-null mast cells treated with the Src selective inhibitor PP2 failed to degranulate.³⁶

Whether Lyn interactions with FcεRI are essential for mast cell degranulation remains somewhat controversial because various studies have demonstrated a reduced response,^{29,37} a normal response,^{33,38} or an enhanced response.^{34,36} One possible explanation for the apparent discrepancies is the genetic background of the mice and mast cells derived thereof. For example, it has been demonstrated that the SV129 strain, most commonly used to generate a genetically altered mouse, is skewed toward dominant T_H2 responses, whereas the C57/BL6 strain, the most common strain used for backcrossing SV129 mice for effective breeding, is skewed toward T_H1 responses.³⁹ Thus the effects of a particular genetic deletion are likely to be influenced by the complex genetic makeup of the strain used. In most studies published to date, the mice or mast cells thereof are of mixed background (SV129 and C57/BL6). Regardless, all studies demonstrate that the loss of Lyn does not completely ablate mast cell degranulation, and thus one can conclude that other early signals, which function independently of Lyn, are required to drive normal mast cell degranulation.

Relationship of Fyn and Lyn with Syk kinase

Considerable evidence has accumulated in support of FcεRI-mediated and Lyn-dependent activation of Syk (Fig 2).^{40,41} Lyn-null bone marrow-derived mast cells (BMMCs) showed a severe defect in the onset and extent of FcεRI-mediated Syk activation.^{32,33,38} However, the relationship between Fyn and Syk in mast cells remains enigmatic. The loss of Syk in mast cells results in a severe FcεRI-nonresponsive phenotype,⁴² but it is not known whether Fyn is active or inactive under these conditions. Recently, it has been proposed that Fyn is required for optimal activation of Syk.⁴³ If so, this places Fyn upstream of Syk activation (Fig 2). However, inactivation of Fyn had no detectable effects on the phosphorylation of Syk

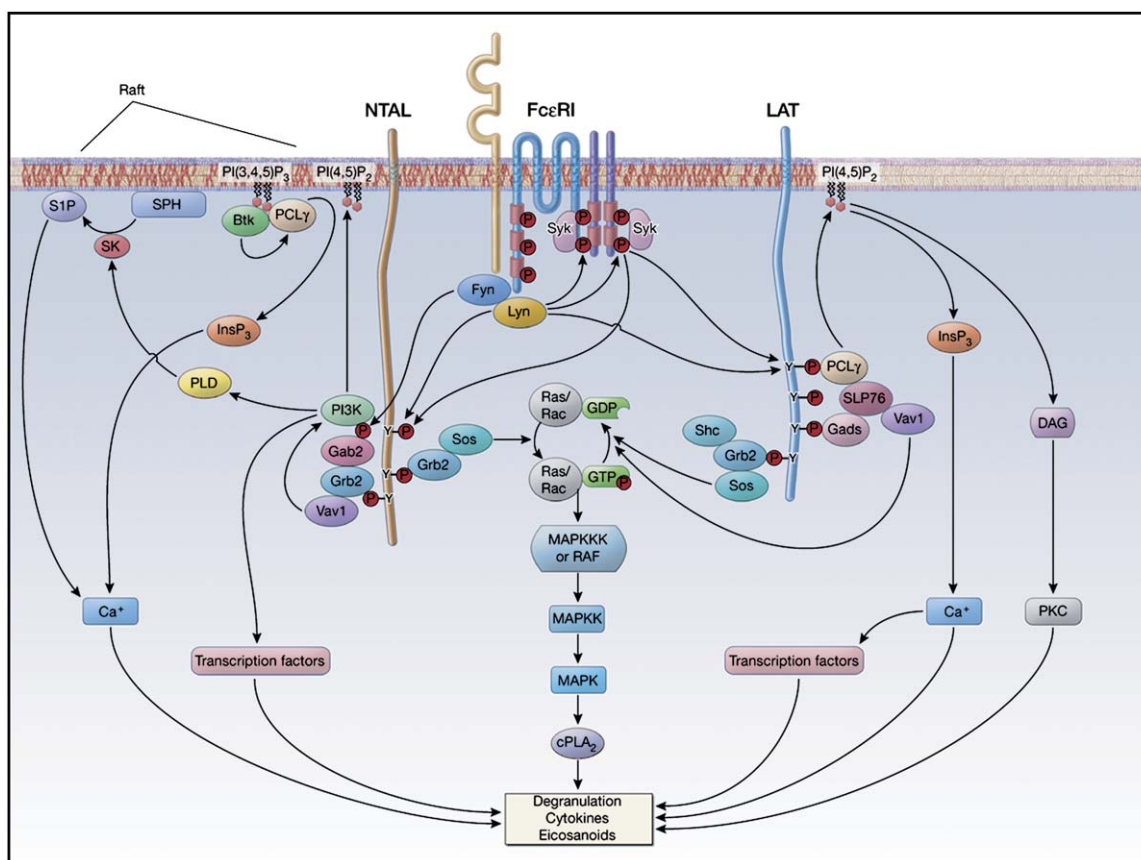


FIG 2. Simplified scheme of FcεRI signaling events in mast cells. Engagement of FcεRI results in inclusion in lipid rafts, phosphorylation (P) of receptor ITAMs by Lyn kinase, and activation of Syk kinase through ITAM binding. Fyn kinase is also activated and is important for phosphorylation of the adaptor known as Gab2 and activation of PI3K activity. Lyn regulates the activation of Syk and phosphorylation of several adaptor proteins called LAT and NTAL. These proteins function as scaffolds and organize other signaling proteins that can affect Ras activation, PLCγ activation through the coordinated function of Gads/SLP-76/Vav1 and Tec family kinases, PI3K activity, and calcium responses. PLCγ activation can also be regulated independently of LAT through a PI3K/Btk-dependent pathway. MAP kinase and transcription factor activation is dependent on LAT (and possibly NTAL) leading to mast cell cytokine production and eicosanoid production through cPLA₂ activation. PLCγ-generated DAG is key for early activation of PKC and mast cell cytokine production and degranulation. PI3K activity is also required for activation of PLD and SphK1. SphK generates S1P from sphingosine (Sph), which influences calcium mobilization and mast cell effector responses through cell-surface receptors for S1P (see text). The coordination of these molecular events is intrinsically regulated by both positive and negative functions of many of the components in the signaling cascade.

substrates (eg, the linker for activation of T cells [LAT] or the guanine nucleotide exchange factor Vav1³⁵), whereas Lyn or Syk deficiencies caused marked reduction in the phosphorylation of these proteins.^{32,33,36,38,42,44} Thus it is possible that other kinases, such as Itk, Bruton's tyrosine kinase (Btk), or even other Src PTKs, can more effectively target LAT and Vav1 in the absence of Syk activity (Fig 2). Regardless, because Fyn interactions with Syk have been reported in other cell types,⁴⁵ the finding of reduced Syk activity in Fyn-null mast cells⁴³ merits further investigation.

Fyn kinase as a key regulator of PI3K and mast cell degranulation

Several studies have demonstrated that Fyn deficiency results in reduced mast cell degranulation.^{32,35,36} The

basis for this observation is not yet well understood. Fyn, however, is thought to mediate its effect through Grb2-associated binding protein 2 (Gab2)-dependent regulation of PI3K (Fig 2).⁴⁶ Loss of Fyn caused decreased phosphorylation of this adaptor, thus inhibiting the binding of PI3K and membrane targeting.^{32,46} It has been suggested that this might be an indirect effect because Lyn or Syk deficiency was shown to abrogate Gab2 phosphorylation, and because Fyn deficiency was found to cause reduced Syk activation, Gab2 phosphorylation was proposed to be indirectly affected by Fyn.⁴³ In contrast, we and others^{32,34} have demonstrated either normal or enhanced Gab2 phosphorylation in Lyn-null mast cells, which were demonstrated to poorly activate Syk phosphorylation. Thus it is unclear whether Fyn is acting directly

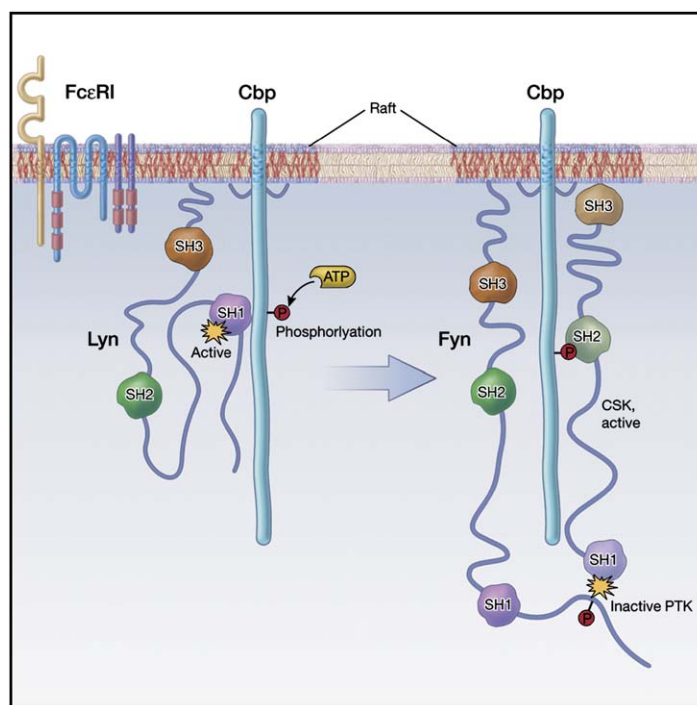


FIG 3. Mechanism for regulation of Src family kinase activity in mast cells. The Csk-binding protein (Cbp) is a target of Lyn kinase activity in mast cells. Cbp is localized in lipid raft domains and is phosphorylated constitutively by Lyn in these domains and interacts with the Csk, a kinase that phosphorylates Src family kinases and inactivates them. FcεRI stimulation increases the phosphorylation of Cbp, causing additional recruitment of Csk that then controls the activity of the Src family kinase Fyn by phosphorylating its negative regulator tyrosine at the C-terminus. The loss of Cbp phosphorylation and Csk recruitment in the absence of Lyn results in a constitutive increase of Fyn activity that is associated with enhanced mast cell degranulation.

or indirectly on Gab2, but common to all studies, these proteins affect PI3K activity.

Negative roles of Lyn and Fyn in mast cell activation and allergic responses

In vivo studies revealed that Lyn-deficient mice have atopic-like allergic disease³⁶ and showed a severe, persistent inflammatory asthma-like syndrome with lung eosinophilia, mast cell hyperdegranulation, intensified bronchospasm, hyper-IgE, and T_H2-polarizing dendritic cells.³⁹ One might expect that this could be a consequence of hypersensitive mast cells, as previously reported.^{34,36} However, this remains to be formally demonstrated because Lyn is expressed in basophils, eosinophils, macrophages, and neutrophils, cells that might have a role in allergic inflammation. Although Lyn might assume a positive or negative role in mast cell activation depending on the stimulus strength,³⁰ considerable evidence exists for Lyn as a dominant negative regulator with regard to mast cell homeostasis and effector responses.^{34,36} Lyn is required for phosphorylation of the lipid raft-localized COOH-terminal Src kinase (Csk)-binding protein and thus for membrane targeting of Csk (Fig 3). Csk negatively regulates Src family kinases, such as Fyn. We now know that this regulatory step is dependent on Lyn localization to lipid rafts⁴⁷ and is required to downregulate

Fyn kinase activity.^{36,47} This negative role of Lyn appears to be independent of its association with FcεRI.²⁹

In contrast to the apparent negative role of Lyn, Fyn-deficient and Lyn/Fyn-double-deficient mice showed defective passive systemic anaphylaxis responses, indicating a positive role for Fyn in promoting mast cell degranulation *in vivo*.³⁶ Fyn deficiency caused the loss of mast cell degranulation and leukotriene and cytokine production.^{32,35} However, exceptions to the role of Fyn as a positive regulator have been noted. Fyn deficiency increased IL-13 production, and the phosphorylation of the mitogen-activated protein (MAP) kinase family member extracellular signal-regulated kinase (ERK) was unaltered by deletion of this gene.³⁵ Additionally, gene expression profiling studies demonstrated increased FcεRI-dependent expression of mouse mast cell protease 4, granzyme B, and cathepsin D in the absence of Fyn, suggesting negative control of gene expression for some granule-localized enzymes that contribute to the inflammatory response of mast cells.

REGULATORY FUNCTION OF MOLECULAR ADAPTORS

As alluded to above, activation of mast cells requires the formation of a multimolecular signaling complex

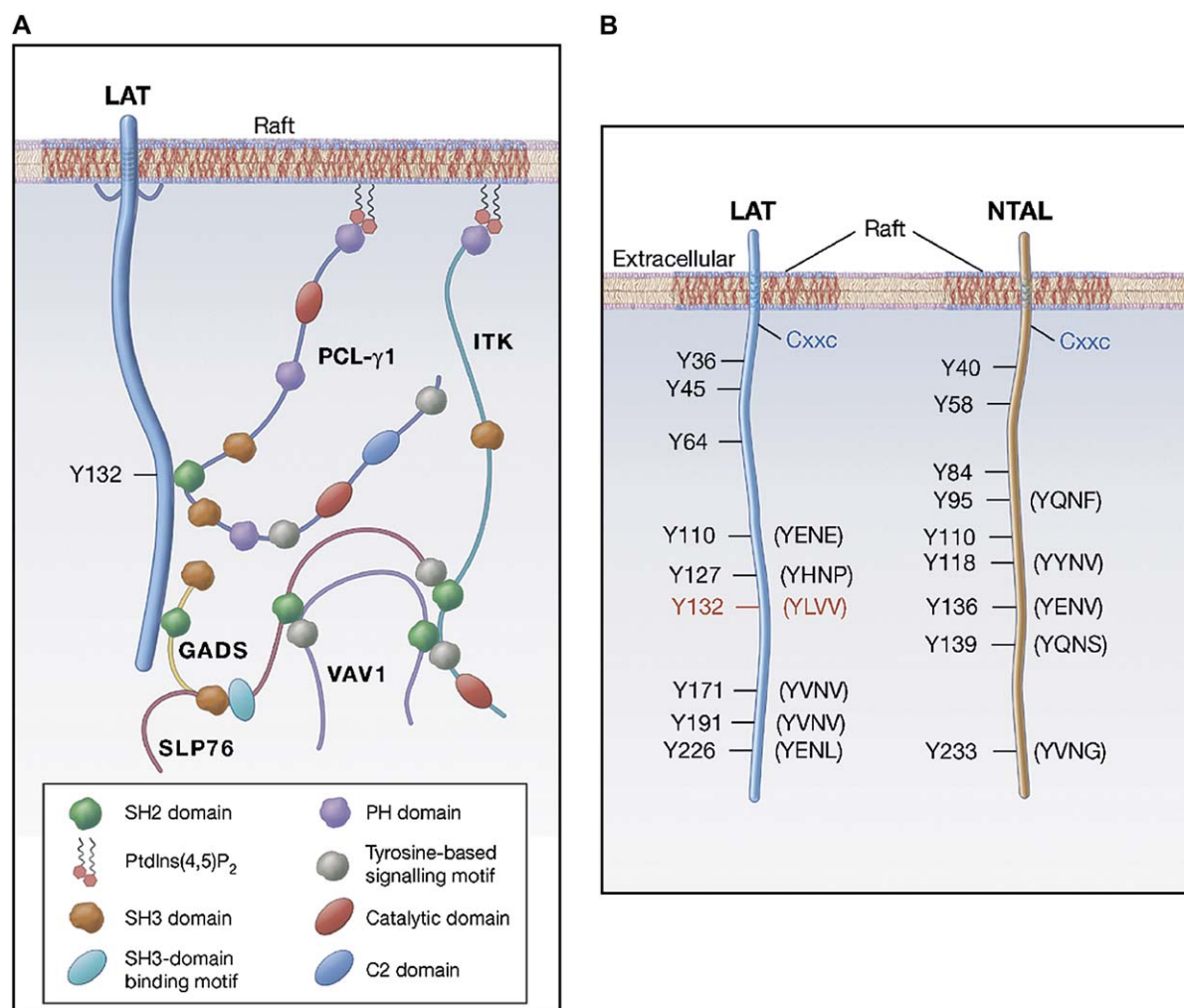


FIG 4. Cooperativity in the LAT-scaffolded signaling complex and structure of LAT and NTAL with potential or known interaction sites. **A**, LAT is a membrane-localized adaptor protein that is concentrated in lipid rafts. It binds both PLC γ and the Gad/Grb2 family of adaptor proteins, which coordinate the binding of other adaptor proteins or functional proteins, such as SLP-76 and Vav1. Interactions of these proteins with the Tec family kinases, such as Itk, are also key in stabilizing the complex. Phosphoinositides play an important role for membrane targeting through PH domains and can also function as substrates for PLC γ -dependent calcium signals (inositol trisphosphate) and PKC activation (DAG). Cooperativity is demonstrated by loss of normal complex function when a single protein is deleted from the complex. **B**, NTAL/LAB and LAT show high structural domain homology. Both adaptors localize to lipid rafts and encode short extracytoplasmic regions and transmembrane domains. Both also possess a conserved palmytoylation motif (Cxxc) at the transmembrane cytosolic interface. Depicted are the conserved tyrosine residues (Y) of the human adaptors. Also shown are binding sites for the Grb2 and Gads family of adaptors (YxN). The location of the tyrosine residue and the consensus motif (YLVV) of PLC γ binding to LAT is shown.

conceptually termed signalosome, which contains the necessary machinery to regulate downstream cellular processes. These signaling complexes (Fig 2) must be localized to specific regions within the plasma membrane that allow interactions with proteins or novel lipids generated by aggregation of surface receptors.⁴⁸ Such events are coordinated by specific proteins termed adaptor (also termed linker or scaffolding) molecules, the primary function of which is to provide docking sites for components of the receptor-signaling complex within the signalosome. The protein-protein and protein-lipid interactions are

inducible or constitutive and are defined by the nature of the specific signaling motifs contained within the associating molecules and the recognition sites for these motifs, which are contained within the proteins with which they interact (Fig 4, A).

LAT, non-T-cell activation linker, SLP-76, and MIST/Clnk: Cooperativity in function

The initial interactions of signaling proteins after phosphorylated Fc ϵ RI-mediated events within the lipid rafts are coordinated around the resident transmembrane

adaptor molecules LAT and, potentially, non-T-cell activation linker (NTAL). These molecules have been proposed to have both positive and negative regulatory control of mast cell activation and have been the topic of recent reviews.^{49,50} The role of NTAL (Fig 4, B), however, is less clear than that of LAT,⁵¹⁻⁵⁵ with the molecular interactions by which NTAL might mediate either inhibitory or activation pathways remaining unclear. To date, there are no reports of an association of phosphatases, such as SHP-1, SHP-2, or SHIP, with NTAL, which could explain the inhibitory regulation by NTAL. However, one proposed scenario is that it could compete with LAT for lipid raft occupancy, thus possibly downregulating LAT function.⁵⁵

In contrast, it is clear that LAT deficiency markedly attenuates mast cell responsiveness. However, LAT might also serve to integrate both positive and negative signals, depending on the tissue origin of the mast cell and on the stimulus.⁵⁶ The primary signaling pathway that LAT regulates is that leading to activation of phospholipase C γ (PLC γ ; Fig 4, A). This is not only dependent on the ability of PLC γ to directly bind LAT (Fig 4) after its phosphorylation but also through increased stability in this complex by cooperative binding of SLP-76 (Fig 4, A), which acts as a linker between PLC γ_1 and LAT-bound Gads.⁵⁷ Consistent with this view of functional cooperativity, LAT-deficient and SLP-76-deficient mast cells also display a reduced calcium response and a reduced capacity to degranulate and generate cytokines in response to antigen.^{58,59}

A SLP-76-related adaptor molecule, mast cell immunoreceptor signal transducer or cytokine-dependent hemopoietic cell linker (MIST/Clnk), has also been implicated in Fc ϵ RI-mediated mast cell activation.^{60,61} As with SLP-76, this molecule becomes tyrosine phosphorylated after Fc ϵ RI aggregation and becomes part of a complex that includes LAT, Gads/Grb2, Vav1, and PLC γ (Fig 4, A). A role for MIST/Clnk in mast cell activation was suggested by the observations that Fc ϵ RI-mediated calcium mobilization, nuclear factor for activation of T cells (NFAT) activation, and degranulation were blocked by a dominant negative form of MIST/Clnk expressed in mouse BMMCs.⁶¹ Because LAT phosphorylation was also inhibited by this construct, this suggested that in contrast to SLP-76, MIST/Clnk might function upstream of LAT. MIST/Clnk associates with 2 other phosphorylated adaptor molecules expressed in mast cells, adhesion and degranulation promoting adaptor protein (ADAP; originally termed Fyn-binding protein or FYB and SLAP-130) and SKAP55.⁶² ADAP appears to be dispensable for the regulation of mast cell degranulation. However, the demonstration of a MIST/Clnk-ADAP-SKAP55 complex associating with Lyn and preferentially with Fyn⁶² provides possible clues for its role in mast cell activation because it might serve to integrate signals from these 2 kinases. In contrast, it should be noted that genetic deletion of MIST/Clnk had no apparent effect on the normal development of the immune system, nor were there notable effects on immune cell activation, including Fc ϵ RI-mediated mast cell degranulation or cytokine production.⁶³

PI3K, A CENTRAL PLAYER IN MAST CELL ACTIVATION AND FUNCTION

By catalyzing the production of phosphatidylinositol 3, 4, 5-trisphosphate (PIP₃), PI3K plays a critical role in mast cell degranulation and cytokine production.^{32,46,64-66} A large number of key signaling molecules contain a pleckstrin homology (PH) domain that binds PIP₃ and serves to target these molecules to membranes and enhance their activity. Some key regulatory molecules, such as protein kinase C (PKC), PLC γ , PI3K-dependent protein kinase 1 (PDK1), Btk, phospholipase D (PLD), and others (Fig 2) appear to directly or indirectly require PIP₃ for their activity.⁶⁷

Several class IA PI3K isoforms exist in mast cells. Genetic deletion of the class IA p85 α regulatory subunit inhibited c-KIT stimulation of mast cells but not on Fc ϵ RI-mediated responses.⁶⁸ A more direct effect on Fc ϵ RI-mediated mast cell responses has been described for the class IA catalytic subunit p110 δ .⁶⁶ In this study a point mutation of this subunit caused a marked decrease in protein kinase B (Akt/PKB) phosphorylation and in the degranulation response of mast cells. Because Fyn- and Gab2-null mast cells also showed impaired Akt phosphorylation,^{32,46} the findings suggest that the p110 γ catalytic subunit of PI3K might be the isoform linked to Fyn-dependent signals in mast cells. Similarly, p110 δ was demonstrated to function downstream of G-protein-coupled receptors in mast cells and provided a contributory (synergistic) effect in Fc ϵ RI-dependent mast cell activation.⁶⁹

Counterregulation of PI3K function

The importance of PI3K activity in mast cells is further supported by the consequences of dysregulation of this pathway. PI3K-dependent pathways are finely regulated by lipid phosphatases that target its product, PIP₃. SHIP family members seem to play a central role in regulating the Fc ϵ RI-inducible levels of PIP₃. SHIP targets the 5' position of PIP₃ for dephosphorylation and thus opposes PIP5 kinase while creating PI-(3,4)P₂, which can bind to the PH domain of a variety of signaling proteins.⁷⁰ SHIP-1-null BMMCs showed increased responsiveness to Fc ϵ RI stimulation and increased degranulation and cytokine production,⁷¹ demonstrating the importance of SHIP-1 in maintaining control of mast cell responsiveness. Importantly, additional studies^{72,73} demonstrated that the influence of SHIP-1 extended beyond Fc ϵ RI because the minimal degranulation elicited by c-KIT engagement is greatly enhanced by the loss of SHIP-1.⁷³

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) directly opposes PI3K by dephosphorylating PIP₃ at the 3' position. Its product, PI-(4,5)P₂, is the well-known substrate for PLC γ (see details below). PTEN is recognized as a tumor suppressor and is a key regulator of cell growth and apoptosis.⁷⁴ The loss of PTEN in mast cells revealed that PIP₃ levels were substantially increased, even in the absence of Fc ϵ RI stimulation. PTEN deficiency also caused loss of homeostatic control of the MAP kinases c-Jun N-terminal

kinase and p38 because their activation was similar to that observed in FcεRI-stimulated cells. Strikingly, this was accompanied by constitutive secretion of cytokines from these cells. This reveals several interesting features of PTEN function: (1) PTEN appears to function to regulate the PI3K activity required for mast cell homeostasis; (2) PTEN appears to selectively control the MAP kinase pathways; and (3) PTEN controls the transcription and constitutive secretion of cytokines (IL-8 and GM-CSF were measured in these studies) independently of FcεRI, suggesting that these cytokines are regulated by increased PIP₃ (and possibly decreased PTEN activity) on FcεRI stimulation. Thus the accumulated data clearly demonstrate the importance of PIP₃ in mast cell-mediated responses and promote the view that the levels of PIP₃ are finely regulated by both positive (PI3K) and negative (SHIP and PTEN) regulators under constitutive and activated conditions.

PHOSPHOLIPASES IN MAST CELL FUNCTION

Molecules derived from the hydrolysis of phospholipids provide both substrates for the production of eicosanoids and secondary signaling molecules for the regulation of the pathways that lead to the release of these and other classes of inflammatory mediators from activated mast cells. These reactions are catalyzed by phospholipase (PL) A₂, PLC, and PLD, which are activated after aggregation of FcεRI and ligation of other receptors, such as Kit, expressed on the mast cell surface. Phospholipases are intimately associated with the activation of PKC, which is required for mast cell activation and responses. As elegantly reviewed elsewhere,⁷⁵ phospholipase A₂ (PLA₂)—catalyzed reactions provide the arachidonic acid required for the production of leukotriene C₄ and prostaglandin D₂, whereas PLC and PLD contribute to the signaling cascade, leading to mast cell activation. We discuss these latter 2 in the context of their relationship with PKC and sphingosine kinases (SphKs) and the regulatory function of these molecules.

PLC and PKC

PLC activation requires both phosphorylation of a critical tyrosine residue (Y783) within its catalytic domain and membrane translocation to allow access to its membrane-associated lipid substrate, PI-(4,5)P₂. The resultant formation of diacylglycerol (DAG) and IP₃,⁷⁶ respectively, promotes PKC activation and an increase in cytosolic calcium levels after liberation of calcium from cellular stores. The isoforms of PLC that are responsible for FcεRI-mediated events are PLCγ₁ and PLCγ₂.^{77,78} By monitoring the aforementioned indices of PLCγ activation, it has been demonstrated that both isoforms are rapidly activated in mast cells after FcεRI aggregation.⁷⁷

Two possible signaling pathways might be responsible for PLCγ activation in stimulated mast cells: a PI3K/Btk-independent mechanism and a PI3K/Btk-dependent

mechanism.^{77,79-81} The PI3K-independent mechanism, as described previously, involves the direct and indirect (through Gads and SLP-76) binding of PLCγ to LAT after FcεRI-dependent LAT phosphorylation, where it is subsequently phosphorylated in a Lyn/Syk-dependent manner (Fig 4, A).⁸¹ The PI3K/Btk-dependent mechanism requires translocation and binding of both Btk and PLCγ to the plasma membrane, where PLCγ is subsequently phosphorylated by Btk.⁷⁹ Studies have suggested that the PI3K-independent, LAT-dependent activation of PLCγ is responsible for the initial calcium signal that is required for degranulation and that the subsequent maintenance of this signal, however, is dependent on both PI3K and Btk.^{5,77,79-81}

As with the Src PTKs and adaptor molecules, PKC activation that follows the generation of DAG by PLC (Fig 2) can both positively and negatively regulate mast cell degranulation, arachidonic acid metabolite production, and cytokine gene transcription. Multiple PKC isoforms are expressed in mast cells, and the differential requirements for their activation implies that these isozymes can, to a certain extent, be independently regulated and thus control different processes in mast cells. Mast cell studies provide evidence that the βI, βII, δ, ε, and θ isoforms⁸²⁻⁸⁷ contribute to the signaling cascades, leading to FcεRI-mediated degranulation, cytokine production, and/or arachidonic acid metabolite generation. Data from PKCδ^{-/-} BMMCs, however, have suggested that rather than promote FcεRI-mediated degranulation, PKCδ might downregulate this response.⁸⁸ The ability of PKCδ to downregulate degranulation of mast cells has been linked to its phosphorylation of, and thus activation of, the inositol phosphatase SHIP.⁸⁸ Alternatively, this might be mediated through the interaction and regulation of Lyn kinase activity by PKCδ⁸⁹ because decreased Lyn kinase activity inactivates SHIP and promotes enhanced mast cell degranulation.^{29,34} PKCα and γ isozymes also appear to be inhibitory for mast cell activation, whereas the ε isozyme might be both a negative regulator of pathways leading to degranulation and arachidonic acid metabolite release and a positive regulator of pathways that regulate cytokine gene transcription.^{82,90-92}

With respect to degranulation, calcium-dependent histamine release from RBL 2H3 cells correlates with PKC-dependent phosphorylation of the myosin light chain.⁹³ This observation and the reports that the fusion proteins synaptosome-associated protein (SNAP)-23, SNAP-25, and syntaxin 4 are targets for PKC-mediated phosphorylation, albeit in other cell types,⁹⁴⁻⁹⁶ suggest that PKC might play a role in the terminal granule mobilization and membrane fusion events associated with the degranulation process. PKC might also positively regulate degranulation of mast cells upstream of these events by virtue of its influence on other signaling process, such as PLD (also see the next section).^{97,98}

Reconstitution experiments conducted in RBL 2H3 cells also indicate a role for PKCs in cytokine production. PKCβ and PKCε in the FcεRI-mediated induction of the AP1 transcription factors Fos and Jun.⁹² In contrast, PKCα, βI, and βII appear to regulate cytokine production

upstream of these events, partially through a MEKK2/ERK5-dependent pathway.⁹⁹ These isozymes might also contribute to Ras activation after phosphorylation by Syk and recruitment of Grb2.¹⁰⁰ Furthermore, PKC β 1 activation, as mediated by Syk-dependent Btk activation, might control IL-2 and TNF production through the c-Jun N-terminal kinase pathway,¹⁰¹ and this pathway might also be regulated by PKC β II through the phosphorylation of Akt.¹⁰² Finally, activation of both PKC δ , downstream of Rac1,⁸⁶ and PKC θ ⁸⁵ was associated with Fc ϵ RI-mediated ERK1/2 activation, which, by leading to PLA₂ activation, would account for the ability of PKC to regulate arachidonic acid metabolite release from activated mast cells.⁸⁶

PLD and SphK

PLD hydrolyses phosphatidylcholine at the sn3 position to yield phosphatidic acid (PA) and free choline.¹⁰³ The PA liberated through this route can be further metabolized to produce DAG through the action of PA phosphohydrolase.¹⁰⁴ Indeed, the DAG produced by this mechanism accounts for the majority of DAG produced in mast cells after Fc ϵ RI aggregation.¹⁰⁵ Two mammalian forms of PLD have been cloned,¹⁰³ PLD₁ and PLD₂, and both of these forms are expressed in mast cells.^{98,106,107} However, these forms are differentially compartmentalized, with PLD₁ being primarily localized to the granule membranes and intracellular vesicles^{108,109} and PLD₂ being localized to the plasma membrane.^{98,106} Both PLD₁ and PLD₂ are activated after Fc ϵ RI aggregation in mast cells,^{107,108} resulting in the subsequent production of both PA and DAG.

Blocking the PLD-dependent production of PA and DAG with the primary alcohols ethanol or l-butanol or the conversion of PA to DAG with d,l-propranolol^{104,107,110} effectively inhibits antigen-mediated degranulation, arachidonic acid metabolite production, or both. More selective gene knockdown studies, which demonstrated that silencing RNA targeted against PLD₁ and PLD₂ effectively blocks antigen-mediated DAG production and degranulation in RBL 2H3 cells, further support a role for these enzymes in Fc ϵ RI-dependent mast cell activation.¹⁰⁷ In contrast, a dominant negative form of PLD₁ was reported to enhance Fc ϵ RI-mediated degranulation and early signaling responses, from which the authors concluded that PLD₁ negatively regulates signals for degranulation upstream of the calcium response.¹¹¹ However, because PLD contains a number of binding sites, including a PH domain and Phox domain,^{112,113} it is possible that this over-expressed construct also competes for binding to plasma membrane lipids with other critical signaling molecules.

Several mechanisms might account for the ability of PLD to regulate Fc ϵ RI-mediated mast cell arachidonic acid metabolite liberation and degranulation. With regard to the generation of arachidonic acid metabolites, it has been proposed that the PLD/PA phosphohydrolase-dependent production of arachidonyl DAG from phosphatidylcholine might provide a major source for the PLA₂-dependent production of free arachidonic acid.¹⁰⁴

PLD might help regulate degranulation by virtue of its contribution to the activation of PKC,¹⁰⁷ potentially through the production of DAG. However, data suggest that this is likely truer for the later stages rather than the initial stages of PKC activation, which are likely more dependent on PLC.⁹⁷ Alternatively, PLD₁ might help regulate the migration of secretory granules to the surface of mast cells, where PLD₂ might participate in the calcium-dependent fusion with the plasma membrane.¹⁰⁸ The sustained production of PA by PLD after Fc ϵ RI aggregation might also play a role in the cytoskeletal reorganization associated with mast cell activation.¹¹⁴

Finally, it has been proposed that PLD might contribute to the calcium signal required for degranulation by regulating the activation of SphK (Fig 2), leading to the production of the calcium-mobilizing lipid sphingosine-1-phosphate (S1P).^{115,116} How this might be accomplished is not clear; however, the calcium-mobilizing properties of S1P might be a consequence of targeting a yet-undefined intracellular receptor¹¹⁶ or through transactivation of cell-surface receptors for S1P (S1P₁ and S1P₂) expressed on mast cells.¹¹⁷ Transactivation of S1P₁ was demonstrated to be important for mast cell chemotaxis, whereas transactivation of S1P₂ contributes to mast cell degranulation.¹¹⁷ In both cases these receptors mobilize calcium. That SphK activation is essential in Fc ϵ RI-mediated mast cell responses is also reflected by the proximal relationship of the 2 isoforms (SphK1 and 2) with the Fc ϵ RI proximal kinases Lyn and Fyn (Fig 2).^{118,119} Both Lyn and Fyn contribute to the activation of SphKs, with Fyn playing an essential role and Lyn contributing to the early phase of SphK activation and translocation. Importantly, this work revealed that SphK2 required Fyn-dependent signals that are independent of pathways needed for PLD activity.¹¹⁸ Therefore although the consensus of studies support a role for both PLD₁ and PLD₂ in Fc ϵ RI-mediated mast cell activation, further studies with gene knockout or knockdown approaches are required to define the individual molecular processes governed by each isoform and their respective contributions in both positively and negatively regulating mast cell responses.

CLOSING REMARKS

Evident from the remarks herein, our increase in the knowledge of the events regulating mast cell activation has revealed an increasing complexity that underlies the presumed simplicity of a mast cell's response to a stimulus. Several lessons can be learned. First, for a given response, such as degranulation, many molecules function in a coordinated manner to promote and control the rate and extent of this response (Fig 2). Molecular redundancy is evident because only a few molecules seemingly can be classified as "essential," where in their absence the degranulation response is ablated, whereas most molecules contribute to the regulation of the response. From a therapeutic perspective, this places strong emphasis on identification of key molecules.

Second, multiple molecular checks and balances that control the extent, persistence, or both of the response are invoked on stimulation of the mast cell. These include ITAM motifs, kinases, phosphatases, adaptors, and lipids-lipases that might act as positive and negative regulators. This diverse array of molecules provides an intrinsic regulatory network in which molecules act coordinately to achieve the desired response and limit the possible injurious effect of a persistent or excessive response. Understanding how this network is coordinated might also allow its disruption in a manner that could be therapeutically beneficial.

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