Mechanisms controlling mast cell activation and allergic responses: Proteins and Lipids in harmony

J. Rivera, A. Olivera

Molecular Inflammation Section, Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, 20892, USA.

ABSTRACT

Mast cells are a key component of innate immunity and serve to amplify adaptive immunity. This latter role is mediated through the binding of antigen-specific immunoglobulin to Fc receptors expressed on their cell surface. Deregulation of the adaptive response makes the mast cell a central player in allergy and asthma through the binding of IgE antibodies to substances that are normally innocuous. Thus, the necessity of controlling mast cell activation is evident. While both activating and inhibitory cell surface receptors on mast cells are important in determining the outcome of a mast cells encounter with a stimulus, once activated, multiple intracellular molecules determine the type and extent of the mast cell response. In vitro and in vivo studies on the coupling of the high affinity IgE receptor (FcεRI) to mast cell effector responses has identified the Src family kinases Lyn and Fyn as having negative and positive roles in mast cell responses. This is in part modulated through the impact of these protein kinases on the cellular levels of phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). The crucial role for PIP3 could be demonstrated by downregulation of PTEN expression, a phosphatase that regulates cellular levels of PIP3, which caused increased levels of PIP3 and deregulation of mast cell homeostasis and activation. The importance of lipid mediators in mast cell function is further demonstrated by the close link between Lyn and Fyn activity and activation of other lipid kinases, like sphingosine kinases (SphK). By producing sphingosine-1-phosphate (S1P), SphKs contribute to mast cell chemotaxis and degranulation. These studies reveal a previously unrecognized cooperation of proteins and lipids that is likely to contribute in allergic disease.

KEY WORDS: FcεRI/ Lyn/ Fyn/ IgE/ Mast cell/ PTEN/ Degranulation/ Sphingosine kinase.
INTRODUCTION

Control of mast cell homeostasis and activation requires a highly coordinated molecular machinery that is capable of distinguishing the appropriate response to a given stimulus\(^1\)-\(^4\). Deregulation of these molecular events may manifest in unregulated mast cell responses that may hamper the ability of mast cells to function in host defense or could lead to disease (Fig. 1). Of particular interest to our research efforts has been the study of regulatory events preceding and following crosslinking by multivalent antigen of the IgE bound to the high affinity IgE receptor (Fc\(\epsilon\)RI). These events are likely determinants of the responsiveness of a mast cell\(^5\)-\(^10\).

In the past five years, significant advances have been made in understanding the molecular events that occur upon engagement of Fc\(\epsilon\)RI on mast cells (Fig. 2). The findings reflect the importance of two Src family kinases, Lyn and Fyn, in the initiation of downstream signaling events and a close collaboration of proteins and lipids in exquisite (and redundant) control of mast cell homeostasis and activation, which is key to the role of mast cells in adaptive immunity. Lyn kinase and cholesterol-enriched membrane microdomains (lipid rafts) are crucial for Fc\(\epsilon\)RI phosphorylation, which initiates mast cell activation. However, Lyn also plays a dominant role as a negative regulator of mast cell effector responses\(^5\),\(^6\),\(^9\). In contrast, Fyn functions to positively regulate mast cell responsiveness through its role in regulating the activation of phosphatidylinositol 3-OH kinase (PI3K)\(^11\),\(^12\) and the levels of its product phosphatidylinositol (3,4,5)-trisphosphate (PIP\(_3\)) (Fig. 2). By artificially manipulating the intracellular levels of PIP\(_3\), through downregulation of the expression of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) this lipid second messenger (LSM) was demonstrated to function as «gatekeeper» of mast cell activation\(^13\). Another product of phosphatidylinositol metabolism, namely phosphatidylinositol (4,5)-bispophosphate (PIP2), is a substrate of phospholipase C\(\gamma\), which generates inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG) that are key in activating calcium responses and the serine/threonine

Figure 1. Mast cells express the high affinity receptor for IgE (Fc\(\epsilon\)RI) and participate in acquired immunity. Crosslinking of the IgE bound to the receptor by a specific antigen triggers numerous signaling cascades that culminate in the massive secretion into the extracellular media of preformed mediators (a process called degranulation) within minutes after activation. This also triggers the de novo synthesis and secretion of cytokines, chemokines, growth factors and lipid mediators, which occurs at later times after activation. Because mast cells can immediately release mediators that invoke hypersensitivity, they constitute primary effector cells in acute IgE-associated allergic reaction. Mast cells also contribute to late phase of IgE-associated allergic reactions and chronic inflammation and are important players in anti-parasitic immunity, mostly by recruiting and promoting the activation of other immune cells and by orchestrating local inflammation and remodeling in the affected tissues. Mast cells are also central components of innate host defense against bacteria and participate in the initiation of acquired immune reactions (i.e. phagocytosis, expression and presentation of antigen) through receptors other than Fc\(\epsilon\)RI (not depicted in this figure).
protein kinase C (PKC), respectively, thus regulating mast cell responsiveness. Additionally, an intimate relationship between the generation of another LSM, sphingosine-1-phosphate (S1P) (an autocrine/paracrine regulator of mast cell chemotaxis and degranulation), and the FcεRI-proximal Src PTKs, Fyn and Lyn, reflects the importance of cooperation between the kinases and this LSM.

THE HIGH AFFINITY RECEPTOR FOR IgE (FcεRI) AND RECEPTOR PROXIMAL SIGNALING EVENTS

Mast cells can be activated through a wide variety of receptors. However, the principal receptor involved in allergy and asthma is FcεRI, the receptor for IgE (Fig. 1). This receptor, which is expressed on mast cells and basophils, is tetrameric. It is comprised of an IgE-binding α chain, a membrane tetraspanning β chain that functions to amplify the receptor’s signaling ability, and a disulfide-linked homodimer of γ chains that provides the receptor its signaling competence. Both the β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAMs), which have been demonstrated as essential for amplifying and signaling competence of immunoreceptors. The ITAM of the FcεRIβ contains a noncanonical tyrosine residue that is situated between the two canonical tyrosines found in conventional ITAMs. FcεRI lacks intrinsic tyrosine kinase.
activity and phosphorylation of the tyrosine residues in the ITAM motifs occurs through transphosphorylation by Lyn, which interacts with the β chain of the receptor. This initial phosphorylation requires liquid-ordered phase domains in the plasma membrane, which are enriched in cholesterol, sphingolipids and other saturated phospholipids (lipid rafts), and serve to concentrate Lyn in this region of the membrane. At present, the data support a role for lipid rafts in maintaining the phosphorylated state of the FcεRI rather than being essential for the initiation of its phosphorylation. Phosphorylated ITAMs can bind to a variety of proteins that can act as positive and negative regulators of signal amplification. The interactions so far identified are those with the tyrosine kinase Syk (Fig. 2), an essential kinase for the propagation of signals, and the tyrosine phosphatases SHP-1/2, although the temporal aspects of these interactions are poorly understood.

THE ARCHITECTURE OF CELL SIGNALING DOWNSTREAM OF FcεRI

The formation of a multi-molecular signaling complex (termed signalosome) is a necessary step in regulating downstream cellular processes. These signaling complexes (Fig. 2) must be localized to specific regions within the plasma membrane (like lipid rafts) that allow interactions with proteins or novel lipids generated by aggregation of surface receptors. The formation of signalosomes is coordinated by specific proteins termed adaptor molecules (also termed linkers or scaffolds) that function to provide docking sites for other signaling proteins. The protein-protein and protein-lipid interactions that occur in these signalosomes can be constitutive or inducible and are mediated by specific structural motifs in the interacting molecules.

Immediately following FcεRI stimulation, the lipid raft resident transmembrane adaptor molecule Linker for Activation of T cells (LAT, Fig. 2) is phosphorylated by Syk in multiple tyrosine residues, becoming an anchor for the organization of signaling proteins. A second lipid raft resident adaptor termed Non-T cell Activation Linker (NTAL, Fig. 2) is also rapidly phosphorylated after engagement of FcεRI. These molecules have been proposed to have both a positive and negative regulatory control of mast cell activation and have been the topic of recent reviews. Whereas it appears that LAT is crucial for degranulation and cytokine production, the role of NTAL is not completely clear as it has been proposed to mediate both inhibitory and activating functions. A role for NTAL in promoting mast cell degranulation was evident in studies that downregulated NTAL expression by siRNA. In contrast, genetic deletion of NTAL resulted in increased mast cell degranulation. A possible competition with LAT for lipid raft occupancy could explain the observed results.

since in the absence of NTAL expression more LAT might partition into lipid rafts, augmenting mast cell responses, as LAT-deficiency markedly attenuates mast cell responsiveness.

LAT may also serve to integrate both positive and negative signals depending on the tissue origin of the mast cell and on the stimulus. Upon phosphorylation by Syk, LAT regulates the activation of phospholipase Cγ (PLCγ) and the mobilization of calcium responses. This is dependent on the ability of PLCγ to directly bind LAT (following LAT phosphorylation) and also through the increased stability of this complex by cooperative binding of the SH2-containing leukocyte protein of 76 kDa (SLP-76), another adaptor protein which acts as a linker between PLCγ, and LAT-bound Gads. Mutational studies show that the multiple tyrosine residues in LAT contribute to mast cell responsiveness, demonstrating cooperativity of these protein binding sites. 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.

Other adaptor proteins are also activated and recruited upon FcεRI stimulation. These include Shc, Grb2, MIST/Clnk, ADAP, and other proteins detailed in previous reviews. Of particular note, the adaptor Grb2-associated binder 2 (Gab2, Fig. 2) has been implicated in binding the p85 regulatory subunit of PI3K in mast cells and is a key adaptor in the activation of PI3K activity in various cell types. This adaptor protein appears to function downstream of Fyn activation; both Fyn and Gab2-deficient mast cells showed defective PI3K activity as well as impairment of cytokine production and degranulation. Recent work places Gab2 downstream of Syk and suggests that Fyn regulation of Syk activity is important for the normal phosphorylation of Gab2. This implicates Fyn as a regulator of Syk activity, which merits further investigation, as Syk would play a dominant role in regulating adaptor function (through phosphorylation) in mast cells.

THE INVOLVEMENT OF LIPIDS IN MAST CELL SIGNALING AND FUNCTION

In addition to the complex regulation of signals by protein kinases, phosphatases and adaptor proteins, the coordinated activation of lipid kinases, phosphatases, and phospholipases results in the formation of lipid mediators that contribute to the intricate array of signals regulating mast cell function. As briefly mentioned above, lipid rafts may provide the environment where active FcεRI-induced signaling complexes occur. The exact contributions of these lipid domains to the organization of FcεRI signaling is still a matter of
investigation and has been extensively reviewed elsewhere(37-39). Following stimulation of FcεRI, there are considerable changes in the overall lipid composition of lipid rafts(40) and this may play an important role in determining mast cell responsiveness. New evidence for this view is provided by several recent studies. A murine model of Smith-Lemli-Opitz syndrome, a disease where the gene for 7-dehydrocholesterol reductase is mutated resulting in cholesterol deficiency, demonstrated the importance of lipid raft stability in controlling mast cell sensitivity to a stimulus(7). Mast cells from these mice showed reduced levels of Lyn expression in the lipid rafts, increased Fyn kinase and Akt activity reflecting increased PIP3 levels, and enhanced degranulation(7). Mast cells deficient of Lyn, SHIP, or PTEN, all of which caused increased intracellular levels of PIP3, also caused enhanced mast cell degranulation(10, 13), whereas inhibition of PIP3 production caused mast cell non-responsiveness(41). The levels of diacylglycerol, a key regulator of protein kinase C activity in mast cells(42) is also a determinant of mast cell responsiveness and changes in the concentration of sphingolipids are now known to both positively and negatively influence mast cell responses(4). The production of LSMs is dependent on a variety of proteins such as phospholipases A2 (PLA2), C and D (PLD) as well as PI3Ks and sphingosine kinases (SphKs). We detail some of the events demonstrating the close harmony of receptors, kinases, and other signaling proteins with LSMs.

Phosphatidylinositols and diacylglycerols in the regulation of signaling and responses

Regulation of phosphatidylinositol metabolism is crucial for effective mast cell signaling and effector responses through FcεRI. This cycle is regulated by both positive and negative protein regulators such as PI3K, PIP5K, PLCγ and SHIP or PTEN, which can act to control the levels of the multiple (phosphatidylinositol)s formed in the cell(43). Here we focus on those events known to impact mast cell activation and function.

PIP(4,5)P₂ acts as a signaling lipid in mast cells by directly binding regulatory proteins and components of the

Figure 3. Schematic representation of the dominant lipid pathways and protein partners in mast cells. After the engagement of the FcεRI both Fyn and Lyn kinases contribute at multiple levels to regulate lipid messengers that positively or negatively influence mast cell responses. Shown are the contribution of protein kinases, which phosphorylate and modify the function of multiple proteins; adaptor proteins, which bring activator proteins and lipid enzymes together in the proximity of the membrane; and lipid enzymes, which produce or remove lipid messengers. Lipid messengers are important in the targeting, activation and regulation of the function of signaling proteins. All these pathways weave a selection of dynamic signals that adjust spatio-temporally to fine-tune mast cell responses.
endocytotic/exocytotic machinery. In addition, through the action of the phosphodiesterases phospholipase Cγ1 and Cγ2 (PLCγ1 and PLCγ2) and the lipid kinase PI3K, two major lipid messengers are produced from PI(4,5)P2: Diacylglycerol, which is released together with a soluble messenger IP3, and PI(3,4,5)P3 produced by the phosphorylation in the 3'-position of the inositol moiety by PI3K (Fig. 3). IP3 is essential for calcium mobilization from intracellular stores, and calcium and DAG are both required for the activation of classical PKCs, like PKCβ, whose activity has been demonstrated as important for mast cell degranulation (Fig. 2 and 3)\(^{(44)}\). Pharmacological stimulation of mast cells with both calcium ionophore and DAG analogs, like phorbol 12-myristate 13-acetate (PMA), results in potent degranulation, whereas DAG analogs alone are insufficient to induce this response\(^{(45)}\). Studies using BMMC derived from PKCδ-deficient mice have demonstrated an important role for PKCδ in mast cell degranulation and cytokine production\(^{(46)}\). In contrast, deficiency in PKCβ enhanced mast cell degranulation, particularly when the cells were weakly stimulated\(^{(47)}\). No obvious defects in development or function were noted for PKCε-deficient mast cells suggesting a function that may be redundant with other isoforms\(^{(48)}\).

The phosphoinositide 3-kinase (PI3K) family, on the other hand, utilizes PI(4,5)P2 to generate PIP3 (Fig. 3)\(^{(49)}\). PIP3 binds effector proteins containing pleckstrin homology (PH), Phox homology (PX) and epsin N-terminal homology (ENTH) domains. The PLC and PI3K-regulated signaling pathways are interdependent, not only because both enzymes act on the same substrate, but because PIP3 binds the PH domains of certain Tec family tyrosine kinases, such as Btk, localizing them to the membrane where they promote maximal activation of PLCγ (Fig. 3 and 4). Furthermore, PI3K is also involved in the activation of PKC by activating PI3K-dependent protein kinase (PDK)-1\(^{(49)}\), which phosphorylates the activation loop sites of PKC isoforms, enhancing their activity (Fig. 3). A role of PI3K in mast cell degranulation was initially demonstrated using PI3K inhibitors such as wortmannin or LY294002, which blocked FcεRI-mediated mast cell degranulation as well as cytokine production\(^{(11, 50)}\). There are three basic classes of PI3K (I, II and III). The class I PI3K family is subdivided into two subclasses, IA and IB, on the basis of molecular structure and activation mechanisms. Class IA PI3Ks are heterodimeric kinases consisting of a regulatory subunit (p85α, p55γ, p50γ, p85δ, or p55γ) and a catalytic subunit (p110α, p110β, or p110δ). The p85 regulatory subunit of PI3K interacts with phosphorylated Gab2, a process that is mediated by Fyn after the engagement of FcεRI. Genetic deletion of either Fyn or Gab2 resulted in marked decreases in PI3K activity and inhibition of mast cell responses\(^{(11, 35)}\), suggesting that Class IA PI3Ks are the isoforms directly involved in FcεRI signaling (Fig. 2). In support of a role for type IA PI3K, mast cells derived from mice expressing p110δ\(^{(39,10)}\), a loss of function allele of the PI3K isoform p110δ, showed deficient mast cell degranulation and cytokine production\(^{(41)}\), and mice carrying this mutation, similarly to Fyn or Gab2-deficient mice, had diminished anaphylactic reactions. This remarkable similarity between Fyn-, Gab2-deficient and p110δ mutant mice, suggests the possibility that p110δ is the key PI3K isoform functioning downstream of Fyn and Gab2. Surprisingly, mast cells from mice lacking the p85 subunit of PI3K, the most abundant of the regulatory type IA class, have intact degranulation and cytokine responses induced upon FcεRI stimulation. These mice exhibit normal passive systemic anaphylaxis as well\(^{(51)}\). This suggests redundant function for some PI3K isoforms. Interestingly, class IB PI3K p110γ, which lacks the p85-binding domain, but is recruited to G-protein coupled receptors, was shown to play a role in IgE-mediated anaphylaxis and mast cell degranulation\(^{(52)}\). The activation of p110γ did not appear to occur directly downstream of the FcεRI, but could involve autocrine loops of G-protein coupled receptor agonists.

The relevance of the regulation of the levels of PIP3 on mast cell responsiveness is further highlighted in studies performed in mast cells derived from the lipid phosphatase SHIP-1-deficient mice or after downregulation of the expression of the lipid phosphatase PTEN\(^{(10, 13)}\) (Fig. 4). In either case, the levels of PIP3 were elevated and rendered mast cells hyperreactive. SHIP-1 regulates the FcεRI-dependent production of PIP3 by dephosphorylating the 5' position to generate PI(3,4)-P2, whereas PTEN opposes PI3K function by dephosphorylating the 3' position of PIP3, yielding PI(4, 5)-P2. In both SHIP-1- and PTEN-deficient mast cells, FcεRI-dependent calcium mobilization and degranulation were enhanced. However, the respective phenotypes differed in that PTEN-deficiency caused a constitutive phosphorylation of Akt in human mast cells\(^{(13)}\). The increased Akt phosphorylation was associated with constitutive activation of the MAP kinase family members, c-jun N-terminal kinase (JNK) and p38, phosphorylation of the transcription factor ATF2 as well as IL-8 and GM-CSF secretion. This differed from SHIP-null murine bone marrow-derived mast cells, where resting cells showed minimal phosphorylation of Akt and constitutive secretion of cytokines was not observed\(^{(53)}\). Thus, Akt, JNK, and p38 MAPK appear to be highly dependent on PIP3 production for their activation and can drive the production and secretion of some cytokines independently of FcεRI engagement. Lyn is required for phosphorylation of SHIP-1\(^{(10, 59)}\), an event important for appropriate targeting.
The phosphorylation of SHIP-1 was found to be defective and PIP3 levels were increased as demonstrated by direct measurement or indirectly by activation of Akt. This suggests the possibility that PTEN is required for control of PI3K in mast cell homeostasis and activation, whereas SHIP-1 is primarily active when mast cells are activated through FcεRI.

**Sphingosine-1-phosphate; a pleiotropic regulator of mast cell function and possible allergic mediator**

Metabolites of sphingomyelin, including ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (SIP) are also bioactive lipids, that mediate broad-range cellular responses such as cell growth, survival, differentiation, calcium homeostasis and chemotactic motility (Fig. 2 and 4)[4, 54]. SIP is involved in the positive and Sph and Cer in the negative regulation of mast cell responsiveness[4, 55]. Notably, SIP is rapidly produced after crosslinking of the FcεRI by two mammalian sphingosine kinases (SphK1 and SphK2). Inhibition of SphK activation, and thus SIP generation, by either competitive analogs of Sph in the RBL tumor mast cell line[56] or by antisense SphK mRNA in human mast cells[57] prevented IgE-triggered calcium responses and inhibited degranulation. This was independent of Syk phosphorylation or inositol 3, 4, 5-trisphosphate (IP3) production. The involvement of SIP in calcium release by a PLCγ-independent route implies its direct or indirect action on an unidentified channel, as has been suggested for other cell types[58]. SIP is a ligand for a family of five G-protein coupled receptors, termed sphingosine-1-phosphate receptors (SIP1-5) (Fig. 5)[4]. Each of these S1PRs couples to a different subunit of heterotrimeric G proteins αi, αq, and α12/13, and therefore they trigger an array of signaling pathways, including activation of Src kinases, small GTPases, MAPK cascades, phospholipases, PKC and calcium mobilization[59]. The role of these receptors is varied, but considerable attention has been placed on the role of SIP1, because of its requirement for thymocyte emigration and lymphocyte recirculation[60] as well as for vascular morphogenesis[61].
Mast cells express two of the five receptors for S1P, S1P1 and S1P2 (Fig. 5), and FcεRI-induced S1P formation results in the transactivation of these receptors, which is necessary for mast cell degranulation. However, the nature of the intracellular calcium stores targeted by S1P has not been determined, although in this figure it is illustrated as an ER calcium pool. S1P is secreted by activated mast cells to the extracellular media by mechanisms that have not yet been elucidated. Furthermore, generated S1P is able to rapidly bind and activate its receptors S1P1 and S1P2 on the plasma membrane. S1P1 induces cytoskeletal rearrangements leading to the movement of mast cells towards an antigen gradient, while transactivation of S1P2 enhances the degranulation response. Mast cell secreted S1P can also promote inflammation by activating and recruiting other immune cells involved in allergic and inflammatory responses. Since S1P also profoundly affects endothelial cell function, induces contraction and proliferation of airway smooth muscle cells, and its levels are elevated in the bronchial lavage of asthmatic individuals after antigen challenge, secretion of S1P by mast cells could be of relevance in this pathology. Mast cell granules are illustrated as black circles and the process of degranulation as granules get in contact with the plasma membrane emptying their content (smaller black dots). The thick, solid black arrow represents the intracellular actions of S1P, the dotted arrows represent the release of S1P to the extracellular media, and the dashed arrows, the signaling pathways activated via S1P receptors. Reprinted from the Journal of Immunology 2005;174:1153-1158. Copyright The American Association of Immunologist, Inc.
mast cells, which was partially corrected by the addition of exogenous S1P upon Ag stimulation\(^{(15, 16)}\).

Notably, S1P is one of the mediators that mast cells secrete upon antigen crosslinking of FcεRI (Fig. 5)\(^{(15, 16)}\). Unlike many other cells (T cells, B cells, etc.), mast cells secrete a substantial amount of S1P suggesting that it is an important mast cell mediator. This is further supported by the finding that S1P was highly elevated in the airways of asthmatic individuals\(^{(8)}\), and in the joints of arthritic individuals\(^{(65)}\). Both asthma and rheumatoid arthritis are inflammatory conditions in which mast cells have been demonstrated to be important effector cells, the latter primarily in a mouse model\(^{(66, 67)}\). The fact that S1P receptors are ubiquitously expressed in a variety of cell types and that S1P dramatically alters the function of endothelial, epithelial and smooth muscle cells, raises the possibility that this LSM is also a paracrine mediator involved in the pathophysiology of asthma or other allergic and/or inflammatory diseases (Fig. 5).

**Phosphatidic acid and mast cell degranulation**

PLD is a phosphodiesterase that hydrolyzes membrane phosphatidylcholine (PC) to generate PA and free choline (Fig. 2-4)\(^{(68)}\). PA is involved in a wide range of physiological processes including cytoskeletal rearrangements, vesicle trafficking, exocytosis and proliferation. The evidence for the involvement of PA in cellular processes comes mostly from studies exploiting the unique ability of the PLD enzymes to catalyze a transphosphatidylation reaction in the presence of primary alcohols, generating, instead of PA, phosphatidylalcohols, which are presumed to be functionally inactive. To date, two mammalian PLD isoenzymes, PLD\(_1\) and PLD\(_2\), have been cloned\(^{(68)}\). Both isoenzymes are membrane-associated in mast cells: PLD\(_1\) is localized to perinuclear endosomes and trans-Golgi sites and PLD\(_2\) is found predominantly at the plasma membrane\(^{(69)}\). Both isoforms are activated upon antigen stimulation\(^{(70)}\) and a role for PA in degranulation has been established by the use of primary alcohols and siRNAs directed against PLD\(_1\) and PLD\(_2\). Further evidence for a role of these enzymes in mast cell degranulation is provided by experiments demonstrating that expression of a catalytically inactive mutant of PLD\(_1\) or PLD\(_2\) in mast cells blocked the migration of granules to the cell periphery and degranulation\(^{(69, 71)}\). Other studies have involved PLD\(_1\) in the regulation of the basal levels of PA in mast cells. A decrease in basal levels of PA was correlated with an increased fraction of FcεRI in lipid rafts and enhanced degranulation\(^{(72)}\). The precise mechanism by which PLD affects degranulation is not known. Formation of PA, a cone-shaped lipid, at the expense of lysoosphatidic acid, an inverted-cone-shaped lipid, was found to participate in the pinching off of membranes during endocytosis by inducing a negative membrane curvature \(^{(73)}\). Thus, regulation of PA may alter membrane properties facilitating membrane fusion in mast cells. Other possibilities include direct signaling by PA, signaling through its metabolic conversion to DAG mediated by PA phosphohydrolase (PAP) or through conversion to lysophosphatidic acid (another lipid messenger) mediated by PLA\(_2\). PA, as other acidic phospholipids, can bind protein’s regulatory domains affecting their activity and ultimately cell functions. PA was shown to activate PKCs\(^{(74)}\), SphK\(^{(16, 75)}\), and type I PIP 5-kinase, which regulates PIP\(_2\) levels\(^{(76)}\), and the GTPase activating proteins (GAPs) that regulate small GTPases\(^{(77)}\). DAG derived from PC has also been proposed to regulate PKC activity (Fig. 4) analogous to DAG derived from PIP\(_2\). Inhibition of PLD by 1-butanol but not tertiary-butanol inhibited the PKC translocation to the membrane fraction normally induced by FcεRI. Similarly, transfection with siRNA for PLD\(_1\) or PLD\(_2\) blocked translocation of DAG-dependent forms of PKC\(^{(78)}\).

Although multiple pathways can regulate PLD activation, the precise mechanism for its activation via FcεRI is not known. Both Fyn and Fgr could induce PLD\(_2\) phosphorylation, a process that correlates with PLD activation\(^{(79)}\). Syk activity and other signals such as calcium, PKC and PI3K\(^{(80)}\) have also been implicated in PLD stimulation. Additionally, in vitro studies suggest that PIP\(_2\) is an allosteric regulator of both PLD\(_1\) and PLD\(_2\); and receptor-mediated regulation of PLD seems to be partly mediated by the small GTPases ARF and Rac\(^{(70, 81)}\). Collectively, the data are consistent with multiple inputs for PLD activation and with the concept of feedback regulatory control, since many of the signals required for PLD activation (such as PKC activity) are in turn regulated by PLD activity. This may reflect the importance of these steps in eliciting and governing mast cell function.

**PROTEINS AND LIPIDS IN THE REGULATION OF TRANSCRIPTION**

In addition to the FcεRI-mediated degranulation of mast cells, which releases pre-formed granule-stored mediators, stimulation of these cells leads to the secretion of de novo synthesized lipid mediators such as leukotrienes, prostaglandins and platelet activating factor (PAF). A wide array of cytokines (IL-1-IL-6, IL-9–IL-14, IL-16, IL-18, MIF, TNF, GM-CSF, LIF, etc) and chemokines (IL-8, MCP-1–MCP-4, MIP-1, MIP-3, RANTES, etc) can also be produced and secreted (Fig. 1)\(^{(2)}\). Signals triggered by FcεRI are sufficient to activate most enzymes in the synthetic pathway of lipid mediators leading to their formation, whereas the de novo production of cytokines...
and chemokines requires gene transcription. The pattern of expression of cytokines depends on the strength of the stimulus and the environment mast cells (i.e. presence of additional signals).

The assembly of transcription factors and DNA-binding proteins onto specific DNA regulatory regions controls gene transcription of cytokines or chemokines. Transcription factors are modified by multiple signals and translocate to or are present in the nucleus where they interact with the promoter regions of genes. Multiple transcription factors are known to regulate cytokine expression in mast cells. Among these, nuclear factor κB (NFκB) and the nuclear factor of activated T cells (NFAT) play major roles in the production of a number of cytokines by mast cells. The NFAT family of transcription factors is known to bind cooperatively with other transcription factors like AP-1, to composite DNA sites. AP-1 is a hetero- or homodimeric complex composed of members of the Fos and/or Jun families of transcription factors. In mast cells, as well as in T cells, the NFAT complex contains Fos and Jun proteins. Depending on whether or not both transcription factors are concomitantly activated, distinct sets of genes may be activated, eliciting different patterns of cytokines. The major pathways for activation of the NFAT and AP-1 transcription factors are distinct, although crosstalk may occur. AP-1 activation is regulated at multiple levels by activation of MAP kinases, although in mast cells PKC has also been implicated. In contrast, a dominant determinant of NFAT binding activity in the nucleus is calcium mobilization. Jun proteins are phosphorylated by MAP kinases (e.g. JNK-1/2, Erk-1/2), while the activity of Fos family members is regulated by a rapid induction of their de novo synthesis, which appears to be regulated by PKCβ and ε in mast cells. Both the increased synthesis of Fos and the phosphorylation of Jun lead to the full activation of the AP-1 complex.

Another possible partner of c-Jun in the AP-1 complex is activating transcription factor 2 (ATF-2), which is also regulated by MAP kinases and as indicated above by the intracellular levels of PIP3.

The MAP kinase family in regulation of mast cell gene expression

MAP kinases play prominent roles in the transcriptional regulation of activated mast cells (Fig. 2). The family of MAP kinases include extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and extracellular signal regulated kinase-5 (ERK5; also called Big MAP kinase-1 (BMK1)). These kinases have to some extent overlapping substrate specificities. MAP kinases regulate NFκB, NFAT and AP-1 complexes, they phosphorylate and activate the immediate early gene regulator, Elk-1, and their translocation into the nucleus results in phosphorylation of other kinases such as the ribosomal S6 kinase (RSK) and the mitogen- and stress-activated protein kinase (MSK1) both of which contribute to gene expression by phosphorylating histones and regulatory proteins. MAP kinases are activated by dual phosphorylation in serine/threonine and tyrosine mediated by a MAP kinase kinase (MKK), whose activation is dependent on phosphorylation by a MAP kinase kinase kinase (MKKK) (Fig. 2). The most well studied of the family members is ERK, whose activation is primarily dependent on MKK and MKK2. In contrast, JNK phosphorylation is dependent on MKK4 and MKK7 whereas p38 MAP kinase is normally a target of MKK3, MKK4, and MKK6. In mast cells, the paradigm of the above required hierarchy and specificity is seemingly maintained. A key component is the adaptor protein LAT because it provides the link for activation of the small monomeric GTP binding protein Ras (Fig. 2). Activated Ras initiates the MAP kinase cascade by the phosphorylation of Raf (a MKKK). Deficiency in LAT or in a LAT-associated adaptor termed SLP-76 causes a marked deficiency in activation of ERK and JNK. NTAL has also been shown to contribute to MAP kinase activation (Fig. 2) but this is less well understood. Vav1, which is part of the LAT scaffold complex, is also involved in the activation of JNK, as Vav1-deficient mast cells revealed a defect in its activation. In contrast, a mast cell deficiency in NTAL caused increased activation of ERK, JNK, and p38 MAP kinase suggesting that NTAL may control the extent of their activation. However, mast cells deficient in both LAT and NTAL showed a more marked defect than LAT-deficient mast cells in the activation of all MAP kinases. This suggests that the role of NTAL in MAP kinase regulation might be both positive and negative. Importantly, a dichotomy has been observed in Lyn-deficient mast cells where ERK activation appeared relatively normal even when LAT phosphorylation was substantially reduced. This suggests that other pathways (possibly NTAL-mediated) are able to compensate for the absence of Lyn and the reduced LAT phosphorylation. In contrast, Lyn-deficiency had no effect on ERK phosphorylation but instead caused a defect in the activation of JNK and p38 MAP kinase. This is consistent with the aforementioned role of PIP3 in regulating the activation of these two MAP kinases as observed by downregulation of PTEN in human mast cells. However, because in mast cells JNK activation is affected by both Fyn and LAT deficiencies, crosstalk in the signals generated by these proteins must occur. In support of this view, both Fyn- and LAT-deficient mast cells showed defective production of IL-6 and TNF, suggesting that...
they may be important in inducing signals required for transcription of these genes.

**NFkB; an essential regulator of proinflammatory cytokines in mast cells**

NFkB is an essential regulator of IL-6 and TNF production in mast cells. NFkB proteins include NFkB1 (p50 and its precursor p105), NFkB2 (p52 and its precursor p100), RelA/p65, RelB, and cRel. The predominant form of NFkB in many cell types is a p50:p50 heterodimer, however, in mast cells the predominant forms are not known. NFkB is sequestered in the cell cytoplasm in a form bound to a family of inhibitory proteins (inhibitor of kB (IkBa), IkBβ, and IkBγ) that mask its nuclear localization signal (Fig. 6). Phosphorylation and ubiquitination of IkB lead to its degradation, allowing NFkB to translocate to the nucleus and to bind DNA thus promoting gene expression. Central to the phosphorylation of IkB is the IkB kinase (IKK) complex (Fig. 6). The IKK complex, which includes two catalytic subunits, IKKa (or IKK1), and IKKβ (or IKK2), and a regulatory subunit, IKKγ (or NEMO), can also be activated by a variety of signals to induce IkB phosphorylation. Among these, the MAP kinases, P38, and PKC seem dominant in many cell types. In mast cells, the mechanism by which FcRRI induces NFkB activation is still unclear, probably because of the redundancy of signals ensuring the activation of this central transcription factor.
Nonetheless, some clues are emerging as phosphorylation of IKK was defective in Fyn-deficient mast cells(99) and this defect resembles the defective IKK activation and NFkB activity in Src PTK triple-deficient (Blk, Fyn, and Lyn) B cells(102). In contrast, disruption of the interaction between FcεRI with Lyn (through mutation of the receptors β subunit ITAM) caused enhanced IKK phosphorylation and enhanced cytokine production(89). The link between Fyn or Lyn and NFκB activation is not clear, but the data supports a model whereby Fyn is required for normal activation of this pathway and receptor-associated Lyn controls the extent of IKK activation. The role of LAT or NTAL in mast cell NFκB activation is also unclear, however, given the defective production of IL-6 and TNF in LAT-deficient mast cells it is likely that NFκB activity is diminished by the absence of LAT. Proteins that may be likely candidates in regulating NFκB downstream of Fyn and Lyn include JNK, PI3K and PKC. In mast cells, these signaling proteins require Fyn for their full activation(11, 99) and they are crucial for NFκB activation in various cell types. Of particular interest are the PKCδ and θ isoforms, which play a major role in IKK phosphorylation and in cytokine production in B and T cells, respectively(100, 104). The mechanism for their key role in NFκB activation was recently defined(105). These PKC isoforms are in the phosphorylation of an adaptor protein CARMA1 important (caspase recruitment domain (CARD)-containing member of the membrane associated guanylate cyclase (MAGUK) family-1) that scaffolds a complex of proteins that lead to IKK activation (Fig. 6). Once phosphorylated, CARMA1 interacts with an adaptor protein Bcl10 through its CARD domain engendering an oligomerization that is essential for activation of IKK once Bcl10 associates with a third adaptor protein, Malt1. This complex can now cause activation of IKK, however, the exact mechanisms by which this happens is not known. In mast cells (Fig. 6), it was recently demonstrated that Bcl10 and Malt1 are both needed for the activation of NFκB and for IL-6 and TNF production(100). Interestingly, deficiency in either of these proteins had no substantial impact on mast cell degranulation or eicosanoid production demonstrating that the bifurcation of these responses from cytokine production occurs upstream of these two proteins(107). At the moment it is unclear if CARMA1 is expressed in mast cells. However, based on the demonstrated requirement for a scaffolding protein for Bcl10 oligomerization and Malt1 function it is likely that a member of the CARMA family of proteins plays such role in mast cells(107).

Summary and Perspectives

The findings summarized herein demonstrate the existence of multiple signaling pathways regulating FcεRI-dependent mast cell responses. A key feature is the complex relationship of signaling proteins and their lipid partners, which are important in targeting, activating, and regulating the function of these proteins. Several lessons can be learned from these studies: 1. Some lipids can directly modulate the activity of proteins independently of a need for complementary signals generated through receptor engagement. An excellent example is provided by the findings on the shRNA silencing of PTEN in human mast cells, which demonstrated that increased PIP, alone can activate Akt and MAP kinases resulting in mast cell cytokine secretion. 2. We have also learned that lipid-mediated regulation occurs by modulating intracellular signaling pathways as well as by engagement of cell surface receptors. An example of this is provided by studies on SphKs whose function in mast cells is dependent on the production of S1P that causes intracellular effects (such as calcium regulation) but also engages the S1P receptors expressed on mast cells to modulate both chemotaxis and degranulation. 3. Finally, although the data is far from definitive, we also learn that PLD production of PA may be essential in regulating the function of key proteins required for mast cell degranulation (such as PKC). PLD production of PA may also impact the ability of granules to fuse with membranes, thus providing regulation for a key signal as well as the actual process of exocytosis (upstream and downstream regulation). Interestingly, the findings demonstrate that this protein-lipid harmony extends well beyond the mast cell degranulation response alone, since cytokine gene expression is also influenced by this interplay.

This protein-lipid mode of regulating FcεRI-dependent mast cell effector responses is not limited to these cells but is a fundamental process by which all cells are likely to be regulated in response to the surrounding stimuli. However, understanding these relationships in the mast cell has revealed several unique features, which suggests that several steps in this process may be considered as possible therapeutic targets in mast cell-related diseases like allergy. For example, the relationship between p110δ and the Fyn-Gab2 complex needs to be further explored as the data strongly suggest that this module may be a key component for mast cell responses downstream of FcεRI engagement. In particular, if FcεRI-associated Fyn is responsible for driving the Gab2-PI3K(p110δ), disruption of this interaction may be beneficial and achievable through targeting of receptor sequences required for the association of Fyn. Because FcεRI is primarily a mast cell and basophil specific receptor, its therapeutic targeting achieves the desired selectivity and avoids the undesired consequences of targeting Fyn kinase activity, an enzyme expressed in a variety of cell types. Alternatively, the targeting of p110δ interactions, or its activity, may provide the needed specificity
since this kinase isoform is not expressed in all cell types. Regardless, the in-depth analysis of the mechanisms underlying FcRI-mediated mast cell activation are likely to provide new insights on the pathophysiological aspects in disease. Examples of this are provided by in vitro findings that show that Lyn-deficient mast cells are hyperactive⁶️. Similarly, in vivo data show that Lyn-deficient mice develop an atopic allergic-like disease and demonstrate an exacerbated asthmatic response(⁵️). Given that Lyn is the enzyme required for FcRI phosphorylation (the initiating step in IgE-dependent mast cell activation), these results were unexpected and once again teach us the unpredictability of a biological system. It is essential that future efforts focus on moving the knowledge gained in vitro to suitable in vivo models that offer the opportunity to determine if the in situ milieu (both proteins and lipids) is a determinant in the observed response.

ACKNOWLEDGEMENTS

Much of the research reported herein was supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health.

CORRESPONDENCE TO:
Juan Rivera
NIAMS/NIH, Building 10, Room 9N228
Bethesda, MD, 20892-1820.
Phone: 301-496-7592. Fax: 301-480-1580
Email: juan_rivera@nih.gov

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