

A Molecular Understanding of Mast Cell Activation and the Promise of Anti-Allergic Therapeutics

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Abstract: Mast cells are central to allergic disease. Their immediate (exocytosis of granule-stored allergic-mediators) and delayed (de novo synthesis of inflammatory mediators) response to an allergen underlies the symptoms seen in acute and chronic allergic disease. Thus, intervention in the allergen-mediated activation of mast cells is a long sought after goal in the treatment and management of allergic disease. The recent gain in deciphering the molecular mechanisms underlying immunoglobulin E (IgE)-mediated mast cell activation has provided optimism for the development of new therapeutic strategies. Among the most promising is the use of humanized anti-IgE antibodies that inhibit binding of IgE to its high affinity receptor (FcεRI) on the mast cell. Other strategies target molecules proximal to FcεRI, whose activities are central in mast cell activation. One such molecule, Syk kinase, has been targeted by various approaches including a small molecule inhibitor that specifically abrogates mast cell degranulation. More recently, various molecules that function to promote protein-protein interactions (adapters) were demonstrated as essential to mast cell degranulation and cytokine production. It remains to be seen if these molecules hold therapeutic promise for disease intervention. Additional studies identifying molecules required for mast cell granule fusion and content exocytosis also bodes well for discovery of new therapeutic targets. While our understanding of IgE-mediated mast cell activation is still at its inception, the modest success in identifying molecules essential to this process affords some confidence for better treatment of allergic disease.

Keywords: Allergy, Mast Cells, Allergen, IgE, FcεRI, Therapeutics, Molecular Mechanisms, Degranulation.

INTRODUCTION

Allergic disease is a consequence of exposure to normally innocuous substances that elicit the activation of mast cells through immunoglobulin E (IgE)-dependent or -independent mechanisms. The physiological response to these substances is thought to be a consequence of evolutionary adaptation of the immune system in the absence of endemic parasitic or bacterial challenges and/or the loss of tolerance to these substances because of the absence of early childhood exposure [1-3]. Allergic disease is thus more prevalent in developed countries where a strong public health emphasis has eliminated endemic exposure to parasites and to many substances normally found in the environment [4]. The effective prevention and treatment of allergies is a long sought after objective since some allergies can be lethal, or burden the individual with a poor quality of life, and are a multi-billion dollar economic burden on society. Until recent years the strategies used in treating allergies aimed at treating the symptoms or at preventing initiation of disease by reducing the allergen exposure risk. However, for the most part, these strategies have proved ineffective or impractical since the causes and symptoms vary among (such as dust mites) in daily life is a difficult and impractical task. More recently, there has been a growing

allergic individuals and prevention of exposure to allergens emphasis on developing strategies that intervene with initiation of the immunologic and allergic responses based on our growing knowledge of the molecular mechanisms underlying allergic disease. In this article we briefly outline some of the new strategies that are based on targeting the activation of a mast cell. Some of these are well-developed strategies that are currently in clinical trials whereas others are newly discovered molecular mechanisms that hold promise for possible therapeutic intervention in disease. Regardless, we hope that this collective view will provide a sense of the importance of the relationship between mechanistic understanding of function and development of more specific therapeutics in allergic disease.

BACKGROUND

Because of their central role in allergic disease, mast cells have long been studied with a focus on understanding the molecular basis by which they are primed for activation or become activated. In allergic disease, mast cells are most frequently activated by an IgE-dependent mechanism (see Fig. 1a). Allergens encountered by antigen presenting cells, like dendritic cells, are processed and presented to T cells eliciting a T helper 2 response (production of IL-4 and IL-13) that causes B cells to produce allergen specific IgE [5]. The high affinity receptor for IgE (FcεRI) on mast cells binds the allergen specific IgE (Fig. 1b) in an essentially irreversible manner [6,7]. Once bound IgE dissociates slowly from FcεRI (the half-life of cell-bound IgE is in the order of

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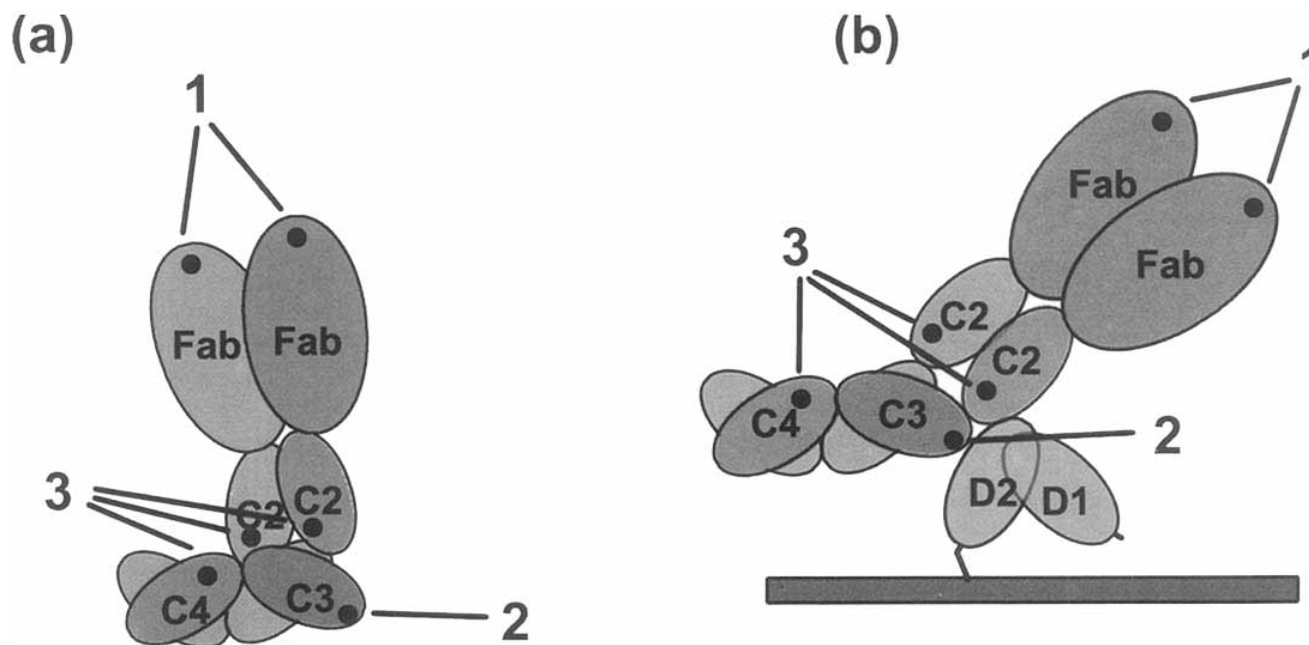


Fig. (1). Structure of IgE and conformational change upon binding to its high affinity receptor (FcεRI). (A) Bent structure of soluble IgE indicating constant domains C2-C4. Specific interactions of IgE with an allergen may be blocked in the Fab portion at site 1 by haptens or bivalent ligands with long rigid spacers. Both of which fail to elicit FcεRI aggregation and mast cell degranulation. Site 2 is the primary site on IgE that interacts with FcεRI α. Humanized monoclonal anti-IgE antibody rhuMab-E25 targets this region. Targeting of sites indicated as 3 is likely to interfere with the quaternary structure of IgE and cause a decrease in the affinity of IgE binding and/or increase in dissociation from FcεRI. (B) Open conformation of IgE bound to FcεRI. Site 2 on the C3 domain interacts with the D2 domain of FcεRIα. The C2 domain facilitates the conformational changes required for the binding of the C3 domain to FcεRIα and thus is likely to contribute to stable IgE binding.

days [7]), therefore sensitization of these cells is persistent when serum IgE levels are increased. While this high affinity monomeric IgE binding to FcεRI has some biological consequences [8,9], it is not until the cell bound IgE antibody interacts with the specific allergen that a mast cell is fully active and able to release histamine, serotonin, and a variety of proteases stored in preformed granules (degranulation). This elicits the immediate hypersensitivity reaction observed upon encountering an allergen. Mast cells also produce a variety of lymphokines and lipid mediators, such as leukotrienes and prostaglandins, that are major contributors to the late or chronic phase of allergic disease because of their potent ability to elicit a generalized immune cell response leading to allergic inflammation [10,11].

Because of its importance in IgE binding, mast cell activation, and thus allergic disease, the structure and function of FcεRI has been intensively studied (see Fig. 2). FcεRI is a tetrameric receptor and a member of the multichain immune recognition receptor (MIRR) family [12]. The FcεRI contains an IgE binding α chain, a signal amplifying β chain [13], and two disulfide linked γ chains that provide signaling competence to this receptor [14]. This competence is afforded by an amino acid motif called the immunoreceptor tyrosine-based activation motif (ITAM [15]) that contains canonical tyrosine residues that are phosphorylated when the receptor is occupied by IgE and the allergen. Allergen-aggregation of the IgE bound to the α

subunit of the FcεRI results in the phosphorylation of the FcεRI ITAMs by the Src family protein tyrosine kinase (PTK) Lyn [16,17]. Phosphorylation of the two γ chain ITAMs creates a novel binding site for another PTK called Syk that serves to amplify the initiated signals by phosphorylating various substrates [18,19]. Additionally, allergen-IgE-dependent aggregation of FcεRI activates another SrcPTK, Fyn, whose function, while not yet fully understood, contributes to mast cell degranulation [20]. Among the substrates targeted by Lyn-Syk or Fyn activities are the adapter molecules LAT and Gab2, respectively. These adapters function to physically organize and coordinate molecules whose activities when brought together contribute to mast cell activation and responses [21]. Of primary importance is the role of these adapters in the upregulation of intracellular calcium levels and activation of protein kinase C (PKC) [21], as these events are required for mast cell degranulation. As might be expected, considerable effort has been made towards understanding the molecular steps required for the aforementioned events.

ALLERGEN and IgE ANTIBODY INTERACTIONS

The critical step in IgE-mediated mast cell activation is allergen-dependent aggregation of IgE antibody bound to FcεRI. Therefore, one possibility for therapeutic intervention in allergic disease is to prevent allergen-dependent aggregation of the IgE-FcεRI complex by binding of a

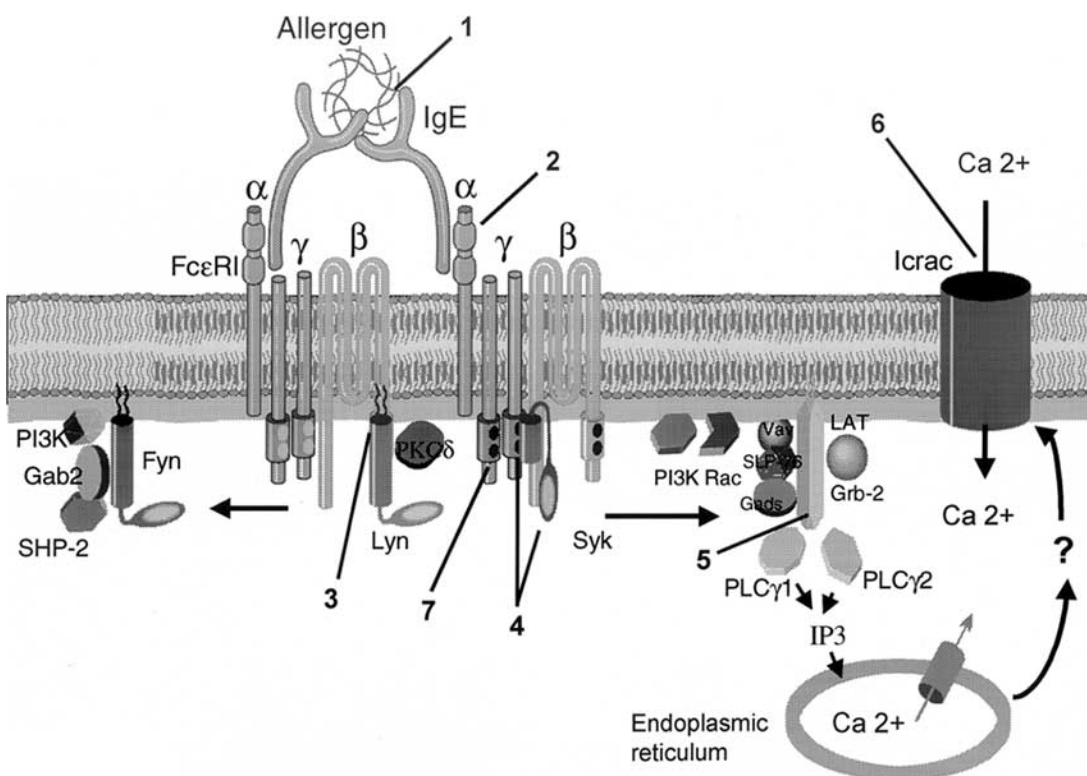


Fig. (2). Possible sites for therapeutic intervention in early steps of IgE-mediated mast cell activation. Aggregation of FcεRI by IgE-allergen interactions causes initiation of signaling events described in the text. (1) Antagonist monovalent hapten or bivalent ligands (such as those with rigid spacers) compete with allergen binding to IgE antibody and thus abrogate degranulation. (2) Anti-IgE antibodies, soluble FcεRI, and peptides that encode the FcεRI binding site on IgE or vice versa can disrupt IgE binding to FcεRIα. (3) Overexpression of peptide encoding the unique domain of Lyn kinase decreases histamine and cytokine release. (4) Compounds like ER-27319 disrupt Syk kinase interaction with ITAMs (defined in text) and inhibit degranulation whereas others directly inhibit its catalytic activity. (5) Absence of LAT abrogates degranulation. PLCγ binding region seems to be critical for degranulation. How FcεRI and LAT communicate or interact is unknown but this molecular step may serve as a means for disruption of mast cell degranulation. (6) Inhibition of the influx of calcium (Ca^{2+}) from the extracellular environment abrogates mast cell degranulation. Disodium cromoglycate is thought to function to block calcium influx but does not show selectivity for Icrac channels. (7) Interference with PKCδ-mediated phosphorylation of FcεRIγ ITAM on a threonine residue inhibits Syk activation and may provide some selectivity in inhibiting Syk activation.

monovalent allergen (hapten) or a bivalent allergen that is unable to effectively aggregate the IgE-FcεRI complex (see Fig. 1 for location of allergen binding sites and Fig. 2 for the steps blocked by allergen antagonists).

Haptens can be comprised of overlapping non-anaphylactic fragments of an allergen, which means they must have a single epitope that binds monovalently and with high affinity to the IgE antibody. Binding of a monovalent hapten removes IgE reactivity to the multivalent allergen by blocking specific allergen binding sites on the IgE molecule. Because allergen multivalency (binding of the allergen to more than one IgE antibody) is required for aggregation of the IgE-FcεRI complex, the hapten effectively blocks aggregation and thus mast cell activation. Interestingly, the cloning of common allergenic epitopes has revealed that some allergenic epitopes may be cross-reactive [22]. Thus, the development of hapten-based therapies for one allergen may provide wider benefits. The hapten approach may also provide for a reduction in the harmful anaphylactic reactions that can occur in the commonly used approach of allergen-specific immunotherapy [23-25].

It is now recognized that low affinity allergens may trigger early stages of mast cell activation without causing the release of most allergic mediators [26]. However, while on the surface this might appear as a possible therapeutic approach, it was also shown that a low affinity allergen, at a concentration that is ten fold higher than a high affinity allergen, failed to effectively inhibit the binding of the high affinity allergen [26]. Therefore, the inability to prevent high affinity allergen binding to the IgE antibody does not bode well for the use of low affinity allergens as a therapeutic approach. On the other hand, several studies indicate that relatively subtle differences in the proximity of- or the conformation achieved by- aggregated receptors following ligand binding could have significant consequences on the cells activation [27]. It was recently shown that high affinity bivalent ligands (an allergen equivalent) with the long rigid spacers of 68-102 Å could effectively bind with high affinity to cell-bound IgE antibody or to IgE antibody in solution. However, unlike a flexible bivalent allergen these rigid ligands were unable to effectively trigger mast cell degranulation [28]. The rigid ligands, however, stimulated robust tyrosine phosphorylation responses but only limited

Ca²⁺ mobilization, indicating that a step upstream of this essential process, for mast cell degranulation, was affected by the rigid nature of the ligand. It was suggested that the rigid nature, and perhaps the longer length, of the ligand did not allow the occurrence of all the signaling events required for the degranulation response. These findings suggest that the development of rigid bivalent high affinity ligands, which can compete for binding to IgE antibodies of known allergenic specificity, may be an effective immunotherapeutic approach towards blocking the release of allergic mediators.

IgE BINDING TO FcεRI

Many patients with an allergic disorder have elevated serum IgE antibody levels and thus their mast cells contain high levels of FcεRI-bound allergen-specific IgE. Binding of monomeric IgE to its high-affinity receptor, in the absence of allergen-induced receptor aggregation, is able to up regulate expression of FcεRI on the cell surface [29-31], which enables the cells to bind more IgE and thus become more sensitive to activation by lower concentrations of an allergen. Monomeric IgE binding also suppresses mast cell apoptosis, which enhances mast cell survival leading to increased mast cell numbers [8,9]. Under these circumstances many mast cells are primed to degranulate and thus upon encountering the allergen are immediately capable of initiating an allergic response. Therefore, the interaction of IgE antibody with FcεRI provides an attractive target for the inhibition of IgE-mediated allergic disease. Development of drugs to interfere with this interaction presents a formidable challenge because of the high affinity of IgE-FcεRI interactions.

Nonetheless, several reasonable therapeutic strategies can be deduced from the recently published crystal structure of IgE [32-34]. IgE shares the overall structure of other classes of antibodies, but it is distinguished, similarly to IgM, by the presence of four constant immunoglobulin domains, C1–C4, one more than is found in IgG, IgA or IgD. The two COOH-terminal constant domains of IgE, C3 and C4 are homologous to the C2 and C3 domains in IgG. The C3 domain contains a site that is critical for binding to FcεRI (Fig. 1a). The high affinity binding of IgE is primarily a consequence of hydrogen bonding, hydrophobic, and electrostatic interactions between the C3 domain of IgE and FcεRI, although the C4 domain also contributes to the conformational changes that facilitate the binding [33]. The C2 domain of IgE is part of the immunoglobulin hinge region, which in IgG mediates flexibility between the antigen-binding Fab and the Fc portion. Interestingly, IgE is less flexible than IgG. Structural analysis of the IgE- FcεRI complex revealed that the C2 domain is folded back onto and interacts with the C3 and C4 domains, causing an acute bend in the IgE molecule. Upon binding to FcεRI a conformational change occurs in the C2 and C3 domains of IgE that allows a flexing forward so that both C3 domains are accessible for binding with FcεRI (Fig. 1b). Thus, the C2 domain contributes to the stability of IgE binding to FcεRI, since deletion of the C2 domain from the IgE Fc fragment leads to an increased rate of dissociation [35].

One approach to prevent IgE binding to FcεRI is to interfere with the C3 domain binding site of IgE (Fig. 1b

and 2). Mutational analysis has revealed that the NH₂-terminal sequence of C3 is essential for FcεRI binding [36]. Alternatively, interfering with the conformational changes in the C2 and C3 domains could also lead to altering the quaternary structure of IgE [37,38]. If the IgE molecule could be locked into the bent structure, only one part of the site for receptor binding would be accessible and the interaction of FcεRI with C2 would be impossible. A peptide vaccine containing residues from the C4 domain, which form a salt bridge and several van der Waals interaction with residues Lys 497 and Ser 501 in C2 domain of the bent structure of IgE, decreases the ability of IgE to undergo conformational changes and prevents the C2 domain interactions required for effective receptor binding [39]. Thus targeting of these individual sites would likely result in an IgE molecule with a low affinity or with a significantly faster off-rate that would render it incapable of persistent mast cell sensitization and activation. This approach has already been tested in several ways. Among them the use of non-agonist antibodies that are capable of interfering directly with the IgE-binding site on the FcεRI α chain or with the FcεRI binding site(s) of the unbound IgE molecule is showing considerable promise in the treatment of allergies.

Humanized monoclonal anti-IgE antibody (rhuMab-E25) was engineered to prevent interaction of IgE with receptor [40,41]. This antibody has specificity for the receptor-binding region on the C3 domain of IgE, but is unable to cause aggregation of cell-bound IgE and thus fails to trigger mast cell activation. The half-life of rhuMab-E25 in serum is much longer than that of IgE and its affinity for IgE is sufficiently high to enable it to compete with and block soluble IgE binding to FcεRI. The anti-IgE-IgE complex formed is presumably removed by degradation since the levels of serum free IgE decreases to less than 10% of the pretreatment values within approximately 24 h of subcutaneous administration of the anti-IgE antibody. An important secondary benefit of continued anti-IgE administration is that the decrease in serum free IgE is accompanied by a reduction in FcεRI expression on mast cells and basophils. Therefore, this therapeutic approach not only reduces sensitivity of a mast cell to an allergen by reducing the levels of circulating IgE but also by reducing FcεRI expression.

Prevention of IgE-FcεRI interaction might also be achieved by targeting the IgE-binding sites on the FcεRIα subunit. The overall structure of the FcεRIα molecule resembles an inverted V shape (Fig. 1b). It is composed of two highly bent tandem immunoglobulin domains (D1 and D2) which presents a large convex surface for IgE ligand binding. There are two distinct binding sides for IgE. One on D2 domain around Tyr131 (binding side 1) and second, which includes four tryptophan residues, on the top of the receptor (binding side 2) [33]. A molecular inhibitor of FcεRI interaction with IgE should be able to contact both binding sides, especially the residues that mediate contact with IgE (Trp 87 and Tyr 131). It has been demonstrated that mutations of the aforementioned residues decreases the affinity of IgE binding [42,43]. Several peptide inhibitors were designed based on structure of D2 domains and demonstrated to inhibit IgE binding and mast cell degranulation in vitro [44,45]. However, these peptides mimic only a part of the IgE- FcεRIα interface and are not

able to compete effectively with high affinity IgE- Fc ϵ RI α interactions. Better success was obtained using larger portions of Fc ϵ RI α or a soluble Fc ϵ RI α that encodes the entire extracellular domain [46]. Soluble Fc ϵ RI α inhibited IgE binding to the cell surface and abrogated the mediator release from RBL-2H3 cells. Investigation of several animal models showed that soluble Fc ϵ RI α is able to completely abolish passive cutaneous anaphylaxis when administered to IgE sensitized rats [47]. It also suppressed recurrent allergic reactions and passive anaphylactic shock in IgE-sensitized mice when the appropriate antigen was introduced intravenously or orally [48-50]. Similarly to the anti-IgE antibody, soluble Fc ϵ RI α also suppressed IgE synthesis. Thus, soluble Fc ϵ RI α or a portion of the Fc ϵ RI α that binds IgE with high affinity might be a valuable therapeutic agent that not only inhibits mast cell sensitization but also suppresses IgE synthesis.

Fc ϵ RI PROXIMAL PROTEIN TYROSINE KINASES

As mentioned above Fyn, Lyn, and Syk are tyrosine kinases central to the early stages of mast cell activation and thus to degranulation (Fig. 2). Because of its central role in Fc ϵ RI phosphorylation one might predict that Lyn-deficiency would cause abrogation of mast cell degranulation. However, Lyn-deficient mast cells showed a hyperresponsive degranulation phenotype whereas Fyn- and Syk-deficient mast cells showed a defect in mast cell degranulation [20,51,52]. In fact, degranulation of Lyn-deficient mast cells is still dependent on the activity of other Src family kinases, as it can be inhibited by Src-selective inhibitors, like PP1 or PP2 [20,53]. Preliminary data suggests that Fyn activity is constitutively increased in Lyn-deficient cells (Sandra Odom and JR, unpublished observation) and thus would fit a model consistent with Fyn kinase as essential for degranulation [20]. Nonetheless, the high homology between members of Src family kinases, the ubiquitous expression of some Src kinases, as well as their important role in controlling cellular homeostasis and proliferative responses causes difficulty in specifically targeting only Fyn kinase in mast cells. Interestingly, and in apparent contradiction to the studies in Lyn-deficient mast cells, it was shown that overexpression of the Lyn unique domain (see Fig. 2), which weakly associates with Fc ϵ RI β [54], prevented association of Lyn with receptor, reduced receptor phosphorylation and decreased both histamine release and production of TNF- α [55]. While this apparent discrepancy is unresolved, the inhibition of Lyn association with Fc ϵ RI may differ from Lyn-deficiency by not affecting the negative feedback role that Lyn seemingly exerts on Fyn (Sandra Odom and JR, unpublished observation). Thus, because Fyn activity is not increased by inhibiting the interaction of Lyn with Fc ϵ RI, Lyn is still able to exert negative control on Fyn activity but, at the same time, fails to activate Syk because of the absence of Fc ϵ RI ITAM phosphorylation. Regardless, it remains to be seen whether loss of the interaction of Lyn with Fc ϵ RI is indeed inhibitory for mast cell degranulation as it may be possible that the unique domain of Lyn may inhibit Fyn-Fc ϵ RI interactions by a competitive mechanism. These experiments show that one may target the interactions of Src family kinases with Fc ϵ RI and possibly with other proteins and

that this inhibits the activation of mast cells and allergic response. It will be of interest to determine how Fyn interacts with Fc ϵ RI and whether inhibition of this interaction has some promise for therapeutic intervention in allergic disease.

The allergen-dependent activation of Syk requires the interaction of its two amino terminus SH2 domains with the tyrosine-phosphorylated ITAMs of the γ subunits of Fc ϵ RI (Fig. 2). This interaction induces a conformational change in Syk that results in autophosphorylation and activation. Because of Syk's central role in mast cell activation and degranulation the inhibition of Syk-ITAM interactions may be an effective approach towards intervention in the allergic response. In fact, oligomeric peptidomimetics based on the Fc ϵ RI γ ITAM that bind to the Syk SH2 domains have been developed [56], although their effectiveness remains to be determined. Fc ϵ RI γ ITAM-Syk interactions are of relatively high affinity and critical for mast cell activation but the importance of this interaction is less for Fc γ RI- and Fc γ RIIIA-mediated phagocytosis, where Syk also plays a critical role [57]. Thus, interfering with the interaction of Fc ϵ RI γ with Syk may hold promise for the development of a specific and effective therapeutic agent.

Small molecule inhibitors are ideally suited as therapeutic agents. The synthetic compound ER-27319 was described as a potent and selective inhibitor of allergen or anti-IgE-mediated degranulation of rodent and human mast cells [58]. ER-27319 pretreatment of mast cells had no effect on Lyn kinase activity nor on the allergen-dependent phosphorylation of the Fc ϵ RI. In contrast, it effectively inhibited the tyrosine phosphorylation of Syk and thus its activity. Interestingly, ER-27319 had no effect on activity of pre-activated Syk and did not bind directly to Syk. This suggested that ER-27319 was binding to the Fc ϵ RI γ ITAM (Fig. 2). Most importantly, pretreatment of Jurkat T cells with ER-27319 did not inhibit anti-CD3-induced tyrosine phosphorylation of ZAP-70 and PLC- γ 1. Additionally, anti-IgM-induced phosphorylation of Syk in human peripheral B cells was also unaffected by pretreatment with ER-27319. This suggests a unique selectivity for Fc ϵ RI ITAM's. These properties make this compound a good choice for further development as an anti-allergic agent.

Other small molecule inhibitors have been described that may serve as parental compounds for drug development, although their mechanism of action is less clear. Piceatannol, a hydroxystilbene, has been used as a selective inhibitor of Syk activation and mast cell degranulation and was demonstrated to effectively inhibit Syk activation but not Lyn activity in vitro [59]. However, a closely related analog resveratrol was shown to be an inhibitor of the Src family kinase, p56Lck. Given that we now know that other Src family kinases are important in Fc ϵ RI mast cell activation [20] the selectivity of piceatannol remains in question. Recent studies also suggest that piceatannol inhibits human basophil degranulation at very low concentrations that do not inhibit the activity of Syk as measured by the phosphorylation of downstream substrates of Syk [60]. Moreover, piceatannol also inhibits NF κ B activation through inhibition of I κ B α kinase and p65 phosphorylation in a Syk-independent manner [61]. Thus, while piceatannol appears to inhibit Syk at high

concentrations, its inhibitory effect on other targets may cause undesirable outcomes like immunosuppression in the treatment of allergic disease. Another small molecule inhibitor, 2,4,6-trihydroxy- α -p-methoxyphenylacetophenone (compound D-58), was shown to inhibit Fc ϵ RI-triggered mast cell-mediated allergic responses *in vitro* and *in vivo* [62]. Compound D-58 abrogated Syk and Btk activation as well as calcium mobilization, degranulation and leukotriene (LT) C (4) release in mast cells and vascular permeability in an *in vivo* model of passive cutaneous anaphylaxis. Even though the exact mechanism of compound D-58 inhibition is not known, these results show *in vitro* and *in vivo* efficacy and thus some potential as a parental compound.

ADAPTER MOLECULES IN THE ALLERGIC RESPONSE: TARGETS FOR THERAPY?

Among the many events that ensue upon activation of mast cells is the assembly of at least two macromolecular signaling complexes that function to coordinate the activity of multiple proteins required for the generation of second messengers and mast cell degranulation. These macromolecular complexes are scaffolded by proteins specialized as molecular adapters by virtue of motifs or domains (some of which can be post-translationally modified by phosphorylation) whose sole function is engendering the interaction with a recognized interaction motif on another protein. Numerous adapters have been shown to participate in IgE-dependent mast cell activation. These include proteins like 3BP [63], CLNK/MIST [64,65], SLAP-130/Fyb/ADAP [66,67], SKAP-55 HOM [68], and NTAL [69], some of which are known to contribute to mast cell degranulation. On the other hand, molecular adapters with an essential role in mast cell degranulation have also been described (Fig. 2). The adapters Gab2 [70] and LAT [71], scaffold the assembly of distinct macromolecular signaling complexes that form upon Fc ϵ RI stimulation of mast cells [21]. Another adapter molecule, SLP-76, occupies a central role in the LAT-scaffolded complex where it serves to bring together signaling molecules that participate in regulating calcium responses in mast cells, as well as in other cells [72-74]. Studies on genetically-altered mice deficient in expression of these adapters have shown that the absence of Gab2, LAT, or SLP-76 causes an *in vivo* abrogation of mast cell degranulation as measured by a systemic anaphylactic challenge. *In vitro* studies on mast cells derived from Gab2-, LAT-, or SLP-76-deficient mice showed a dramatic inhibition ($\geq 80\%$) of IgE-dependent mast cell degranulation. Interestingly, deficiency of these adapter molecules was also shown to cause a defect in lymphokine production in mast cells. This suggests that the function of these adapters is in the regulation of mast cell activation, thus, inhibition of their function might serve to intervene in allergic responses.

The therapeutic targeting of adapter molecules in mast cells is currently only an area of consideration because our knowledge of their function in mast cells is at its infancy. While Gab2, LAT, and SLP-76 are expressed primarily in hematopoietic cells, the expression of these molecules in monocytes/macrophages, platelets, and T cells presents

significant challenges in developing strategies to specifically intervene with their function in mast cells. Nonetheless, there is hope based on the findings that point to specific functions for these molecules in a given cell type. Thus, Gab2-deficient mice showed a specific loss of mast cells from the gut [75] whereas the tissue distribution of other cell types appeared to be normal. LAT-deficient mice showed a defect in the development of T cells, however, other cell types developed normally [76]. Platelet activation by integrins showed a greater dependence on SLP-76 function than on LAT function as SLP-76 is essential for platelet aggregation and, unlike LAT, is phosphorylated downstream of fibrinogen activation of the integrin α IIb β 3 [77]. Thus, understanding the particular role of adapter proteins in a given cell type may be an important step towards specifically intervening with their function in a given cell type.

A recent strategy in developing therapeutics is on the basis of understanding the function of a particular structural domain of a protein and determining whether this function is essential for the activity in which one wants to intervene. Studies aimed at understanding the function of structural motifs for adapter molecules are well underway. For example, mutational studies of four tyrosine residues of LAT that bind PLC γ , Grb-2, or Gads demonstrated that mutation of a single tyrosine (Y136F), which is essential for PLC γ binding, causes the abrogation of mast cell degranulation to a similar extent as seen in the absence of LAT or by mutation of all four tyrosine residues [78]. This suggests that targeting of the PLC γ binding site alone would be sufficient to stop mast cell degranulation. However, as this site serves a similar function in binding PLC γ in other cell types expressing LAT (T cells, monocyte/macrophage, platelets), intervention in PLC γ binding would likely have undesirable consequences. A more reasonable strategy might be to intervene in the ability of Fc ϵ RI to activate LAT phosphorylation and function (Fig. 2). Recent high resolution electron microscopic studies showed that LAT resides in plasma membrane regions that are distinct from those in which Fc ϵ RI is found [79]. Nonetheless, aggregation of Fc ϵ RI resulted in a rapid coalescence of LAT to form large plasma membrane regions where multiple LAT molecules are concentrated. Whether this concentration of LAT is required for LAT function, and how Fc ϵ RI aggregation could cause the concentration of LAT molecules, is presently unknown. However, this demonstrates the ability of Fc ϵ RI to communicate its aggregation into the concentration of LAT and also promotes the idea that intervention with Fc ϵ RI's ability to promote this step merits some consideration in inhibiting LAT function in an Fc ϵ RI-dependent manner. Yet, we have much to learn about the critical role of adapter molecules in mast cell degranulation since it is not clear as to why multiple adapters that scaffold distinct signaling complexes can individually show such a significant inhibitory effect on mast cell degranulation [20,21].

CALCIUM AND PROTEIN KINASE C

The requirement of increased intracellular calcium and activation of protein kinase C (PKC) for mast cell

degranulation has long been recognized [80-82]. The flux of calcium from intracellular stores is a consequence of allergen-mediated Fc ϵ RI aggregation [80,83]. However, the flux from intracellular stores alone is not sufficient to sustain mast cell degranulation and calcium influx from the extracellular environment must occur [80,83] (Fig. 2). It is clear that inhibiting calcium entry, by the use of lanthanum or by the depletion of extracellular calcium with EGTA, causes a complete abrogation of mast cell degranulation [80]. The postulation of store-operated calcium (SOC) channels [84], which open when the calcium stored in the endoplasmic reticulum is depleted, led to the demonstration of a similar calcium release-activated calcium I (Icrac) current in mast cells [85,86]. Dissodium cromoglycate (cromolyn), which is widely employed in treatment of allergic asthma, inhibits mast cell degranulation by a mechanisms that inhibits calcium uptake [87]. Cromolyn functions as an calcium channel blocker but shows no selectivity in blocking these channels versus chloride or potassium channels [88]. While reports of a cell surface cromolyn binding protein (CBP) as a calcium channel exist [89,90], this claim was not substantiated as the responsible protein was not identified though substantial efforts were made by various groups. Thus, identification of the Icrac channel responsible for mast cell degranulation remains a priority and its identification may likely provide another area of emphasis in the development of anti-allergic drugs.

The activation of PKC and its role in mast cell degranulation and cytokine production has also been extensively studied. This stems from the seminal observation that PKC activity and calcium synergized in stimulating mast cell degranulation and that either alone was insufficient to induce a normal degranulation response [81,82]. The discovery that PKC is a family of enzymes led to identification of two isoforms (β and δ) that were able to induce degranulation in vitro by PKC depletion and reconstitution experiments in permeabilized cells [82]. One of these isoforms (PKC δ) was also shown to phosphorylate Fc ϵ RI γ on a threonine residue within the ITAM [91] and this phosphorylation was demonstrated as critical for Syk activation and degranulation [92] (see Fig. 2). However, more recent analysis of mast cells derived from genetically disrupted mice provides confirmation of the importance of the β isoform for degranulation but contradictory evidence for the δ isoform. PKC β -deficient mast cells derived from null mice showed a significant (50%) inhibition in degranulation [93]. In contrast, PKC δ -deficient mast cells showed a hyperresponsive degranulation phenotype suggesting a negative regulatory role for this isoform in degranulation [94]. Although this latter finding appears to be contradictory to the previous studies [82,92,95] an explanation for the apparent discrepancy may lie in the role for PKC δ in Lyn activation [96]. Several studies show that PKC δ and Src kinases (like Lyn) interact and that these enzymes are capable of modulating each others activity [96,97]. Therefore, it is possible that the absence of PKC δ could result in the loss of Lyn activation and, as demonstrated in Lyn-deficient mast cells, the loss of Lyn results in a hyperresponsive degranulation phenotype [20]. It remains to be seen if PKC δ /Lyn interactions are critical for Lyn activity and for regulation of mast cell degranulation.

Regardless, the direct therapeutic targeting of PKC β or δ as an approach for intervention in allergies is not simple nor necessarily, on first impression, an effective approach. These PKC isoforms are ubiquitously expressed and thus specific mast cell targeting must be developed. Second, PKC β -deficient mast cells showed only a 50% inhibition of degranulation whereas PKC δ -deficient mast cells are hyperresponsive and thus targeting either isoform could be either non-effective or counterproductive. Third, both isoforms seemingly play a role in cellular homeostasis [98]. An alternative approach might be based on targeting the substrates of PKC β or δ that participate in degranulation. For example, the observation that threonine 60 (T60) in the cytoplasmic domain ITAM of Fc ϵ RI γ is a target of PKC δ and is required for normal Syk activation and degranulation [99] provides a possible and specific target for intervening in the activation of Syk (Fig. 2). As the amino acid sequence surrounding this threonine is specific to the Fc ϵ RI γ , one might expect that blocking its phosphorylation by PKC δ would prove effective in abrogating Syk activation and thus in the allergic response. This remains to be demonstrated.

THE MOLECULAR APPARATUS FOR GRANULE EXOCYTOSIS

The release of histamine and other allergic mediators stored in preformed granules found in the mast cell cytoplasm occurs by a process in which the granule membrane and the plasma membrane fuse, allowing extrusion of granule content [100]. Recent advances in the identification of molecular components involved in granule fusion has led to a rapid growth in our understanding of the molecular machinery responsible for the docking and fusion of granules with the plasma membrane [100]. Some of the discovered components functionally respond to increases in intracellular calcium and/or are targets of phosphorylation by PKC. In mast cells, proteins like munc 18-2 [101], rab3d [102], SCAMP2 [103], SNAP 23 [104], syntaxin 4 [105], and synaptotagmins [106] were experimentally implicated as part of the machinery that contributes to mast cell degranulation. These proteins are involved in vesicle trafficking and form part of the essential complex for vesicle docking and fusion. However, it is interesting to note that overexpression of these proteins or their functional domains results in only a modest inhibitory effect (30-50%) on the exocytotic response. This suggests the possibility of functional redundancy and thus might explain why multiple isoforms of many of these proteins are expressed in mast cells. Nonetheless, the effect of disruption of the respective genes, encoding these proteins, on the in vivo anaphylactic response and degranulation remains to be seen. Most of the molecules identified in mast cell granule docking and fusion are also expressed in other cell types. However, the growth in the knowledge of how exocytosis occurs has identified some apparent cell specificities. For example, the recent identification of a calcium-dependent kinase activity associated with the vesicle localized GTPase, rab3d, in mast cells, rat adipocytes and pancreatic cells, suggests that the induction of the granule fusion process has molecular components specific to secretory competent cells [107]. Moreover, the indirect coupling of rab3 proteins with specific calcium channels through the interaction of rab3-

interacting molecules (RIMs) with RIM binding proteins (RBPs) suggests that specificity may also exist in the interaction of a calcium channel with a given RBP or RIM, and possibly with a particular rab3 protein [108]. Nonetheless, our knowledge of the events leading to membrane-membrane fusion in mast cells is still elementary and only with further study will one be able to define if there are molecular events in this process amenable to therapeutic intervention in allergic disease.

CLOSING REMARKS

In brief we have summarized the current knowledge on IgE-mediated mast cell activation and how this knowledge affords hope for development of therapeutics in treatment of allergic disease. This review is not all inclusive and many additional investigative efforts with promise for development of therapeutics in allergic disease are underway. Nonetheless, based on the included material, it is evident that progress has been made in understanding mast cell activation and that this progress bodes well for the future of rational drug design. One reasonable expectation is that better management of allergic disease will be a near future outcome of the gain in knowledge.

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