

Signal Transduction by the High-Affinity Immunoglobulin E Receptor Fc ϵ RI: Coupling Form to Function

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I. Introduction

Mast cells are activated by cross-linking of the high-affinity Fc ϵ RI receptor, initiated by the binding of multivalent antigen to prebound IgE molecules in the receptor complex. Once activated, mast cells elicit three types of biological responses: (i) secretion of preformed granules, (ii) synthesis and secretion of cytokines, and (iii) synthesis of lipid mediators. These responses form the basis of one of the most potent effector systems in immunity—immediate hypersensitivity—which includes, in the most extreme cases, anaphylaxis. The speed and efficiency of this immune response are facilitated by immunoglobulin E (IgE) antibody, which is prebound by its Fc portion to the Fc ϵ RI receptor in a 1:1 ratio with high affinity ($K_d = 10^{-9}$ to 10^{-10}) (Keown *et al.*, 1998; Metzger, 1992, 1999).

The high-affinity Fc ϵ RI receptor is expressed on the surface of mast cells and basophils as a multimeric $\alpha\beta\gamma_2$ complex (Blank *et al.*, 1989; Kuster *et al.*, 1992; Ra *et al.*, 1989b). The α -chain, responsible for binding to the Fc portion of IgE, is extensively N-glycosylated and contains two extracellular Ig-related domains, a transmembrane region and a short cytoplasmic tail which has no signaling capacity (Alber *et al.*, 1991; Kinet *et al.*, 1987; Kochan *et al.*, 1988; Letourneur *et al.*, 1995; Ra *et al.*, 1989b; Shimizu *et al.*, 1988). The β -chain, which consists of four transmembrane domains with cytoplasmic N- and C-termini (Kinet *et al.*, 1988; Kuster *et al.*, 1992; Ra *et al.*, 1989b), and a disulfide-linked γ -chain homodimer constitute the signal-transducing portion of the receptor (Fig. 1) (Blank *et al.*, 1989; Kuster *et al.*, 1990; Ra *et al.*, 1989b). Upon antigen challenge, the receptors are cross-linked and the β - and γ -chains play a central role in the initiation of signaling cascades by serving as phosphoacceptor sites for intracellular tyrosine kinases.

The high-affinity Fc ϵ RI receptor belongs to the Ig superfamily (Kinet, 1999; Metzger, 1991; Ravetch and Kinet, 1991; Turner and Kinet, 1999). The modular nature of the related Fc receptor and antigen receptor structures is evidenced by the presence of similar phosphoacceptor sequences found in the cytoplasmic tails of the receptor chains known as immunoreceptor tyrosine-based activation motifs (ITAMs) Fig. 1 (Cambier, 1995a,b;

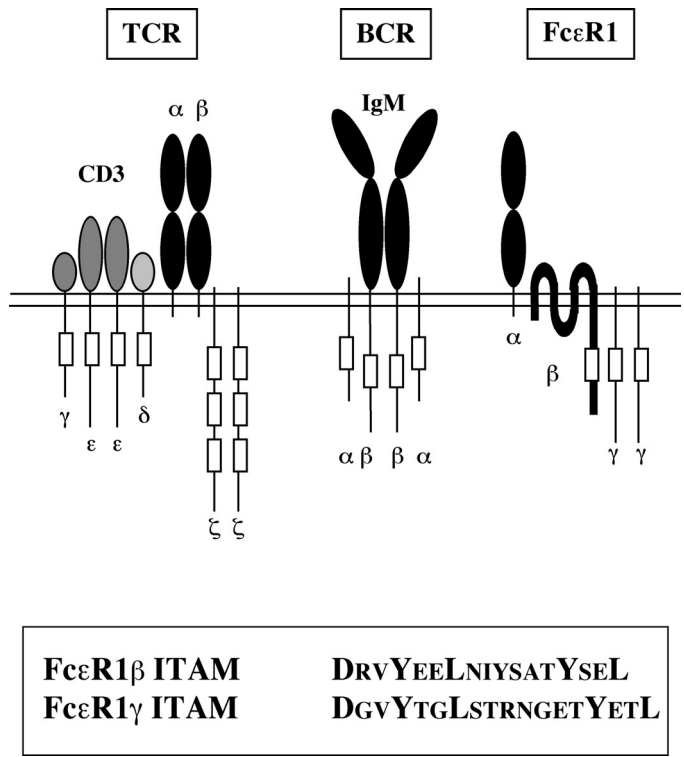


FIG. 1. ITAM-containing immunoreceptors. The tetrameric FcεR1 is shown in the context to related immunoreceptor family members. The sequences of the FcεR1 β- and γ-chain ITAMs are also shown with canonical residues in bold type. TCR, T cell antigen receptor; BCR, B cell antigen receptor; IgM, immunoglobulin M; ITAM, immunoreceptor tyrosine-based activation motif. White boxes represent ITAMs; black ovals represent immunoglobulin domains.

Daeron, 1997; Isakov, 1997; Reth, 1989). Signals generated from these related antigen receptors involve many of the same secondary molecules found in FcεR1 cascades and result in changes in calcium flux, gene activation, and cellular activation. Furthermore, the β- and γ-chains of FcεR1 are also shared by other receptor complexes. The β-chain is present as a subunit of the low-affinity IgG receptor (FcγRIII) (Dombrowicz *et al.*, 1998; Kurosaki *et al.*, 1992), while the γ-chain is found in FcγRIII, FcγRI, FcαR, and the T cell antigen receptor (TCR) (Anderson *et al.*, 1990; Ernst *et al.*, 1993; Hibbs *et al.*, 1989; Kinet, 1992; Kurosaki and Ravetch, 1989; Lanier *et al.*, 1989; Morton *et al.*, 1995; Orloff *et al.*, 1990; Pfefferkorn and Yeaman, 1994; Ra *et al.*, 1989a; Scholl and Geha, 1993).

Overall, these similarities in structure and the presence of β - or γ -chains in other complexes suggest redundancies in the function of ITAMs in other cell types.

II. Signal Initiation: The Central Importance of ITAM

The FcεRI receptor does not contain intrinsic catalytic activity. Instead, it associates with nontransmembrane protein tyrosine kinases (PTKs) that phosphorylate tyrosine residues found in the ITAMs of β - and γ -chains (Bolen, 1995; Daeron *et al.*, 1995; Paolini *et al.*, 1991). The ITAM is present in both the β - and γ -chains and is centrally important to signal initiation (Fig. 1). Phosphorylated tyrosine residues within the ITAMs serve as docking sites for secondary signaling proteins, which in turn propagate the signal, leading to increases in cellular tyrosine phosphorylation, calcium flux, secretion, and gene activation. Mutation of the tyrosine residues in the β - and γ -chain ITAMs abolishes signaling (Jouvin *et al.*, 1994; Letourneur and Klausner, 1991; Lin *et al.*, 1996). Furthermore, experiments have shown that the ITAM sequence of the γ -chain can be attached to unrelated extracellular domains and engaged to activate intracellular signaling cascades (Letourneur and Klausner, 1991; Romeo and Seed, 1991). Taken together, these studies show that the ITAM portions of β - and γ -chains are required for FcεRI signaling.

Although there are many similarities among ITAMs, there are structural differences and nuances in the FcεRI complex that may contribute to the unique signaling events mediated by this receptor. Importantly, the β - and γ -chain ITAMs differ: the β -chain has a shorter spacing region between the first and second tyrosines of the consensus motif and contains an additional third tyrosine that the γ -chain ITAM lacks (Fig. 1). There are also functional differences between the β - and γ -chains. Experiments have shown that the γ -chain alone is sufficient to initiate FcεRI signaling cascades (Letourneur and Klausner, 1991; Romeo and Seed, 1991). Despite the fact that the β -chain also contains an ITAM, chimeras of the β -chain do not signal efficiently (Jouvin *et al.*, 1994). Instead, the β -chain serves as an amplifier of γ -chain signaling (Dombrowicz *et al.*, 1998; Lin *et al.*, 1996). The unique structural features of the β -chain ITAM may contribute to this novel function. Indeed, LYN preferentially associates with the β -chain compared to the γ -chain, which suggests a molecular explanation for its role as an amplifying molecule: the addition of more LYN within the receptor complex by β -chain could increase tyrosine phosphorylation events of several substrates, which in turn would lead to a greater activation signal. Further structural features of the β -chain may also contribute to its unique function, since truncation of the non-ITAM-containing N-

terminal tail of the β -chain diminishes its amplifying potential (Lin *et al.*, 1996). To date, this amplifier property of the β -chain is unique among antigen receptors.

III. Phosphate Transfer and PTKs

In the context of a receptor that lacks intrinsic catalytic activity, such as Fc ϵ RI, the initiation of an antigen receptor signal results in the recruitment of secondary signaling molecules through inducible protein–protein interactions. The phosphorylated tyrosine residues in the ITAMs act as docking sites for Src homology 2 (SH2) domain–containing proteins. Specifically, tyrosine phosphorylation of the Fc ϵ RI γ -chain ITAM results in the recruitment of SYK. The subsequent recruitment of further secondary signaling molecules also involves SH2 domains. Some SH2 domain–containing proteins are enzymes, some are transcription factors, and other are adapters that lack intrinsic catalytic activity and consist mainly of SH2 and SH3 domains, which bind to proline-rich regions. Various adapter proteins that contain both SH2 and SH3 domains are critical in linking signaling molecules together. These interactions result in increased tyrosine phosphorylation events and in increased enzymatic activity of secondary signaling proteins at or near the receptor complex. In addition, protein–lipid interactions are also essential for signal propagation and are mediated in part by pleckstrin homology (PH) domains, which bind directly to lipids, localizing signaling proteins to the plasma membrane. Overviews of Fc ϵ RI receptor proximal signaling events and the signaling molecules involved are shown in Figs. 2 and 3.

A. LYN AND SYK

Receptor aggregation establishes the earliest events in Fc ϵ RI signaling, which involve activation of two tyrosine kinases that act in a two-step process to initiate the signal. Several studies have come together to establish the following sequence of events. The Src family PTK LYN is the earliest kinase activated by Fc ϵ RI receptor engagement (Eiseman and Bolen, 1992). LYN associates with the β -chain and phosphorylates tyrosine residues within the ITAMs of both β - and γ -chains upon activation (Jouvin *et al.*, 1994; Paolini *et al.*, 1991; Yamashita *et al.*, 1994). SYK then binds to the receptor complex at the phosphorylated ITAMs of the γ -chain via its SH2 domain, where it is further activated by LYN (Scharenberg *et al.*, 1995). The phosphorylation of SYK by LYN fully activates SYK, and it has been demonstrated that these tyrosine phosphorylation events, which occur in the activation loop of SYK, are essential for intracellular signaling (El-Hillal *et al.*, 1997; J. Zhang *et al.*, 1998). The position of LYN upstream

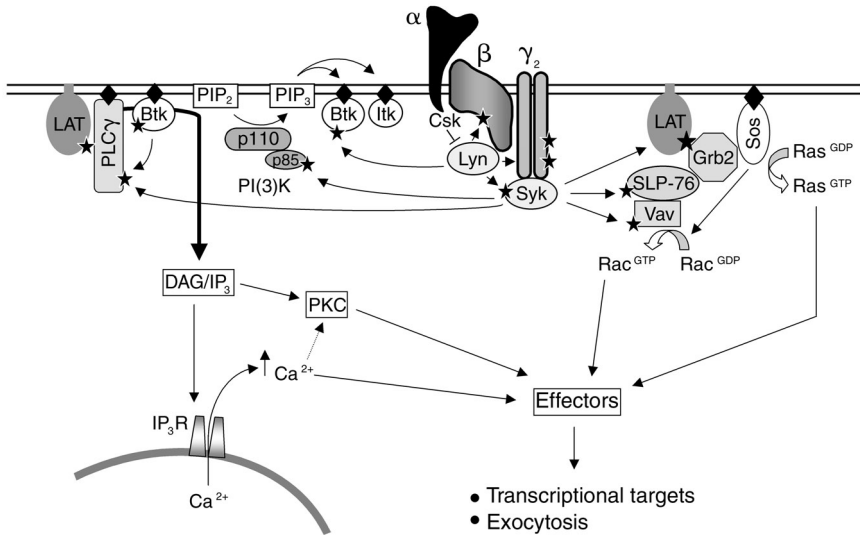


FIG. 2. FcεRI activation induces a complex network of proximal signaling events. FcεRI aggregation is induced by cross-linking of bound immunoglobulin E by polyvalent antigen. Information is then passed to the β and γ signaling chains of the receptor, activating intracellular signaling pathways that control mast cell effector functions, ultimately leading to allergic responses. LYN phosphorylates the β - and γ -chain ITAMs, recruiting SYK, which is in turn phosphorylated and activated by LYN. LYN and SYK phosphorylate and activate a number of adapter proteins and protein tyrosine kinases, including LAT, the p85 regulatory subunit of PI3K, and the Tec family kinase BTK (Bruton's tyrosine kinase). FcεRI activation of phosphatidylinositol-3-kinase (PI3K) elevates plasma membrane phosphatidylinositol-3,4,5-trisphosphate (PIP₃) levels, recruiting pleckstrin homology (PH) domain-containing proteins such as BTK to the plasma membrane. BTK contributes to the activation of phospholipase C γ (PLC γ), which in turn is localized to the membrane through the interaction of its Src homology 2 domain with the phosphorylated LAT adapter. PLC γ metabolizes the membrane inositol phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which regulate calcium mobilization and activate members of the protein kinase C (PKC) family of serine-threonine kinases, respectively. IP₃ binds to the IP₃ receptors (IP₃R) on the surface of endoplasmic reticulum calcium stores, leading to store depletion and elevation of cytosolic calcium levels. Activation of the FcεRI also regulates signaling pathways controlled by GTPase proteins. The phosphorylated LAT adapter molecule anchors the Grb2 and SLP-76 adapters, which in turn couple to guanine nucleotide exchange factors for Ras and Rac GTPases (Sos and Vav, respectively). GTP loading of these GTPases induces a wide range of effector responses, including activation of various transcription factors and changes in cell morphology. Csk, c-Src kinase. Black diamonds represent PH domain-phospholipid interactions; stars indicate phosphorylation events.

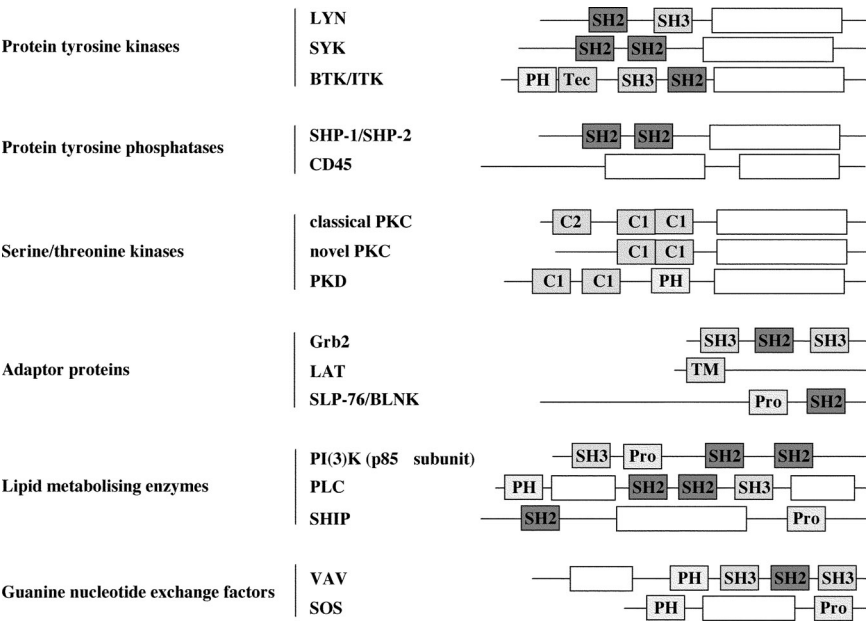


FIG. 3. Molecules involved in FcεRI signaling pathways. Schematic representation of the signaling molecules discussed in the text, depicted to highlight their modular structures. Domains the enzymatic or nucleotide exchange factor activity are shown in open rectangles. C1, cysteine-rich domain (diacylglycerol binding); C2, calcium binding domain; PH, pleckstrin homology domain (lipid interactions); PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PKD, protein kinase D; PLCγ, phospholipase Cγ; Pro, proline-rich domain (SH3 binding); SH2, Src homology 2 domain (phosphotyrosine binding); SH3, Src homology 3 domain (proline-rich region binding); Tec, Tec kinase homology domain; TM, transmembrane domain.

of SYK has been confirmed by genetic studies: LYN^{-/-} mast cells exhibit neither β nor γ phosphorylation and therefore do not recruit SYK to the γ-chain following receptor activation (Nishizumi and Yamamoto, 1997). Importantly, the overall requirement for LYN in FcεRI signaling is illustrated by the finding that IgE-mediated systemic anaphylaxis, a measure of FcεRI function (Dombrowicz *et al.*, 1993), is diminished in LYN-deficient mice (Hibbs *et al.*, 1995). Furthermore, LYN-deficient mast cells have decreased calcium mobilization and tyrosine phosphorylation events (Nishizumi and Yamamoto, 1997). Taken together, these results demonstrate that LYN is indispensable in the FcεRI pathway. SYK is also essential for mast cell activation: in SYK^{-/-} mast cells β- and γ-chain phosphorylation events are intact, but downstream events are abrogated (Costello *et al.*, 1996; Zhang *et al.*, 1996).

The structures of the FcεRI β - and γ -chain ITAMS are distinct. However, the similar roles of ITAMs within the signaling chains of other receptors would suggest a parallel redundant function. Indeed, engagement of these related receptors initiates similar signaling cascades that utilize the same or related signaling molecules. Interestingly, there is genetic evidence for a differential role for LYN in FcεRI versus B cell antigen receptor (BCR) signaling even though both antigen structures share a great deal of similarity. In B cells, the BCR complex is required for B cell activation events that lead to proliferation and secretion of antibodies. LYN plays an initiating role for the BCR similar to the role it plays for FcεRI: it is one of the earliest PTKs activated, and it phosphorylates and activates several positive signaling molecules, including SYK (Kurosaki, 1997; Yamamoto *et al.*, 1993). In addition, LYN participates in the activation of Bruton's tyrosine kinase (Park *et al.*, 1996; Rawlings *et al.*, 1996). However, in B cells, LYN has also been implicated in the phosphorylation of the BCR co-receptor CD22, which recruits the protein tyrosine phosphatase (PTP) SHP-1 (Smith *et al.*, 1998). Interestingly, B cells from LYN-deficient mice show a hyperresponsive phenotype: these mice are IgM hyperglobulinemic (i.e., have increased serum IgM levels) and develop severe glomerulonephritis (Hibbs *et al.*, 1995; Nishizumi *et al.*, 1995). These abnormalities result from the loss of LYN's function as a negative regulator of BCR activation signals. The negative signaling function of LYN is dependent on CD22 and is further compounded by loss of SHP-1 (Cornall *et al.*, 1998). Therefore, in B cells, the predominant function of LYN is the phosphorylation of the co-receptor CD22, which results in subsequent recruitment of SHP-1 into the vicinity of the BCR. In contrast, LYN does not dominantly function as a negative regulator of FcεRI signaling in mast cells.

The opposing effects of LYN in mast cells versus B cells are an example of how the molecular and cellular context determines whether the action of a PTK is positive or negative. Importantly, the differences between the related antigen receptor complexes should be understood to appreciate the impact that specific mutations can have. This is especially true for diseases with complex genetic origins in which there may be several mutations or differential effects caused by the same mutation in different cell types.

B. BRUTON'S TYROSINE KINASE

Another PTK activated by antigen receptor engagement is Bruton's tyrosine kinase (BTK). BTK is a member of the Tec family of tyrosine kinases, which are distinguished by the presence of a PH domain at the N-terminus, an SH2 domain, and an SH3 domain (Fig. 3). PH domains

are important in binding to lipids and thus link the critical upstream protein tyrosine phosphorylation events with lipid metabolism, and in the case of BTK, interaction with lipid localizes it to the antigen receptor complex. Several studies have unraveled the role of BTK in antigen receptor signaling. First, BTK is targeted to the membrane via binding of its PH domain to the lipid phosphatidylinositol-3,4,5-trisphosphate (PIP₃) (Fluckiger *et al.*, 1998; Scharenberg and Kinet, 1998); which is produced by the action of phosphatidylinositol-3-kinase (PI3K) upon receptor engagement. Second, this brings BTK into the vicinity of LYN, which further phosphorylates BTK, providing full activation (Park *et al.*, 1996; Rawlings *et al.*, 1996). Finally, in turn, BTK phosphorylates and contributes to the activation of phospholipase C γ_1 (PLC γ_1), resulting in calcium mobilization (Scharenberg and Kinet, 1998). The importance of this pathway has been illustrated in chicken B cell lines that lack BTK. These cells fail to phosphorylate PLC γ or release calcium upon BCR stimulation, indicating that BTK plays an essential role in antigen receptor signaling, linking upstream phosphorylation events with calcium release (Takata and Kurosaki, 1996).

Genetic analysis has also demonstrated the function of BTK in Fc ϵ RI signaling. X-linked immunodeficient (Xid) mice are characterized by a mutation in BTK that lowers its affinity for PIP₃, decreasing BTK activation (Rawlings *et al.*, 1993). In these mice, B cell development is arrested at the pre-B cell stage, with resulting hypo- or agammaglobulinemia (Conley *et al.*, 1994). In contrast, mast cell development from both Xid mutant and BTK-deficient mice appears to be normal (Hata *et al.*, 1998). However, BTK-deficient mast cells show decreased IgE-mediated anaphylactic responses, impaired degranulation, and reduced cytokine production (Hata *et al.*, 1998). These data suggest that the role BTK plays in Fc ϵ RI signaling is similar to its role in BCR signaling. However, there may be some differences in the dependence on BTK in mast cells. Mice deficient for both BTK and the related Tec family member ITK have a more severe Fc ϵ RI signaling deficiency than that found in single BTK-deficient mice (F. W. Alt, personal communication), suggesting functional redundancy between BTK and ITK in mast cells. The lesser dependence on BTK in Fc ϵ RI signaling in mast cells than in antigen signaling in B cells illustrates that overlapping specificities can lead to differential functional effects upon mutation or ablation of given signaling molecule in different cell types.

IV. Phosphate Transfer and PTPs

Phosphate transfer is a bidirectional process, with the removal of phosphate as important to the regulation of protein function as the addition of phosphate. Importantly, PTPs are not solely antagonists of PTKs. Although

some PTPs oppose PTK action by dephosphorylation of PTKs, PTPs can act as positive or negative regulators in any given pathway on the basis of which phosphotyrosine residues they target. For example, the PTP CD45 is viewed as a positive regulator since its activity is required to dephosphorylate Src family PTKs for the initiation of antigen receptor signaling. In contrast, the SH2 domain-containing PTP-1 (SHP-1) is viewed as a negative regulator, as illustrated by the phenotype of *motheaten* (SHP-1 mutant) mice, in which there are multiple defects in myeloid and lymphocyte populations, leading to hyperproliferation and eventual death (reviewed in Bignon and Siminovitch, 1994; Neel, 1993; Tsui and Tsui, 1994). The diverse impact of the PTPs on cellular signaling should thus be viewed as a regulatory mechanism that is separate but related to that of the PTKs. Like tyrosine phosphorylation, tyrosine dephosphorylation can be activating or deactivating, depending on the molecular context.

The roles of PTPs in mast cells, specifically those involved in FcεRI signaling, are not well characterized. To date, only a few studies have focused on this important but relatively unexplored area of mast cell research. Although well over 70 mammalian PTPs have been identified, only a few have been implicated in immune receptor signaling. There are likely to be more PTPs involved in FcεRI signaling cascades, and as sequencing of the human genome and deduction of coding sequences proceed at an unprecedented pace, the exact size of the PTP family will become evident.

A. CD45

The transmembrane PTP CD45 was one of the first PTPs to be identified and characterized. It contains a large extracellular domain that is variable because of alternate splicing and two tandem intracellular PTP domains, of which only the N-terminal domain shows significant activity. The function of the C-terminal PTP domain is unclear, although it may participate in binding to various signaling molecules (Mustelin *et al.*, 1998; Plas and Thomas, 1998). In addition to a large body of CD45 literature from *in vitro* and *in vivo* studies, the overall importance of CD45 to immune receptor signaling is underscored by the recent report of a human severe combined immunodeficiency syndrome associated with mutation of CD45 (Kung *et al.*, 2000). Classically, the role of CD45 in lymphocytes has been viewed as that of a positive regulator: CD45 opposes the action of c-Src kinase (CSK) by dephosphorylating Src family PTKs on their negative regulatory site. This allows the activation of Src family PTKs, initiating antigen receptor signaling. This mechanism appears to be the same for the FcεRI receptor: studies have shown that inhibiting the function of CD45 with blocking antibodies prevents the release of histamine from activated basophils (Hook *et al.*, 1991). In addition, CD45-deficient mast

cells show defective Fc ϵ RI-induced histamine release and are resistant to IgE-mediated systemic anaphylaxis (Berger *et al.*, 1994). Furthermore, adequate PTK activity in Jurkat T cells that have been reconstituted with Fc ϵ RI requires the presence of CD45 (Adamczewski *et al.*, 1995). Another recent study has also indicated a role for CD45 in the positive regulation of Fc ϵ RI-induced LYN activity (Murakami *et al.*, 2000). Taken together, these results indicate that CD45 plays an important positive role in Fc ϵ RI signaling.

B. SHP-1

In contrast, the SH2-containing PTP SHP-1 has been implicated as a negative regulator of several hematopoietic signaling pathways and in setting the antigen receptor threshold for B and T cell antigen signals (reviewed in Long, 1999; Plas and Thomas, 1998; Siminovitch and Neel, 1998). SHP-1 has a single phosphatase (PTP) domain with two N-terminal SH2 domains and a C-terminus that contains several tyrosine phosphorylation sites. The SH2 domains of SHP-1 bind to phosphotyrosine-containing acceptor motifs located in the cytoplasmic tails of several transmembrane inhibitory receptors. These motifs, known as immunoreceptor tyrosine-based inhibition motifs (ITIMs) serve to target SHP-1 into the vicinity of cellular substrates (Bolland and Ravetch, 1999; Daeron, 1997). Once activated, SHP-1 dephosphorylates target molecules involved in antigen receptor signaling. There is a natural SHP-1 mutant mouse, *motheaten*, which has mutations in SHP-1 that either abrogate its expression or impair its catalytic function (Shultz *et al.*, 1993; Tsui *et al.*, 1993). The overall importance of SHP-1 in lymphocyte signaling is well illustrated by the phenotype of this mouse, which shows prominent abnormalities in many lymphocyte- and myeloid-derived populations (reviewed in Bignon and Siminovitch, 1994; Neel, 1993; Tsui and Tsui, 1994). However, the specific roles of SHP-1 in Fc ϵ RI-mediated mast cell function are only beginning to be explored.

One useful way to probe PTP function is to overexpress catalytically inactive forms of the enzyme that retain the ability to bind to and thus “trap” substrates. Such “trapping mutants” have been described comprehensively, but one caveat of their use is that they can have the unanticipated consequences, including acting as dominant positives by sequestering phosphorylated substrates (Siminovitch and Neel, 1998). SHP-1 has been implicated in Fc ϵ RI signaling with the use of such trapping mutants. Although expression of SHP-1 trapping mutants in mast cells results in increased tyrosine phosphorylation of β , γ , and SYK, suggesting a negative signaling role for SHP-1, these changes do not correlate with enhanced downstream signaling events (Xie *et al.*, 2000). Moreover, the identities of SHP-1 substrates in

mast cells are unknown, although studies in T and B cells have implicated LYN, SYK, and ZAP-70 as substrates of SHP-1 (Dustin *et al.*, 1999; Lorenz *et al.*, 1996; Plas *et al.*, 1996; Somani *et al.*, 1997; Yang *et al.*, 1998). Further work is required to untangle the complexities of SHP-1 action in mast cells.

Many questions remain regarding SHP-1 function, and the complex nature of SHP-1 signaling may be even more involved than originally envisioned. It is interesting to note that a splice variant of SHP-1 was recently isolated from Jurkat T cells (Jin *et al.*, 1999). This variant, SHP-1L, differs from SHP-1 at the C-terminus. The last C-terminal 66 amino acids are different, and the enzyme is 29 amino acids longer. Interestingly, SHP-1L shows increased catalytic activity at physiological pH compared to SHP-1, suggesting that it has a unique role in basal signaling. Although the function of this variant is unknown, the unique features of its altered C-terminus may confer a difference in substrate specificity or localization. It will be interesting to determine whether mast cells utilize SHP-1L or a similar isoform in the FcεRI pathway.

C. OTHER PHOSPHATASES

The related PTP, SHP-2, is also expressed in mast cells and was initially reported to associate with the FcεRI, but subsequent plasmon resonance studies have shown that this is a very low-affinity interaction (Ottinger *et al.*, 1998). It is therefore unclear what role SHP-2 plays in FcεRI signaling. There have also been reports of other PTP activities that are activated upon FcεRI engagement (Mao and Metzger, 1997; Swieter *et al.*, 1995). Cloning and characterization of these PTPs will be of great interest.

V. Other Signaling Molecules

In antigen receptor signaling, coupling of FcεRI proximal phosphorylation events to downstream events is accomplished by various adapter molecules that link these initial events with calcium flux and mitogenic signaling. Recent studies have demonstrated that PLCγ phosphorylation represents an important regulatory point for the control of calcium flux, and several adapter proteins are now clearly implicated in linking upstream PTK activity to PLCγ phosphorylation and activation in various cell types. Genetic evidence for adapter proteins involved in FcεRI signaling has emerged. In addition, the requirement for VAV in FcεRI signaling has been reported.

A. THE ADAPTER PROTEIN LAT

The protein LAT (linker for activation of T cell), which was originally defined in T cells, is essential for FcεRI signaling. LAT is a 36- to 38-kDa

protein that serves as a critical link between activated PTKs and downstream substrates (W. Zhang *et al.*, 1998a). In T cells, LAT becomes heavily tyrosine phosphorylated upon TCR activation, and it has been reported to bind to the SH2 domains of multiple signaling proteins, including Grb2, Gads, Grap, PLC γ , and the p85 subunit of PI3K (Schraven *et al.*, 1999). Mice deficient for LAT are resistant to IgE-mediated passive systemic anaphylaxis, which illustrates the importance of LAT to Fc ϵ RI signaling (Saitoh *et al.*, 2000). In addition, LAT-deficient bone marrow-derived mast cells display decreased Fc ϵ RI-induced tyrosine phosphorylation of SLP-76 and PLC γ , show reduced calcium mobilization, and have defective MAP kinase activation, whereas tyrosine phosphorylation of the β - and γ -chains, SYK, and VAV remains unaltered. Fc ϵ RI-mediated effector functions of degranulation and cytokine production are also diminished in LAT^{-/-} mast cells. Therefore, LAT links the upstream kinase phosphorylation events to calcium signaling via PLC γ in an essential manner required for effector function. An analogous role for LAT also occurs in T cells, where it has been shown to be essential for T cell development and TCR-mediated activation (Finco *et al.*, 1998; Zhang *et al.*, 1999a,b).

B. THE SLP-76 ADAPTER FAMILY

The SH2 domain-containing protein of 76 kDa (SLP-76) is a member of a family of adapter proteins expressed in leukocytes (Jackman *et al.*, 1995). Characterized members of this family are SLP-76 itself, which is expressed in T cells, mast cells, natural killer cells, and platelets, and BLNK (also known as SLP-65/BASH), which is expressed exclusively in B cells (Fu *et al.*, 1998; Goitsuka *et al.*, 1998; Jackman *et al.*, 1995; Wienands *et al.*, 1998). A newer family member, known as cytokine-dependent hematopoietic cell linker, CLNK, is also expressed in cytokine-stimulated hematopoietic cells and is thought to be involved in cross-talk signals between interleukin 2 (IL-2) and IL-3 cytokines and antigen receptors (Cao *et al.*, 1999). This family of adapter proteins is characterized by a common primary structure that includes a C-terminal SH2 domain, multiple N-terminal tyrosine phosphorylation sites, and a central proline-rich region. SLP-76, which was initially characterized as a PTK substrate in T cells, is essential for T cell development (Clements *et al.*, 1998; Pivniouk *et al.*, 1998). It is a substrate for ZAP-70 and SYK and, once phosphorylated, binds to VAV, Gads, Nck, and other proteins, including SLAP-130 (SLP-76-associated phosphoprotein of 130 kDa) (da Silva *et al.*, 1997; Law *et al.*, 1999; Liu *et al.*, 1999; Musci *et al.*, 1997a; Tuosto *et al.*, 1996; Wu *et al.*, 1996; Wunderlich *et al.*, 1999). SLP-76 has been implicated in PLC γ phosphorylation, calcium mobilization, MAP kinase activation, and IL-2 production, as demonstrated by the analysis of SLP-76^{-/-} Jurkat cells (Yablonski *et al.*,

1998). In addition, transient overexpression of SLP-76 augments IL-2 production in wild-type Jurkat cells (Musci *et al.*, 1997b). Importantly, loss of SLP-76 arrests T cell development, resulting in an absence of CD4⁺8⁺ lymphocytes (Clements *et al.*, 1998; Pivniouk *et al.*, 1998). Together, these results demonstrate that SLP-76 is critical in linking upstream PTK events to PLCγ phosphorylation and subsequent downstream signaling events in T cells. In addition, the B cell-specific member of the SLP-76 family, BLNK, has also been implicated in linking upstream PTK activation events to PLCγ phosphorylation and calcium release. Notably, BLNK-deficient B cell lines show reduced PLCγ phosphorylation, lowered inositol-1,4,5-trisphosphate (IP₃) production, decreased calcium flux, and reduced c-Jun NH₂-terminal kinase (JNK) activation (Ishiai *et al.*, 1999). Moreover, BLNK-deficient mice exhibit defects in B cell development and BCR-mediated calcium mobilization and proliferation (Hayashi *et al.*, 2000; Jumaa *et al.*, 1999; Pappu *et al.*, 1999). Overall, these observations indicate a similar function for this family of adapters: SLP-76 and BLNK link PTK activation events to PLCγ phosphorylation and subsequent calcium release.

SLP-76 has also been implicated in playing a vital role in FcεRI mast cell signaling. SLP-76-deficient mast cells appear to develop normally and are present in normal numbers in skin and bronchi. This suggests that SLP-76 is not required for mast cell development as it is for T cell development. Similar to LAT-deficient mice, SLP-76^{-/-} mice are resistant to IgE-mediated passive anaphylaxis (Pivniouk *et al.*, 1999). Furthermore, bone marrow-derived mast cells from SLP-76 mice show reduced PLCγ phosphorylation, demonstrate reduced calcium mobilization, and have defective degranulation responses (Pivniouk *et al.*, 1999). These results indicate that SLP-76 plays an important role in FcεRI signaling in mast cells.

C. VAV

VAV, a substrate of PTKs in antigen receptor-activated T cells, B cells, and mast cells, is essential for coordinating upstream signals to downstream cellular events. VAV functions as a guanine nucleotide exchange factor and regulates the activity of the small GTPase Rac1, promoting the transition from the inactive GDP-bound state to the active GTP-bound form. There are two VAV proteins, VAV1 and VAV2, and they have an overall structure that includes a PH domain, a Dbp domain, a catalytic region specific for Rho family GTPases, one SH2 domain, and two SH3 domains (Cantrell, 1998). VAV contributes to efficient calcium signaling in lymphocytes, as illustrated by the phenotype of VAV-deficient mice which show perturbed antigen receptor-induced calcium mobilization (Fischer *et al.*, 1998; Holsinger *et al.*, 1998; O'Rourke *et al.*, 1998; Turner *et al.*, 1997). In B cells, recruitment of VAV1 to the plasma membrane via the coreceptor

CD19 appears to regulate this process (O'Rourke *et al.*, 1998). VAV has additional functions that are mediated by its various protein binding domains, and its overall importance is underscored by VAV1-deficient mice that have a number of abnormalities in T and B cell development (Turner *et al.*, 1997).

In bone marrow-derived mast cell cultures from VAV-deficient mice, early signaling events are not perturbed, since SYK, LAT, and PLC γ ₁ tyrosine phosphorylation is equivalent to that of wild type-derived cells (Manetz *et al.*, 2000). However, VAV is required for mast cell degranulation and cytokine production (Manetz *et al.*, 2000). Although the signaling mechanism responsible for these alterations is currently unknown, VAV appears to be required for efficient Fc ϵ RI signaling.

VI. Integration of Lipid-Protein Interactions and Calcium Flux

Engagement of antigen receptors leads to an increase in cellular calcium levels that is required for many cellular processes leading to secretion and gene transcription. Upon receptor activation, the calcium that is released from intracellular stores is in a finite amount and is quickly depleted. However, many cellular responses require a sustained calcium flux that is maintained by the influx of extracellular calcium through plasma membrane channels. Emptying of intracellular stores drives the influx of extracellular calcium in a process known as store-operated calcium influx. The plasma membrane channels that underlie calcium influx are currently unknown.

Importantly, there are two aspects of antigen receptor-mediated calcium flux that involve lipid metabolism: IP₃ and PIP₃. First, phosphorylation of PLC γ by SYK is required for its activation, resulting in the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP₂) into IP₃ and diacylglycerol (DAG). IP₃ generated by PLC γ binds to the IP₃ receptor calcium channels on the surface of intracellular stores. Second, sustained calcium flux is regulated by PIP₃, a lipid product of PI3K. As noted earlier, PIP₃ is required to localize BTK to the plasma membrane. BTK then phosphorylates PLC γ , which is important for maintaining a sustained calcium response: PIP₃-BTK kinase interactions increase PLC γ phosphorylation, enhancing its activity and thus generation of IP₃ (Scharenberg and Kinet, 1998).

New genetic evidence is unraveling these signaling interactions in mast cells. PI3K, which generates PIP₃ by phosphorylating the 3' position of PIP₂, is activated upon Fc ϵ RI engagement. PI3K is composed of a 110-kDa catalytic and an 85-kDa regulatory subunit. There are three isoforms of p85, and generation of mice deficient for p85 α has suggested a unique function of p85 α in mast cells. In B cells, p85 α deficiency results in a phenotype similar to that of BTK-deficient mice, implicating p85 α as a

critical regulator of B cell antigen receptor signaling (Fruman *et al.*, 1999). However, a recent study showed that p85 α deficiency does not alter FcεRI-mediated signaling events, whereas other signaling pathways in mast cells are affected (Lu-Kuo *et al.*, 2000). This suggests a differential use of p85 isoforms in mast cells, and future work is required to explore this further.

The inositol phosphatase SHIP breaks down PIP₃ by dephosphorylating the 5' phosphate of the lipid PIP₃ (Huber *et al.*, 1999; Krystal *et al.*, 1999). This in turn decreases the amount of IP₃ produced by PLC γ : decreased levels of PIP₃ result in decreased recruitment of BTK and thus reduced phosphorylation and activation of PLC γ . SHIP is tyrosine phosphorylated and associates with the FcεRI following its activation (Kimura *et al.*, 1997). In SHIP^{-/-} mast cells, the degranulation response is greater than that in wild-type cells (Huber *et al.*, 1998). The lower threshold of FcεRI activation observed in SHIP^{-/-} mast cells suggests that SHIP functions to negatively regulate FcεRI *in vivo*. Similar studies have also implicated SHIP as having a negative signaling role in B cells. Notably, SHIP-deficient B cell lines exhibit increased BCR-induced calcium flux (Hashimoto *et al.*, 1999). Interestingly, SHIP also plays a role in modulating FcεRI responses via the inhibitory FcγRIIb receptor. Coligation of FcγRIIb and FcεRI inhibits IgE-mediated release of inflammatory mediators and cytokines (Fong *et al.*, 1996). This is also analogous to the role played by the FcγRIIb in the regulation of B cell antigen receptor signals: coligation of FcγRIIb with the BCR abrogates calcium influx by recruiting and activating SHIP to break down PIP₃ (Ono *et al.*, 1997; Scharenberg and Kinet, 1998; Scharenberg *et al.*, 1998).

VII. From Lipid Signaling to Serine–Threonine Protein Kinases

A. PROTEIN KINASE C

The protein kinase C (PKC) family of serine–threonine kinases has long been implicated in the regulation of cell growth, differentiation, and apoptosis, and their activity is regulated by DAG, the second lipid product of PLC γ . To date, 10 mammalian PKC enzymes have been identified and are divided into three subgroups on the basis of their structure and regulation: classical PKCs (α , β_1 , β_{II} , and γ) are activated by DAG and phosphatidylserine in the presence of calcium, while the novel PKCs (δ , ϵ , η , and θ) are regulated by DAG and phosphatidylserine but not calcium. In contrast, the atypical PKC enzymes (ζ and λ/ι) are unresponsive to DAG and are instead regulated by ceramide and PIP₂. Mast cells are known to express at least five different PKC isoforms— α , β , δ , ϵ , and ζ (Ozawa *et al.*, 1993)—all of which could potentially function in FcεRI-regulated signaling pathways.

In addition to lipid-induced activation, PKC enzymes are also regulated by specific phosphorylation events. Phosphorylation of three serine–threonine residues located in the kinase domains and C-terminal regions of classical, novel, and atypical PKC isoforms is critical to ensure maximal catalytic competency upon subsequent lipid cofactor binding. The PI3K-regulated protein kinase PDK1 (phosphoinositide-dependent kinase 1) and a complex containing atypical PKC ζ and a rapamycin-sensitive component have been demonstrated to regulate these phosphorylation events *in vivo* (reviewed in Parekh *et al.*, 2000). Furthermore, tyrosine phosphorylation of PKC δ by Src family kinases also occurs in response to a variety of stimuli, including engagement of the Fc ϵ RI (Haleem-Smith *et al.*, 1995; Song *et al.*, 1998). However, the function of these tyrosine phosphorylation events remains controversial, with conflicting reports of effects on PKC activity *in vitro* (Denning *et al.*, 1996; Li *et al.*, 1994, 1996), although tyrosine phosphorylation of PKC θ by the Src kinase LCK has been implicated in TCR signaling (Liu *et al.*, 2000). The overall importance of tyrosine phosphorylation of PKC δ for mast cell function remains to be determined.

Despite recent advances in understanding the upstream regulation of PKCs, the exact function of specific PKC isoforms in intracellular signaling cascades remains unclear. In mast cells, studies using tumor-promoting phorbol esters (which mimic the biological activity of DAG), pharmacological inhibitors, or constitutively active or dominant-negative mutants have implicated roles for classical or novel PKC enzymes in a variety of Fc ϵ RI effector responses. These include activation of MAP kinase cascades (Kawakami *et al.*, 1998; Zhang *et al.*, 1997); the induction of cytokine genes through activation of Elk, NFAT, and AP-1 transcription factors (Baranes and Razin, 1991; Lewin *et al.*, 1996; Razin *et al.*, 1994; Turner and Cantrell, 1997; Turner *et al.*, 1998); and as regulators of mast cell degranulation (Buccione *et al.*, 1994; Chang *et al.*, 1997; Kimata *et al.*, 1999; Ozawa *et al.*, 1993). In addition, Fc ϵ RI activation also involves threonine phosphorylation of the γ -chain ITAM (Paolini *et al.*, 1991; Pribluda *et al.*, 1997), which may be mediated by receptor-associated PKC δ (Germano *et al.*, 1994). Importantly, the γ -chain threonine phosphorylation site, T60, is required for the full activation of SYK upon Fc ϵ RI engagement (Swann *et al.*, 1999). Furthermore, PKC δ phosphorylates the γ -chain on T60 *in vitro* (Swann *et al.*, 1999). Taken together, these data suggest that threonine and tyrosine phosphorylation events are interdependent and linked via PKC. It will be interesting to investigate whether PKC δ phosphorylates the γ -chain T60 site *in vivo* and to determine how this event links into the signaling pathway that regulates SYK and subsequent downstream events that culminate in DAG production and PKC activation.

Recent studies have attempted to define the functional roles of particular PKC enzymes *in vivo*. In mast cells, the *in vivo* function of PKC β has been explored. Analysis of bone marrow–derived mast cells from PKC β -deficient mice has demonstrated significant inhibition of FcεRI- or calcium ionophore–induced degranulation responses, as well as reduced IL-6, but not IL-10, production compared to wild-type cells (Nechushtan *et al.*, 2000). These data support earlier observations from reconstitution and overexpression studies that defined a role for PKC β in FcεRI-regulated exocytosis and cytokine production (Chang *et al.*, 1997; Ozawa *et al.*, 1993). Similarly, the B cell compartment in the PKC β -deficient mice is affected, with reduced numbers of B1 cells, reduced serum IgM and IgG levels, and the inability to mount humoral responses to T cell–independent antigens *in vivo* (Leitges *et al.*, 1996). In contrast, the PKC θ isoform (which is restricted to the T cell lineage) is important for TCR signaling. T cell development is normal in PKC θ knockout mice, but the mature peripheral T cells exhibit defects in AP-1 and NF- κ B activation, IL-2 production, and proliferation in response to TCR activation (Sun *et al.*, 2000). Thus, the PKC β isoform has a critical, nonredundant role in both mast cells and B cells, while PKC θ has a similar role in T cells. At present the *in vivo* significance or functional redundancy of other PKC isoforms in mast cell or lymphocyte effector responses is unknown.

B. PROTEIN KINASE D

The signaling events occurring downstream of individual PKC enzymes remain poorly defined, largely because most cell types express several PKC isoforms and because few downstream targets have been identified. Interestingly, one recently identified target for classical or novel PKC enzymes in mast cells is the serine kinase protein kinase D (PKD) (Valverde *et al.*, 1994), also known as PKC μ (Johannes *et al.*, 1994). PKD was originally classified as a new member of the PKC family based on the presence of a DAG binding cysteine-rich (C1) domain within its regulatory domain (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995). However, unlike PKC enzymes, PKD does not possess an autoinhibitory pseudosubstrate motif upstream of its DAG binding domain nor a calcium binding domain. Furthermore, the kinase domain of PKD shows very low homology to the conserved kinase domains of the PKC family (Valverde *et al.*, 1994), and PKD displays a unique peptide substrate specificity *in vitro* (Valverde *et al.*, 1994; Van Lint *et al.*, 1995). PKD, unlike all known PKC enzymes, also contains a PH domain that serves to negatively regulate its catalytic activity (Iglesias and Rozengurt, 1998). Thus, PKD, together with the closely related protein kinase PKC ν (Hayashi *et al.*, 1999), constitute a distinct protein kinase family.

Phorbol esters and growth factors stimulate PKD activity in a variety of cells types (Van Lint *et al.*, 1998; Zugaza *et al.*, 1996, 1997). PKD has been implicated in the regulation of NF- κ B transcriptional activity and in the control of Golgi organization in epithelial or fibroblast cell lines (Jamora *et al.*, 1999; Johannes *et al.*, 1998; Prestle *et al.*, 1996), underscoring its overall importance for cellular processes. PKD is highly expressed in mast cells and lymphocytes and is involved in antigen receptor signaling cascades (Sidorenko *et al.*, 1996). Specifically, Fc ϵ RI aggregation rapidly and potently stimulates PKD activity (Matthews *et al.*, 2000), and phosphorylation of two serine residues within the PKD kinase domain is both required and sufficient for activation of PKD (Iglesias *et al.*, 1998). Production of DAG (but not IP₃) is essential for antigen receptor coupling to PKD, as demonstrated by analysis of PKD activity in SYK- and PLC γ -deficient DT40 B cells (Sidorenko *et al.*, 1996). However, mutation of the PKD C1 domain reveals that direct binding of DAG is dispensable for the activation of PKD. Instead, biochemical studies have revealed that classical or novel PKC enzymes control the activation of PKD in response to Fc ϵ RI, BCR, or TCR engagement (Matthews *et al.*, 2000). Therefore, the identification of PKD as a target for DAG–PKC signals reveals a novel aspect of this signaling cascade in mast cells and lymphocytes. Future work is required to address the functional importance of this PKC–PKD connection in mast cells.

Almost invariably, the biological effects of DAG and phorbol esters *in vivo* are attributed to the action of classical or novel PKC enzymes. However, it is important to note that the recent identification of DAG binding domains present in proteins other than PKCs has increased the complexity of this signaling pathway (reviewed in Ron and Kazanietz, 1999). The novel Ras guanine nucleotide exchange factor (GEF) RasGRP is highly expressed in T and B cells, and its activity (as well as that of a related Rap1-specific GEF) is stimulated by DAG or phorbol esters (Kawasaki *et al.*, 1998; O'Ebinu *et al.*, 1998; Tognon *et al.*, 1998). These exchange factors therefore constitute a novel link between DAG- and GTPase-regulated signaling pathways in lymphocytes. Given that many signaling molecules in mast cells parallel those present in lymphocytes, mast cells may also contain novel DAG-regulated proteins that could function in Fc ϵ RI-regulated signaling pathways. Thus, the identification of additional “non-PKC” DAG-responsive proteins highlights the need for caution in interpreting previous and future studies on this signaling pathway. Moreover, future research should be focused on exactly how specific PKC enzymes are selectively activated by DAG in distinct cellular or receptor contexts and also on the identity and function of downstream targets of DAG-regulated proteins.

VIII. Lipid Rafts

Specialized plasma membrane microdomains known as lipid rafts have been proposed to regulate receptor signaling by serving as platforms that control the spatial organization of key signaling molecules (Janes *et al.*, 2000; Kurzchalia and Parton, 1999; Sheets *et al.*, 1999b; Xavier and Seed, 1999). These detergent-insoluble structures contain a high density of sphingolipids and cholesterol, and in addition a higher prevalence of phosphatidylinositol-linked proteins, receptor and nonreceptor tyrosine kinases, G proteins, and various other signaling molecules compared to bulk plasma membrane (Simons and Ikonen, 1997; Xavier and Seed, 1999). Lipid rafts are thought to be crucial for optimal antigen receptor signaling, as illustrated by studies in T cells, where disruption of lipid rafts (using pharmacological agents) is associated with attenuated TCR signaling (Stulnig *et al.*, 1998; Xavier *et al.*, 1998). Indeed, chemical cross-linking of lipid rafts induces signaling events analogous to TCR stimulation (Janes *et al.*, 1999).

In mast cells the FcεRI receptor is associated with both loosely and tightly bound membrane lipids and ester-linked fatty acids (Kinet *et al.*, 1985) and, until activation, is uniformly distributed within the plasma membrane. Upon engagement, the FcεRI transiently redistributes into lipid rafts, along with coordinate spatial reorganization of signaling molecules, as illustrated by the movement of chimeric proteins containing the SH2 domains of SYK and PLCγ fused to green fluorescent protein (Stauffer and Meyer, 1997). Activation-induced compartmentalization of signaling proteins in mast cells has also been observed biochemically: detergent-insoluble membrane fractions from FcεRI-stimulated cells are enriched for activated LYN and tyrosine-phosphorylated β- and γ-receptor chains (Field *et al.*, 1995, 1997). Moreover, cholesterol depletion reversibly disrupts FcεRI–LYN interactions and reduces early tyrosine phosphorylation events in activated mast cells (Sheets *et al.*, 1999a). Together, these data support the hypothesis that FcεRI activation and proximal signaling events occur in specialized membrane microdomains. Similar observations have also been made for antigen receptor complexes in T and B cells (Cheng *et al.*, 1999; Montixi *et al.*, 1998; Xavier *et al.*, 1998).

The description of lipid rafts in mast cells and other cell types has clearly necessitated that the model of FcεRI signaling events include three-dimensional spatial and geometrical considerations. Spatial inclusion or exclusion of specific molecules from a raft or specific lipid modifications to signaling molecules may provide independent means of regulation. For example, observations that CD45 may be excluded from lipid rafts illustrate this type of topological regulation (reviewed

in Thomas, 1999). CD45 initiates antigen receptor signaling by dephosphorylating the inhibitory site of Src kinases. However, why CD45 does not subsequently dephosphorylate activated kinases or adaptor proteins, thus turning off signaling cascades, is unclear. Exclusion of CD45 from activated antigen receptor complexes may provide the explanation. In addition, N-myristoylation and protein palmitoylation are well known to regulate membrane association of key signaling proteins and may facilitate efficient partitioning into rafts. Interestingly, mutation of the palmitoylated cysteine residues of the SRC kinases LYN and FYN and the adapter molecule LAT prevents the association of these proteins with lipid rafts (Shenoy-Scaria *et al.*, 1994; W. Zhang *et al.*, 1998b), although it is difficult to attribute such effects to the lack of palmitate per se. However, it is attractive to speculate that reversible acylation events, such as palmitoylation, may provide an additional level of control for antigen receptor signaling by regulating the spatial organization of signaling proteins. Taken together, mechanisms controlling the spatial organization of lipid rafts will likely represent a novel means for regulation of Fc ϵ RI signal transduction.

IX. Conclusion

Our knowledge of the signaling events that underlie Fc ϵ RI-mediated activation of mast cells is becoming clearer. Most recently, these advances have led to a greater understanding of how upstream PTK activity is integrated into downstream events. Importantly, genetic evidence has illustrated the essential requirement of several signaling molecules and has contributed to a better understanding of the protein–lipid interactions involved in calcium mobilization. However, many questions still exist. Paramount among these is the complete lack of understanding of the plasma membrane calcium channels responsible for calcium influx. To date, the existence of these channels is known only from electrophysiological measurements. Cloning and characterization of calcium influx channels and the signaling pathways that regulate them will clearly be of great importance. Such work will provide a basis for understanding how Fc ϵ RI-mediated proximal signals are integrated into downstream responses leading to gene activation. Other important areas of investigation include characterization of the molecules that negatively regulate Fc ϵ RI signals and elucidating how effector functions are differentially induced and regulated. Future analysis of mast cells deficient for different signaling molecules will no doubt continue to provide critical insights into many of these issues.

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