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The conundrum of inhibitory signaling by ITAM-containing immunoreceptors: potential molecular mechanisms

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Abstract

Immunoreceptor signals must be appropriately transduced and regulated to achieve effective immunity while controlling inflammation and autoimmunity. It is generally held that these processes are mediated by the interplay of distinct activating and inhibitory receptors via conserved activating (ITAM) and inhibitory (ITIM) signaling motifs. However, recent evidence indicates that under certain conditions incomplete phosphorylation of ITAM tyrosines leads to inhibitory signals. This new regulatory function of ITAMs has been termed ITAMi (inhibitory ITAM). Here we discuss the potential molecular mechanisms of inhibitory signaling by ITAM-containing receptors.

Keywords

B cell; Antigen Receptor; CD79; ITAM; ITIM; ITAMI; anergy; SHIP-1; SHP-1; mIg

Introduction

The outcome of antigen receptor signaling is determined by a complex network of activating and attendant regulatory signaling circuitry. The balance of these signals determines cell fate and is essential for generation of effective immunity, while controlling inflammation and autoimmunity. The classical view of immune receptor signaling and its regulation involves the interplay of signals transduced via receptors containing immunoreceptor tyrosine-based activation motif (ITAM) and distinct receptors containing immunoreceptor tyrosine-based inhibition motifs (ITIM). However, recent evidence suggests that more complex mechanisms exist, wherein incomplete phosphorylation of ITAM tyrosines may alter the output of signaling [1]. This inhibitory function has been termed ITAMi (Inhibitory ITAM). Interestingly, studies in our own laboratory suggest that similar mechanisms may be involved in maintenance of B cell anergy. More specifically, the chronic antigen receptor signals that are required to sustain B cell anergy drive monophosphorylation of BCR ITAMs (O'Neill and Cambier, *in preparation).* Importantly, further studies suggest that the distinguishing feature of this inhibitory signaling is the robust activation of Src-family kinases. We suggest that it is monophospho-ITAM ($[P]_1$ITAM)-mediated activation of Src-family kinases rather than some other intrinsic ITAM function that leads to inhibitory signaling. In support of this concept we have found that activation of Lck by aggregation of

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CD4 on T cells leads to inhibitory signaling that negatively regulates TCR mediated T cell activation. In this brief review we will compare and contrast these mechanisms and discuss the potential identity of effectors of these inhibitory signals.

Immunoreceptor tyrosine-based signaling motifs

ITAMs are found in multisubunit immunoreceptors such as B-cell and T-cell antigen receptors (BCRs and TCRs) and activating Fc receptors (FcRs) [2,3]. ITAMs are characterized by content of the consensus sequence YxxL/I-$(x)_{6–8}$-YxxL/I (where x is any amino acid) which mediates signal propagation by activation of Syk or Zap70 tyrosine kinases [3,4]. Aggregation of ITAM-containing receptors leads to phosphorylation of the ITAM tyrosines via Src-family kinases. Dual phosphorylation of the conserved ITAM tyrosines yielding $[P]_2$ITAMs is required to generate a docking site for tandem SH2 domains of Syk and/or ZAP-70 kinases (16). Recruitment and activation of Syk and/or ZAP-70 leads to tyrosine phosphorylation-dependent activation of multiple downstream pathways that drive activation, proliferation, differentiation and survival [5]. ITAM monophosphorylation is not without functional consequence. Aggregation-induced monophosphorylation of BCR ITAMs leads to tyrosine phosphorylation of Lyn and a limited number of its downstream substrates consistent with kinase activation [6].

ITIM-containing receptors are evolutionarily conserved membrane proteins whose origin can be traced to the most primitive metazoa [7]. The inhibitory function of ITIM-containing receptors was first defined in the low affinity immunoglobulin G (IgG) receptor FcγRIIB [8]. Most ITIMs contain the consensus sequence (I/V/L/S)xYxx(L/V) which, when tyrosine phosphorylated, binds to the SH2 domain-containing 5-inositol phosphatase SHIP-1 and SHIP-2 and/or the SH2 domain containing tyrosine phosphatases SHP-1 and SHP-2 [9,10]. While ITIM binding to SHPs strictly requires Y-2 hydrophobic residues due to presence of a hyphobic pocket in the phosphatases, association with SHIPs does not and further prefers L at the Y+2 position [11–13]. Once activated, SHIP-1/2 and SHP-1/2 dephosphorylate inositol phospholipids and tyrosyl-phophorylated proteins, respectively, attenuating cell activation.

Proximal Events in B cell antigen receptor signaling

The BCR transduces signals via a disulfide-bonded heterodimer of immunoglobulin α (Igα or CD79a) and immunoglobulin β (Igβ or CD79b) that is noncovalently associated with membrane bound immunoglobulin (mIg) [14,15]. Aggregation of mIg-Igα/Igβ complexes leads to activation of associated Src-family kinases (Lyn in most mature B cells) which then phosphorylate the two tyrosines found in the single ITAM in each Igα and Igβ chain [14,16]. As noted above, dual phosphorylation of a single ITAM is required to generate the Syk/Zap70 docking site, and thereby promote activation of the kinase [17]. One of the key Syk substrates is the adaptor molecule B-cell linker protein (BLNK) which when phosphorylated serves as a scaffold for phospholipase C gamma (PLCγ), Bruton's tyrosine kinase (Btk), GRB2/VAV and Son of Sevenless (SOS) [18].

PI3K is activated by Lyn and Syk via a parallel pathway. This occurs by tyrosine phosphorylation and PI3K binding to the cytosolic adaptor BCAP [19], and the membrane adaptor/coreceptor CD19 [20]. In addition to its direct function in PI3K activation, CD19 reportedly mediates the processive activation of Lyn creating a positive forward feeding loop [21] and this is predicted to enhance positive signaling by recruiting Btk and VAV [22]. PI3K can also be activated by a process involving direct binding to Lyn, thus the processive CD19 activation of Lyn could further promote PI3K activation [23].

PI3K mediates the production of phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) from PtdIns(4,5)P_2. PtdIns(3,4,5)P_3 is a critical lipid second messenger in BCR signaling, functioning in the recruitment of multiple effectors to the membrane via binding to their pleckstrin homology (PH) domains. These include Btk, VAV, PDK1, Akt and PLCγ. The precise temporal and functional relationship of Btk and PLCg binding to BLNK and PtdIns(3,4,5)P_3 is unclear. It seems likely that this is a stepwise process in which binding to BLNK facilitates phosphorylation and activation by receptor associated kinases, while subsequent binding to phospholipids places them in apposition to their substrates. PLCγ cleavage of PtdIns(4,5)P_2 yields Ins(1,4,5)P_3 and diacylglycerol (DAG) which trigger calcium release and stimulate various isoforms of Protein Kinase C (PKC), respectively. Obviously, generation and accumulation of PtdIns(3,4,5)P_3 is a critical step in the propagation of activating antigen receptor signals.

SHP-1 and SHIP-1 as mediators of both FcgRIIB and feedback inhibition of BCR signaling

In view of the roles of protein tyrosine and inositol lipid phosphorylation in antigen receptor signaling, it is intuitive that this process is regulated by inositol lipid and tyrosine phosphatases. Phosphatases were first implicated in regulation of antigen receptor signaling in studies of the molecular basis of inhibitory signaling by FcgRIIB [24]. It was found that synthetic phosphopeptides constructed based on the cytoplasmic tail sequences of FcγRIIB known to be required for inhibitory function, bound to SHP-1 and SHP-2 [24]. These observations were later extended to SHIP-1 [9,25,26], and it was shown that both SHP-1 deficient (Me^V) and SHIP-$1^{-/-}$ mice exhibited defects in FcγRIIB signaling [9,27]. Interestingly, while SHIP-1 is the primary mediator of this signal, the relative importance of SHP-1 and SHIP-1 appears to depend on the efficiency of receptor crosslinking, with higher order crosslinking evoking more SHP-1 function [12]. This may relate to the fact the SHP-1 (but not SHIP) contains two SH2 domains that may bridge phosphorylated ITIMs on apposing FcgRIIB. Occupancy of both SHP-1 SH2 domains appears to be required for its activation by derepression [11].

The inhibitory activities of SHIP-1 and SHP-1 differ in more than the fact that they have distinct substrates. Studies by Blery et al [28] demonstrated the ITIM-containing receptors that engage SHP-1 function only at short range, inhibiting only receptors with which they are co-aggregated. ITIM-containing receptors that engage SHIP-1 are able to act in trans, inhibiting remotely stimulated receptors. If SHP-1 is activated by derepression as discussed above, inhibition would be limited to the reach of inhibitory receptor-bound enzyme because it would only be active when tethered. Long-range activity of SHIP-1 suggests that this effector is active after dissociation from the inhibitory receptor. In addition to its phosphatase domain, SHIP contains an amino-terminal SH2 domain, a proline-rich region and two NPxY motifs in an extended C terminal region. These domains mediate association with several proteins, including CD150, Shc, SHP-2, Gab and Dok [29]. NPxY motifs of SHIP-1 are phosphorylated upon FcgRIIB stimulation, and bind to the phosphotyrosine-binding domain (PTB) of Downstream of kinase (Dok-1). This binding likely facilitates the subsequent tyrosine phosphorylation of Dok-1. Phosphotyrosines within Dok-1 are known to bind to the SHIP-1 SH2 domain [30]. Thus it is easy to imagine that the consequence of this stepwise recruitment and tyrosine phosphorylation is formation of a bidentate complex of SHIP and Dok. Indeed, Dok is the primary phosphoprotein that co-precipitates with SHIP-1 in stimulated lymphocytes (J.C.C. and P. Waterman, unpublished).

The question then arises, does Dok play a role in SHIP-1-mediated trans-inhibitory signaling? It is noteworthy that Dok-1 contains a PH domain that binds to PtdIns(3,4,5)P_3 and could therefore mediate targeting of SHIP-1/Dok-1 complexes to areas of plasma

membrane enriched in PtdIns(3,4,5)P_3 [31]. This localization could facilitate SHIP-1 hydrolysis of PtdIns(3,4,5)P_3 yielding PtdIns(3,4,)P_2. Consistent with this hypothesis, we have found in ongoing studies that expressed fusion proteins containing only the Dok-1 PH domain and the SHIP-1 catalytic domain function as constitutive inhibitors of BCR signaling (Akerlund and Cambier, unpublished).

Although SHIP-1 and Dok-1 phosphorylation can be achieved by co-aggregation of FcgRIIB with BCR, it is also seen, though to a much lesser extent, following aggregation of the BCR alone [9,32,33]. Furthermore, BCR stimulation leads to transient SHIP-1 translocation to the plasma membrane[34]. These finding suggested that in addition to its role in active FcγRIIB mediated inhibition, SHIP mediates feedback inhibition of antigen receptor signaling.

It is unclear how the SHIP-1/Dok-1 circuit is activated by antigen receptor aggregation. Studies of cKit signaling indicate that Lyn can associate with Downstream of kinase (Dok) [35]. This may facilitate Dok phosphorylation, followed by SHIP recruitment and phosphorylation, leading ultimately to generation of a mobile bidentate effector complex equivalent to that predicted to occur following BCR-FcγRIIB co-aggregation.

As noted above SHP-1 functions as mediator of inhibitory FcγRIIB signals under conditions of efficient receptor coaggregation. Like SHIP-1, SHP-1 also functions as a feedback inhibitor of BCR signaling. SHP-1 is recruited to phosphorylated ITIM tyrosines in membrane adaptors, such as CD22 and CD72, that are phosphorylated by Lyn upon BCR aggregation. Active SHP-1 attenuates BCR signaling via tyrosine dephosphorylation of molecules involved in positive signaling such as Igα, Igβ, CD19, Syk, and BLNK [36–38].

SHIP-1/Dok-1 inhibitory circuit in B cell anergy

B cell anergy is likely maintained by multiple inhibitory signaling effectors including the phospatase and tensin homolog PTEN, SHP-1 and SHIP-1. PTEN expression is uniquely elevated in anergic B cells from MD4xML5 anti-HEL mice in which BCR affinity for autoantigen is extremely high [39]. By association, we hypothesize that PTEN upregulation may be a unique consequence of chronic B cell stimulation by very high affinity antigens. Its function may be uniquely required to reinforce anergy in cells with very high autoantigen affinity/avidity because high avidity antigens are very potent inducers of BCR signaling. B cell targeted PTEN knockout mice do not display an autoimmune phenotype [39]. However, this may be due to the previously described PTEN requirement for immunoglobulin class switching [40–42], since clinical signs of autoimmunity require class switched IgG autoantibody.

SHP-1 functions in maintenance of B cell tolerance, presumably via interactions with ITIM-containing membrane adaptor proteins. B cell targeted deletion of SHP-1 leads to lupus-like disease at 5–6 months of age and glomerulonephritis at 7–11 months of age [43]. Loss of tolerance in these animals appears to reflect loss of anergy especially since the recently defined anergic B cell compartment is reduced in size by approximately 40%.

Mounting evidence supports a more major role for SHIP-1 in maintaining anergy. In support of this, mice with B cell-targeted SHIP-1 gene ablation exhibit early (2–4months) onset lupus-like disease with glomerulonephritis and death by 5–8 months. B cell ablation of SHIP-1 in the Ars/A1 (anti-ssDNA) immunoglobulin transgenic mouse lead to loss of anergy and recovery of antigen receptor mediated calcium signaling. Finally, SHIP-1 and Dok-1 are constitutively tyrosine phosphorylated in naturally occuring anergic B cells, as well as those from both Ars/A1 and MD4/ML5 transgenic mouse models [44,45] (O'Neil and Cambier, in preparation). These findings suggest that chronic activation of the SHIP-1/

Dok-1 circuit maintains anergy by limiting accumulation of PtdIns(3,4,5)P_3 during responses to crossreacting immunogens, chemokine, BAFF and other ligands that signal via activation of PI3k.

Maintenance of anergy requires chronic binding of antigen to BCR [46] suggesting that autoantigen occupied BCR transduce signals that lead to unresponsiveness. Indeed, anergic B cells exhibit elevated intracellular free calcium levels and ERK kinase pathway activition [47], and when autoantigen is removed from BCR by competitive inhibition with monovalent hapten, levels of intracellular free calcium and phosphorylated ERK are reduced to that seen naïve B cells. In addition, after removal of autoantigen the cell lifespan is increased and the ability to mobilize calcium and up-regulate CD86 following BCR stimulation is restored [46]. These data indicate that chronic BCR signaling is required to maintain anergy. By extension, this chronic BCR signaling is probably required to maintain activation of the SHIP-1/Dok-1 inhibitory circuit and resultant unresponsiveness.

The question arises, how does chronic BCR stimulation lead to inhibitory SHP-1 and SHIP-1 signaling in anergic B cells? Ongoing studies in our laboratory are interrogating mechanisms of chronic signaling by BCR in anergic B cells. These studies have revealed increases in basal Ig-α/β ITAM tyrosine phosphorylation in anergic cells (O'Neill and Cambier, in preparation). Most importantly, only monophosphorylation is detectable. This finding resonates with previous observations made in studies of signaling competency of receptors in which only a single ITAM tyrosine is available for phosphorylation. These studies showed that ITAM monophosphorylation induces activation of Lyn but not Syk tyrosine kinases [6]. The resultant bias towards chronic Lyn activation in anergic cells could skew signaling towards activation of SHIP-1 and SHP-1 enforcing unresponsiveness. In support of this possibility, mice lacking Lyn are autoimmune prone, which suggests Lyn is required for stimulation of inhibitory signaling circuitry.

Chronic antigen stimulation-induced monophosphorylation of Igα/Igβ ITAMs in anergic B cells is strikingly similar to the ITAMi signaling described by Blank et.al. [1]. Taken at face value, the major difference in these signaling pathways is that ITAMi signaling is proposed to involve only SHP-1 as the operative effector phosphatase. However, the possible role of SHIP-1 in ITAMi signaling has not been explored.

ITAMi signaling involves specific binding of an ITAM-containing receptor to low affinity, low avidity, or low valency ligands that lead to the recruitment of inhibitory effectors such as SHP-1. For example, FcαRI exhibits ITAMi signaling following monomeric IgA or anti-FcαRI Fab ligation (low valency interaction) that leads to incomplete phosphorylation of the associated ITAM containing receptor FcRγ. This incomplete ITAM phosphorylation favors SHP-1 recruitment over Syk [48,49]. In contrast, multivalent ligand interaction leads to robust phosphorylation of FcRa associated FcRγ ITAMs and recruitment of Syk [50,51]. Preferential SHP-1 recruitment has also been described in T cells following weakly binding TCR ligands [52,53]

SHIP-1/Dok inhibitory circuit activation by CD4 aggregation; the exception that may prove the rule

ITAM phosphorylation is mediated primarily by Src-family kinases, and phosphorylated ITAMs further stimulate Src-family kinase activity in a forward feeding loop. It is unclear whether ITAM phoshorylation plays a role in activation of the SHIP-1/Dok-1 circuit other than activation of Src-family kinase. A solution is suggested by studies of the molecular basis of inhibitory signaling by CD4. It has long been known that aggregation of CD4 leads to generation of molecular signals that inhibit subsequent responses to TCR aggregation[54].

Under these circumstances, CD4 signals via activation of the Src-family kinase Lck with no involvement of ITAMs. This allowed us to determine whether pITAMs are actually required for activation of SHIP-1/Dok-1 inhibitory signaling.

We have found that CD4 aggregation in the absence of TCR induces strong activation of the SHIP-1/Dok-1 inhibitory circuit via Lck, abrogating subsequent TCR signaling (Waterman and Cambier, *in preparation*). This indicates that no intrinsic ITAM (or ITIM) function is required for inhibitory signaling. Rather, the role of monophospho-ITAMs is restricted to activation of Src-family kinases. Thus regardless the mechanism of its activation, Src-family kinase may be expected to stimulate SHIP-1/Dok-1 inhibitory circuit.

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