Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Adv Biol Regul. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Adv Biol Regul. 2021 January ; 79: 100775. doi:10.1016/j.jbior.2020.100775.

Reconsidering Phosphorylation in the Control of Inducible CARD11 Scaffold Activity During Antigen Receptor Signaling

Joel L. Pomerantz*

Department of Biological Chemistry, Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Abstract

Protein phosphorylation is a commonly used regulatory step that controls signal transduction pathways in a wide array of biological contexts. The finding that a residue is phosphorylated, coupled with the observation that mutation of that residue impacts signaling, often forms the basis for concluding that the phosphorylation of that residue is a key signaling step. However, in certain cases, the situation is more complicated and warrants further study to obtain a clear mechanistic understanding of whether and how the kinase-mediated modification in question is important. CARD11 is a multi-domain signaling scaffold that functions as a hub in lymphocytes to transmit the engagement of antigen receptors into the activation of NF-κB, JNK and mTOR. The phosphorylation of the CARD11 autoinhibitory Inhibitory Domain in response to antigen receptor triggering has been proposed to control the signaling-induced conversion of CARD11 from an inactive to an active scaffold in a step required for lymphocyte activation. In this review, I discuss recent data that suggests that this model should be reconsidered for certain phosphorylation events in CARD11 and propose possible experimental avenues for resolution of raised issues.

1. Introduction

Many critical signaling pathways rely on regulated protein phosphorylation for the precise temporal control of the signaling output that determines the cellular response to external stimuli (please see for example (Hirsch et al., 2020; Jhanwar-Uniyal et al., 2019; Rahimova et al., 2020)). A wealth of data collected over many decades has described how the phosphorylation of proteins can elicit inducible changes in protein structure, enzyme activity, protein-protein interactions, and protein degradation, among other effects. Protein phosphorylation is so commonly employed in signal transduction pathways, that its attribution as an important step in a particular context can often be made without thorough

*Corresponding author: Joel L. Pomerantz, PhD, Associate Professor, Department of Biological Chemistry, Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Miller Research Building, Room 607, 733 N Broadway, Baltimore, MD 21205, Voice: 443-287-3100, Fax: 443-287-3109, joel.pomerantz@jhmi.edu.

Credit Author Statement

The author is entirely responsible for all content.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The author declares no conflict of interest.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

evidence for precisely what the phosphorylation event in question accomplishes mechanistically. In such cases, continued reflection on the role of the phosphorylation can yield unexpected insights about the system being studied.

CARD11 is a multidomain scaffold protein that controls lymphocyte activation and proliferation in response to antigen recognition in the adaptive immune response (Bedsaul et al., 2018). The importance of CARD11 in immune system function is well documented by at least three forms of primary immunodeficiency caused by gain- or loss-of function germline mutations in the CARD11 gene which lead to high susceptibility to viral and bacterial infections (Lu et al., 2019). In addition, somatic gain-of-function CARD11 mutations are frequently found in different forms of B and T lymphoma, and can promote the inappropriate lymphocyte proliferation that drives disease (Juilland and Thome, 2016).

In response to antigen receptor engagement on the surface of lymphocytes, CARD11 functions as a signal inducible scaffold that nucleates the formation of a multiprotein complex that activates branching signaling pathways leading to NF-κB, JNK, and mTOR (Bedsaul et al., 2018). The CARD11 signaling cycle has been well described (Wang et al., 2019). In resting T and B cells, CARD11 is kept in a closed, inactive state via the action of an Inhibitory Domain that prevents scaffold activity through four Repressive Elements (REs) that function cooperatively and with redundancy (Jattani et al., 2016a, b). Autoinhibition by the REs occurs in part through intramolecular interactions with other CARD11 domains that prevent the binding of protein signaling partners. Upon antigen receptor triggering, the REs are somehow neutralized, leading to the **Opening Step** in the CARD11 signaling cycle, which converts CARD11 into an open, active scaffold that exposes domain interfaces for the binding of several proteins. In the next step, the **Cofactor Association Step**, a variety of proteins assemble onto CARD11, including the adaptor protein Bcl10, the MALT1 protease, and the E3 ubiquitin ligases HOIP and TRAF6, among several other proteins. It has recently been recognized that the enzymatic activities of MALT1 and HOIP are activated in a step distinguishable from the Cofactor Association Step, the **Enzyme Activation Step** (Wang et al., 2019). During this step, HOIP ligase activity is induced to conjugate Bcl10 with linear ubiquitin chains to form $LinUb_n$-Bcl10, an intermediate that determines the degree of NF-κB activation downstream of CARD11 signaling (Yang et al., 2016). In addition, the MALT1 protease is activated to cleave a collection of substrates that also modulate signaling output (Juilland and Thome, 2018). Subsequently, in the **Complex Disassembly Step**, proteins disassociate from CARD11 and CARD11 returns to the autoinhibited resting state.

The phosphorylation of CARD11 has been invoked as a major regulator of CARD11 activity by positively influencing CARD11 activity, by providing negative feedback, or by inducing CARD11 degradation. The residues that are modified, the kinases responsible, and the regulatory effects associated with these phosphorylations have been discussed extensively in several excellent reviews (Lork et al., 2019; Meininger and Krappmann, 2016; Thome et al., 2010; Thys et al., 2018). Here, I will focus the discussion on recent findings that have modified our view of the proposed role for phosphorylation in the autoinhibitory CARD11 Inhibitory Domain.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2. The case for certain serine phosphorylation events

Seminal studies identified several serine residues in the CARD11 Inhibitory Domain that were important for antigen receptor induced activation of NF-κB, including Serine 564, Serine 567, and Serine 577 (Matsumoto et al., 2005; Shinohara et al., 2007; Sommer et al., 2005). The mutation of any one of these residues resulted in reduced signal-induced CARD11 signaling. The phosphorylation of these sites was proposed to be critically important for their role in the pathway based upon the following evidence in addition to the clear effect of their mutation. For Serine 564 and Serine 577, mass spectrometry data from PMA/anti-CD28 treated Jurkat T cells detected a CARD11 peptide containing the phosphorylated serines (Matsumoto et al., 2005). For Serine 564, *in vitro* experiments with PKCθ or PKCβ enzymes demonstrated that this serine could be phosphorylated by these kinases known to be important in the pathway in either T or B cells (Matsumoto et al., 2005; Sommer et al., 2005). Similarly serine 567 was shown to be phosphorylated *in vitro* by IKKβ, also a critical kinase known to be important in the pathway and a phospho-specific antibody was developed that could detect the phosphorylation of the equivalent serine in stimulated chicken DT40 cells (Shinohara et al., 2007). To my knowledge, the kinase responsible for phosphorylation of 577 has yet to be identified.

These data, and subsequent studies on the role of the Inhibitory Domain in keeping CARD11 in the closed, inactive state in the absence of receptor triggering, led to a model in which the phosphorylation of these residues was required for the neutralization of the autoinhibitory function of the Inhibitory Domain during the Opening Step of CARD11 signaling.

3. Redefining the Importance of these Serine Residues

A recent study from my group has refined understanding of the role of serines 564, 567, and 577 in CARD11 signaling and suggests a reconsideration of whether their phosphorylation is as key to inducing CARD11 activity as the current model proposes (Wang et al., 2019).

A deletion analysis of the CARD11 Inhibitory Domain revealed that serines 564, 567, and 577 reside in a 26-residue region that is required to confer T Cell Receptor (TCR)-inducible activity to CARD11. This region was named Inducible Element 1 (IE1). Scanning mutagenesis confirmed that the individual mutation of these three serines and ten other IE1 residues reduced CARD11-mediated inducible activation of NF-κB. The six strongest IE1 mutations, including the mutation of serines 564, 567, and 577, were each shown to abrogate the inducible binding of Bcl10, MALT1, and HOIP to CARD11, the subsequent generation of $LinUb_n$-Bcl10 and the activation of MALT1 protease activity, consistent with a requirement for these residues in the Opening Step of the CARD11 signaling cycle. Notably, it had been previously shown that the binding of Bcl10, MALT1, and HOIP to CARD11 did not require the IE1 region or any other portion of the Inhibitory Domain (McCully and Pomerantz, 2008; Yang et al., 2016), ruling out a role for IE1 residues in a protein-protein interface between CARD11 and these cofactors.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The surprise came when the mutation of IE1 residues was analyzed in the context of a constitutively open CARD11 variant in which all four Repressive Elements in the Inhibitory Domain had been disabled, termed the repressive element quadruple mutant (reQM). The reQM variant represents the CARD11 protein subsequent to the Opening Step, since it constitutively binds cofactors in the Cofactor Association Step and does not require upstream signaling events including receptor engagement for signaling to NF-κB. Thus, reQM signals to NF-κB independently of upstream kinases like PKCθ in T cells and PKCβ in B cells, the kinases that phosphorylate serine 564 in the model for phosphorylation-induced CARD11 opening. Surprisingly, the individual mutation of IE1 residues, including serines 564, 567, and 577, had the same deleterious effect on signaling to NF-κB in the reQM context as in the wild-type CARD11 context, indicating a clear requirement for these residues in a step following the Opening Step. Further analysis showed that IE1 mutations do not affect the constitutive binding of Bcl10, MALT1 and HOIP to the constitutively open reQM variant. Rather, IE1 mutations in the reQM context prevent the activation of HOIP enzymatic activity, as measured by the production of $LinUb_n$-Bcl10, and the activation of MALT1 proteolytic activity, as measure by the appearance of cleavage products of MALT1 substrates HOIL1 and CYLD. The data revealed that IE1 controls a newly defined Enzymatic Activation Step that is distinguishable from the Cofactor Association Step. In addition, the remarkably similar pattern of effect of individual IE1 mutations in the wild-type CARD11 and reQM contexts suggested that IE1 interacts with the same molecular target during the Opening and Enzymatic Activation Steps.

4. New Possibilities Raised

The requirement for CARD11 serines 564, 567, and 577 in a step distinguishable from the Opening Step raises several new distinct possibilities regarding their phosphorylation that should be considered.

Possibility A. The phosphorylation of serines 564, 567, 577 is not required for signal-inducible CARD11 Opening.

Each of these serines is clearly important for the Opening Step, but it is possible that their action in this step does not depend on their phosphorylation. This possibility is raised most clearly in the case of serine 564, an invoked substrate of PKCθ in T cells. PKCθ is required for inducible CARD11 activity in T cells, but is not required for reQM activity, which signals robustly in the absence of upstream signals emanating from the TCR that activate PKCθ. PKCθ is thus required for the Opening Step, but not for subsequent signaling steps. In contrast, serine 564 is important for CARD11 activity in both the wild-type and reQM contexts, and is therefore operating in signaling independently of PKCθ. Furthermore, the profile of effects of IE1 point mutations in wild-type and reQM contexts strongly suggest that serine 564 is recognized by the same target in both contexts. The possibility exists, then, that serine 564 participates in CARD11 opening as an unphosphorylated residue, and that other PKCθ targets either in CARD11 or in another protein must be phosphorylated by PKCθ to allow CARD11 opening during antigen receptor signaling.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Similarly, serines 567 and 577 may also operate during the Opening Step as unphosphorylated residues. Serine 567 has been invoked as a substrate of IKKβ, a kinase that is thought to become active downstream of the formation of $LinUb_n$-Bcl10 and therefore downstream of the Enzymatic Activation Step. Serine 567 phosphorylation by IKKβ could provide positive feedback on CARD11 signaling strength after an initial turn through the CARD11 signaling cycle, as has been proposed (Shinohara et al., 2014; Shinohara et al., 2007), and this would not be inconsistent with a role for non-phosphorylated serine 567 in opening. The kinase that phosphorylates serine 577 has not been reported and the absence of knowledge regarding how its modification occurs leaves open the possibility that its phosphorylation is not required for the Opening Step.

Possibility B. The phosphorylation of serines 564, 567, 577 is not required for CARD11 signaling at all.

The finding that serines 564, 567, and 577 are all required for signaling by the constitutively open reQM CARD11 variant also opens up the possibility that these residues are operating as unphosphorylated residues throughout the CARD11 signaling cycle. The deleterious effects of their mutation on CARD11 signaling output may solely be due to the prevention of their recognition as unmodified serine residues, even though their phosphorylation under stimulated conditions may be observed by MS analysis or via phospho-specific antibody recognition.

Possibility C. The phosphorylation of one or more of serines 564, 567, and 577 is a negative regulatory step in CARD11 signaling.

A third possibility that should be considered is that these residues function during the CARD11 signaling cycle as unmodified serines and that their phosphorylation at some point following receptor triggering prevents their positive effect on the pathway as a negative feedback mechanism. The interaction between IE1 and the undefined target that binds IE1 during the Opening and Enzymatic Activation steps may be disrupted by the phosphorylation of these serines. There is ample precedent for this notion. Serine 649, elsewhere in the CARD11 Inhibitory Domain has been demonstrated to be phosphorylated in a step that downregulates CARD11 activity (Moreno-Garcia et al., 2009). A variety of other mechanisms have also been described that downregulate CARD11 activity following antigen receptor engagement including Bcl10 (Scharschmidt et al., 2004) and CARD11 degradation (Moreno-Garcia et al., 2010) and the action of inhibitory A20 (Coornaert et al., 2008; Duwel et al., 2009), GAKIN (Lamason et al., 2010) and RNF181 (Pedersen et al., 2016) proteins, among other mechanisms. Clearly many avenues for tuning CARD11 signaling have evolved to regulate pathway output and it is possible that the phosphorylation of the IE1 may be another.

5. Avenues for Addressing Possibilities

The resolution of these possibilities will require detailed study of CARD11 signaling along several fronts that are certain to advance our mechanistic understanding of this important signaling pathway.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Clearly, the identification of the target that recognizes the IE1 element during the Opening and Enzymatic Activation Steps is essential for resolving these issues. IE1 may be recognized by a protein *in trans* or by another domain of CARD11 that supports a conformational change that promotes Opening or Enzymatic Activation. Once this target is identified, it can be tested whether its recognition of IE1 requires the phosphorylation of serines 564, 567, or 577, or alternatively whether their phosphorylation inhibits recognition.

Another fruitful endeavor would be to use *in vitro* kinase assays with PKCθ, PKCβ, and IKKβ and the IE1 to define what other IE1 residues are part of the substrate recognition motifs of these kinases in the IE1 context. If the phosphorylation of serines 564 and 567 is required for signaling then the residues required for *in vitro* phosphorylation should be among those already shown to be important in IE1 for Opening and Enzymatic Activation Steps.

The mutation of serines 564, 567, and 577 to residues other than alanine, including the phosphomimetic aspartate and glutamate, and the assay of these mutations in the WT CARD11 and reQM contexts may also be informative, if it turns out that one of these substitutions unexpectedly does not impair signaling or promotes it.

Further study of precisely how PKCθ, PKCβ, and IKKβ function to promote CARD11 scaffold activity would also help put the potential importance of serine 564 and 567 phosphorylation in context. For example, evidence has been presented that Serine 668, outside of IE1, is also a requisite target of PKC enzymes in inducing CARD11 activity and may account for the requirement for PKCθ in the Opening Step (Shinohara et al., 2007). The identification of the kinase that phosphorylates serine 577 would also allow further resolution of the specific functional relevance of the modification of this residue.

Finally, the three dimensional structure of CARD11, at atomic resolution, in both autoinhibited and activated states would provide important information necessary for understanding the individual steps in the CARD11 signaling cycle, the role of IE1 in these steps, and how serine phosphorylation within IE1 may or may not perturb the autoinhibited conformation or promote the activated conformation of CARD11 during antigen receptor signaling.

Acknowledgments

This work was supported by National Institutes of Health Grant RO1AI143053, Leukemia and Lymphoma Society Translational Research Program Grant 6596-20, and funds from The Johns Hopkins University Institute for Cell Engineering.

References

Bedsaul JR, Carter NM, Deibel KE, Hutcherson SM, Jones TA, Wang Z, Yang C, Yang YK, and Pomerantz JL (2018). Mechanisms of Regulated and Dysregulated CARD11 Signaling in Adaptive Immunity and Disease. Front Immunol 9, 2105. [PubMed: 30283447]

Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, Sun L, Chen ZJ, Marynen P, and Beyaert R (2008). T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. Nat Immunol 9, 263–271. [PubMed: 18223652]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Duwel M, Welteke V, Oeckinghaus A, Baens M, Kloo B, Ferch U, Darnay BG, Ruland J, Marynen P, and Krappmann D (2009). A20 negatively regulates T cell receptor signaling to NF-kappaB by cleaving Malt1 ubiquitin chains. J Immunol 182, 7718–7728. [PubMed: 19494296]

Hirsch E, Gulluni F, and Martini M (2020). Phosphoinositides in cell proliferation and metabolism. Adv Biol Regul 75, 100693. [PubMed: 32008962]

Jattani RP, Tritapoe JM, and Pomerantz JL (2016a). Cooperative Control of Caspase Recruitment Domain-containing Protein 11 (CARD11) Signaling by an Unusual Array of Redundant Repressive Elements. J Biol Chem 291, 8324–8336. [PubMed: 26884335]

Jattani RP, Tritapoe JM, and Pomerantz JL (2016b). Intramolecular Interactions and Regulation of Cofactor Binding by the Four Repressive Elements in the Caspase Recruitment Domain-containing Protein 11 (CARD11) Inhibitory Domain. J Biol Chem 291, 8338–8348. [PubMed: 26884334]

Jhanwar-Uniyal M, Wainwright JV, Mohan AL, Tobias ME, Murali R, Gandhi CD, and Schmidt MH (2019). Diverse signaling mechanisms of mTOR complexes: mTORC1 and mTORC2 in forming a formidable relationship. Adv Biol Regul 72, 51–62. [PubMed: 31010692]

Juilland M, and Thome M (2016). Role of the CARMA1/BCL10/MALT1 complex in lymphoid malignancies. Curr Opin Hematol 23, 402–409. [PubMed: 27135977]

Juilland M, and Thome M (2018). Holding All the CARDs: How MALT1 Controls CARMA/CARD-Dependent Signaling. Front Immunol 9, 1927. [PubMed: 30214442]

Lamason RL, Kupfer A, and Pomerantz JL (2010). The dynamic distribution of CARD11 at the immunological synapse is regulated by the inhibitor kinesin GAKIN. Molecular cell 40, 798–809. [PubMed: 21145487]

Lork M, Staal J, and Beyaert R (2019). Ubiquitination and phosphorylation of the CARD11-BCL10-MALT1 signalosome in T cells. Cellular immunology 340, 103877. [PubMed: 30514565]

Lu HY, Biggs CM, Blanchard-Rohner G, Fung SY, Sharma M, and Turvey SE (2019). Germline CBM-opathies: From immunodeficiency to atopy. The Journal of allergy and clinical immunology 143, 1661–1673. [PubMed: 31060714]

Matsumoto R, Wang D, Blonska M, Li H, Kobayashi M, Pappu B, Chen Y, and Lin X (2005). Phosphorylation of CARMA1 Plays a Critical Role in T Cell Receptor-Mediated NF-kappaB Activation. Immunity 23, 575–585. [PubMed: 16356856]

McCully RR, and Pomerantz JL (2008). The protein kinase C-responsive inhibitory domain of CARD11 functions in NF-kappaB activation to regulate the association of multiple signaling cofactors that differentially depend on Bcl10 and MALT1 for association. Mol Cell Biol 28, 5668–5686. [PubMed: 18625728]

Meininger I, and Krappmann D (2016). Lymphocyte signaling and activation by the CARMA1-BCL10-MALT1 signalosome. Biol Chem 397, 1315–1333. [PubMed: 27420898]

Moreno-Garcia ME, Sommer K, Haftmann C, Sontheimer C, Andrews SF, and Rawlings DJ (2009). Serine 649 phosphorylation within the protein kinase C-regulated domain downregulates CARMA1 activity in lymphocytes. J Immunol 183, 7362–7370. [PubMed: 19917688]

Moreno-Garcia ME, Sommer K, Shinohara H, Bandaranayake AD, Kurosaki T, and Rawlings DJ (2010). MAGUK-controlled ubiquitination of CARMA1 modulates lymphocyte NF-kappaB activity. Mol Cell Biol 30, 922–934. [PubMed: 20008554]

Pedersen SM, Chan W, Jattani RP, Mackie d S, and Pomerantz JL (2016). Negative Regulation of CARD11 Signaling and Lymphoma Cell Survival by the E3 Ubiquitin Ligase RNF181. Mol Cell Biol 36, 794–808.

Rahimova N, Cooke M, Zhang S, Baker MJ, and Kazanietz MG (2020). The PKC universe keeps expanding: From cancer initiation to metastasis. Adv Biol Regul 78, 100755. [PubMed: 33017725]

Scharschmidt E, Wegener E, Heissmeyer V, Rao A, and Krappmann D (2004). Degradation of Bcl10 induced by T-cell activation negatively regulates NF-kappa B signaling. Mol Cell Biol 24, 3860–3873. [PubMed: 15082780]

Shinohara H, Behar M, Inoue K, Hiroshima M, Yasuda T, Nagashima T, Kimura S, Sanjo H, Maeda S, Yumoto N, et al. (2014). Positive feedback within a kinase signaling complex functions as a switch mechanism for NF-kappaB activation. Science 344, 760–764. [PubMed: 24833394]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Shinohara H, Maeda S, Watarai H, and Kurosaki T (2007). IkappaB kinase beta-induced phosphorylation of CARMA1 contributes to CARMA1 Bcl10 MALT1 complex formation in B cells. J Exp Med 204, 3285–3293. [PubMed: 18086859]

Sommer K, Guo B, Pomerantz JL, Bandaranayake AD, Moreno-Garcia ME, Ovechkina YL, and Rawlings DJ (2005). Phosphorylation of the CARMA1 Linker Controls NF-kappaB Activation. Immunity 23, 561–574. [PubMed: 16356855]

Thome M, Charton JE, Pelzer C, and Hailfinger S (2010). Antigen Receptor Signaling to NF-{kappa}B via CARMA1, BCL10, and MALT1. Cold Spring Harb Perspect Biol 2, a003004. [PubMed: 20685844]

Thys A, Douanne T, and Bidere N (2018). Post-translational Modifications of the CARMA1-BCL10-MALT1 Complex in Lymphocytes and Activated B-Cell Like Subtype of Diffuse Large B-Cell Lymphoma. Front Oncol 8, 498. [PubMed: 30474008]

Wang Z, Hutcherson SM, Yang C, Jattani RP, Tritapoe JM, Yang YK, and Pomerantz JL (2019). Coordinated regulation of scaffold opening and enzymatic activity during CARD11 signaling. J Biol Chem 294, 14648–14660. [PubMed: 31391255]

Yang YK, Yang C, Chan W, Wang Z, Deibel KE, and Pomerantz JL (2016). Molecular Determinants of Scaffold-induced Linear Ubiquitinylation of B Cell Lymphoma/Leukemia 10 (Bcl10) during T Cell Receptor and Oncogenic Caspase Recruitment Domain-containing Protein 11 (CARD11) Signaling. J Biol Chem 291, 25921–25936. [PubMed: 27777308]