Intramolecular Interactions and Regulation of Cofactor Binding by the Four Repressive Elements in the Caspase Recruitment Domain-containing Protein 11 (CARD11) Inhibitory Domain**

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The CARD11 signaling scaffold transmits signaling between antigen receptors on B and T lymphocytes and the transcription factor NF-κB during the adaptive immune response. CARD11 activity is controlled by an inhibitory domain (ID), which participates in intramolecular interactions and prevents cofactor binding prior to receptor triggering. Oncogenic CARD11 mutations associated with the activated B cell-like subtype of diffuse large B cell lymphoma somehow perturb ID-mediated autoinhibition to confer CARD11 with the dysregulated spontaneous signaling to NF-κB that is required for the proliferation and survival of the lymphoma. Here, we investigate how the four repressive elements (REs) we have discovered in the CARD11 ID function to inhibit CARD11 activity with cooperativity and redundancy. We find that each RE contributes to the maintenance of the closed inactive state of CARD11 that predominates in the absence of receptor engagement. Each RE also contributes to the prevention of Bcl10 binding in the basal unstimulated state. RE1, RE2, and RE3 participate in intramolecular interactions with other CARD11 domains and share domain targets for binding. Remarkably, diffuse large B cell lymphoma-associated gain-of-function mutations in the caspase recruitment domain, LATCH, or coiled coil can perturb intramolecular interactions mediated by multiple REs, suggesting how single amino acid oncogenic CARD11 mutations can perturb or bypass the action of redundant inhibitory REs to achieve the level of hyperactive CARD11 signaling required to support lymphoma growth.

The activation of the transcription factor NF-κB by antigen receptor signaling is required for antigen-elicted lymphocyte activation during the adaptive immune response (1). Both T cell receptor and B cell receptor complexes signal to NF-κB through the IKK1/2 complex, which inducibly phosphorylates inhibitory IκB proteins leading to their ubiquitylation and degradation and the resultant stable nuclear translocation of NF-κB. CARD11 is a large multidomain scaffold protein that transmits signaling from both the T cell receptor and B cell receptor to the IKK complex (2–10). CARD11 functions as a signal-dependent scaffold. Prior to receptor engagement, CARD11 is kept in an inactive state through an inhibitory domain (ID) that participates in intramolecular interactions with the caspase recruitment domain (CARD) and coiled coil (11). Upon antigen receptor ligation, the autoinhibitory activity of the ID is neutralized, in part, through the phosphorylation of several serine residues within the ID (12–16). This neutralization of the ID converts CARD11 into an active scaffold that allows the CARD and coiled coil to recruit multiple signaling proteins, including Bcl10, MALT1, TRAF6, TAK1, caspase-8, and IKKγ, into a complex that activates IKK kinase activity.

The antigen receptor signaling pathway is frequently dysregulated in the activated B cell-like (ABC) subtype of diffuse large B cell lymphoma (DLBCL), the most common form of non-Hodgkin lymphoma (17). In ABC DLBCL, NF-κB is constitutively activated due to a variety of genetic lesions that generate loss-of-function alleles of pathway inhibitors or gain-of-function alleles of positively acting signaling components (18). Dysregulated NF-κB activity is required for the growth and proliferation of ABC DLBCL cells in culture (19). CARD11 is required for the aberrant signaling to NF-κB in ABC DLBCL (20), and ~10% of human ABC DLBCL biopsies exhibit oncogenic CARD11 alleles with gain-of-function mutations (21).

DLBCL-associated oncogenic CARD11 mutations have been found in the CARD, LATCH, and coiled-coil domains (21–25). Previous studies have shown that these single amino acid mutations hyperactivate CARD11 signaling activity by interfering with autoinhibition by the ID (26, 27). Oncogenic CARD11 mutations in the CARD, LATCH, and coiled coil disrupt intramolecular binding of the ID and lead to the spontaneous recruitment of Bcl10 to CARD11 in the absence of signal-induced ID neutralization, which promotes the dysregulated acti-

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The abbreviations used are: IKK, IκB kinase; ID, inhibitory domain; DLBCL, diffuse large B cell lymphoma; RE, repressive element; NT, non-target; AE, activating element; CARD, caspase recruitment domain; ABC, activated B cell-like; PMA, phorbol myristate acetate; IPLB, immunoprecipitation lysis buffer; SH, Src homology; GUK, guanylate kinase.
vation of the IKK complex and NF-κB (26, 27). However, no oncogenic gain-of-function mutations in the ID itself have been reported in DLBCL. In the accompanying paper (28), we resolved this apparent paradox by defining the existence of four repressive elements (REs) in the CARD11 ID that function cooperatively with redundancy to autoinhibit CARD11 prior to receptor engagement. The redundant properties of the REs make single amino acid mutations in the ID unable to activate CARD11 signaling activity to the extent that point mutations in the CARD, LATCH, and coiled coil can achieve. Here, we investigate which of the four REs participate in inhibitory interactions with other CARD11 domains, map their intramolecular targets, and address whether the REs control signaling cofactor binding and, if so, whether they do so cooperatively. Finally, we investigate how DLBCL-associated oncogenic mutations affect RE function in a manner that can result in the observed robust dysregulated CARD11-mediated signaling to NF-κB.

**Experimental Procedures**

**Cell Lines and Expression Constructs—**HEK293T and human Jurkat T cell lines were cultured as described previously (29). pc-CARD11, which expresses murine CARD11 with an N-terminal Myc tag, CARD11ΔID, and the panel of ΔID double deletions have been described (11), as have ΔID-F130I and ΔID-L232LI (previously referred to as ΔID-F123I and ΔID-L225LI (26)), and ΔID-C49Y, ΔID-F97Y, and ΔID-G123D (27). The ΔIDΔLATCH construct deletes residues 116–149 and 441–671; we note that the LATCH has been previously defined to occupy residues 112–130 (27). CARD11 variants with RE mutations are described in the accompanying paper (28). FLAG-Bcl10, FLAG-TRAF6, FLAG-IKKγ, and FLAG-Casp8 C360S have been described (11). To generate pEBB-FLAG-GST-ID, a DNA fragment of CARD11 corresponding to amino acids 441–671 was cloned into pEBB in-frame with an N-terminal FLAG tag and GST derived from pEBG (11). pEBB-FLAG-GST is a variant of the above construct that encodes a stop codon after GST. To generate FLAG-GST-RE multimers, the full-length ID in pEBB-FLAG-GST-ID was replaced with four copies of the DNA encoding RE1, RE2, RE3, or RE4 corresponding to CARD11 residues 441–493, 586–599, 600–610, or 617–641, respectively.

**HEK293T Immunoprecipitations—**Immunoprecipitations were performed as described previously (27), except for the following. Cells were cotransfected with 4–700 ng of expression vector for each CARD11 variant and 110–400 ng of FLAG-Bcl10, 600 ng of FLAG-TRAF6, 1300 ng of FLAG-IKKγ, or 1350 ng of FLAG-Casp8-C360S. At 40–42 h post-transfection, cells were harvested in 500 μl of immunoprecipitation lysis buffer (IPLB), incubated on ice for 10 min, scraped off, and centrifuged at 17,970 g for 5 min at 4 °C. Eleven microliters or 21 μl of lysate was reserved for Western blot analysis and 440 μl or 420 μl of the remaining lysate was used for immunoprecipitation with anti-FLAG antibody (Sigma F7425). Input and immunoprecipitation elutions were resolved by immunoblot as described previously (27). Results shown are the representative of three assays performed.

**HEK293T Glutathione-Sephrose Pulldowns—**Cells were plated at 5 × 10^5 cells per well in 2 ml of media in 6-well plates 22–23 h prior to transfection. Cells were transfected using the calcium phosphate method with 500–1600 ng of FLAG-GST variants and 50–900 ng of CARD11 variants. In each experiment, additional pcDNA3 and pEBB vectors were transfected such that each well was transfected with equal amounts of pcDNA3 vector and pEBB vector and totaled 2 μg of DNA. Media were changed 22–24.5 h post-transfection and harvested as described previously (11), except as follows. Cells were lysed in 500 μl of IPLB and precleared twice for 30 min with a 10-μl bed volume of protein G-Sepharose Fast Flow (GE Healthcare) with 4 °C rotation. From the precleared lysate, 2 or 3% of lysate was reserved for input. 440 μl of precleared lysate was incubated with a 10-μl bed volume of glutathione-Sepharose (GE Healthcare) for 3 h at 4 °C with rotation. Samples were washed and analyzed as described previously (11). Western blotting was completed using anti-Myc (Santa Cruz Biotechnology, sc-40) and anti-GST (Santa Cruz Biotechnology, sc-459) antibodies. Results shown are representative of two or three assays that were performed.

**Jurkat T Cell Immunoprecipitations—**To stably express Myc-tagged CARD11 variants in Jurkat T cells, cDNAs were cloned into the pCLIP3A vector; retroviruses were packaged, and Jurkat T cells were infected as described previously (27). Infected cells were selected and maintained in the presence of 0.5 μg/ml puromycin. For some constructs, to obtain Jurkat T cells with equivalent expression levels of CARD11 variants, cell clones were isolated by limiting dilution and expanded.

Control Jurkat T cells or Jurkat T cells stably expressing Myc-tagged CARD11 variants were resuspended to a final concentration of 5 × 10^6 cells/ml and treated with or without 50 ng/ml phorbol myristate acetate (PMA) (Sigma) and 1 μM ionomycin (Sigma) at 37 °C as follows. For Bcl10 immunoprecipitations, 7.5 × 10^7 cells were untreated or stimulated for 15 min. To test IKKα immunoprecipitations, 1 × 10^7 cells were untreated or stimulated for 15 min. To assess TRAF6 immunoprecipitations, 9 × 10^7 cells were untreated or stimulated for 10 min. Caspase-8 immunoprecipitations were assessed with 9 × 10^7 cells untreated or stimulated for 12 min. After stimulation, cells were incubated in an ice-water bath for 10 min, spun at 423 × g for 5 min at 4 °C, and lysed in 1.3 ml of IPLB with protease inhibitor mixture (Sigma P8340) or IPLB containing protease inhibitor mixture (Sigma P8340). 2.5 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM β-glycerophosphate. Cells were incubated with lysis buffer for 15 min on ice, and cell debris was cleared by centrifugation at 18,000 × g at 4 °C. Cell lysates were precleared twice with a 14-μl bed volume of protein A-Sepharose (Sigma P9439) or protein G-Sepharose beads (GE Healthcare) for 30 min at 4 °C with rotation. One percent of the precleared lysate was saved for analysis by Western blotting, and the remaining fraction was incubated with 2 μg of anti-Bcl10 (Santa Cruz Biotechnology sc-5273), anti-IKKα (Santa Cruz Biotechnology sc-7606), anti-TRAF6 (Santa Cruz Biotechnology sc-8409), or anti-caspase-8 (Santa Cruz Biotechnology sc-6136) antibody overnight at 4 °C with rotation. A 14-μl bed volume of protein A-Sepharose or protein G-Sepharose that had been blocked with 1% insulin (Sigma I9278) was added to each sample and incubated for 2 h at 4 °C with rotation. The resulting immunocomplex was washed with IPLB four times.
for 5 min at 4 °C with rotation. The samples were boiled in SDS-PAGE loading buffer, resolved on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and analyzed by Western blotting using anti-Myc (Cell Signaling 2278), anti-Bcl10 (Sigma sc-5611), anti-IKKα (Santa Cruz Biotechnology sc-7218), anti-TRAF6 (Santa Cruz Biotechnology sc-7221), or anti-caspase-8 (Santa Cruz Biotechnology sc-7890) antibodies.

Results

REs Cooperatively Promote Intramolecular Interaction between the ID and Other CARD11 Domains—In our accompanying paper (28), we described the presence of four REs in the CARD11 ID, termed RE1, RE2, RE3, and RE4, which function cooperatively to repress CARD11 signaling to NF-κB prior to antigen receptor triggering (Fig. 1).

Previous work demonstrated that under basal unstimulated conditions, the ID as a whole maintains CARD11 in a closed inactive state in part by participating in intramolecular interactions that involve the CARD and coiled coil and prevent the association of CARD11 with the signaling cofactors Bcl10, TRAF6, caspase-8, and IKKγ (11). To probe the contribution of the four REs to the maintenance of the closed inactive state of CARD11, we assessed the ability of a GST-ID fusion to associate with wild-type CARD11, the ΔID, and variants of full-length CARD11 containing all combinations of mutated REs after coexpression in HEK293T cells (Fig. 2). For ease of representation, the presence of a mutated RE in a construct is indicated by lowercase letters (e.g. re1), and the presence of a wild-type RE is indicated by capital letters (e.g. RE1). As shown previously (11), the GST-ID robustly associated with the ΔID but did not associate with wild-type CARD11, presumably because the ID present in cis prevented the association of the GST-ID in trans (Fig. 2A). Mutation of all four REs in the re1 re2 re3 re4 quadruple RE mutant allowed the GST-ID to associate, indicating that one or more of the REs are required for ID-mediated intramolecular interactions (Fig. 2A).

The mutation of any single RE was insufficient to allow robust GST-ID binding, as compared with that observed with the re1 re2 re3 re4 quadruple RE mutant (Fig. 2A), although the mutation of either RE1 or RE4 resulted in detectable binding that was higher than that observed with wild-type CARD11.

The combined mutation of any two REs resulted in GST-ID binding that was greater than that observed with single RE mutants (Fig. 2B), while three of four triple RE mutants displayed binding to the GST-ID that was equivalent to that observed with the re1 re2 re3 re4 quadruple RE mutant (Fig. 2C). Thus, any combination of three wild-type REs in cis was efficient at preventing GST-ID binding in trans (Fig. 2A) and could do so to a greater extent than any combination of two wild-type REs (Fig. 2B). These results indicated that all four REs contribute cooperatively to intramolecular interactions with other domains in CARD11 and that there is not a specific single RE or subset of REs that controls these intramolecular interactions.

REs Control Cofactor Binding to CARD11—The ID has previously been shown to regulate the association of CARD11 with Bcl10 (11). To test whether the REs control this association, we compared the re1 re2 re3 re4 quadruple RE mutant to wild-type CARD11 and the ΔID for binding to FLAG-Bcl10 after coexpression in HEK293T cells and immunoprecipitation with anti-FLAG antibodies. As shown previously (11), FLAG-Bcl10 did not associate with wild-type CARD11 but readily bound the ΔID (Fig. 3A). As shown in Fig. 3A, mutation of all four REs in the re1 re2 re3 re4 quadruple RE mutant allowed Bcl10 to bind CARD11, indicating that the REs prevent its association in the absence of antigen receptor engagement.

To verify that the four REs control Bcl10 binding at physiological levels of expression in T cells, we stably expressed the Myc-tagged re1 re2 re3 re4 quadruple RE mutant and wild-type CARD11 control in Jurkat T cells by infection with Moloney murine leukemia virus-based retroviruses. As expected, wild-type CARD11 did not associate under basal conditions with endogenous Bcl10 (Fig. 3B) but did associate after PMA/ionomycin treatment. In contrast, the re1 re2 re3 re4 quadruple RE mutant readily associated with endogenous Bcl10 in the absence of stimulation (Fig. 3B), verifying that the REs prevent Bcl10 from associating with CARD11 at physiological levels in T cells. Stimulation with PMA/ionomycin also increased the observed association of the re1 re2 re3 re4 quadruple RE mutant with Bcl10. This increase is likely due to the oligomerization of the Myc-tagged re1 re2 re3 re4 quadruple RE mutant with the endogenous untagged wild-type CARD11 and the response of the latter to PMA/ionomycin.

We also tested whether the REs control the binding of CARD11 to IKKγ, TRAF6, and caspase-8, cofactors whose association has also been previously shown to be ID-regulated (11). As shown previously (11), these cofactors did not associate with wild-type CARD11 but readily bound the ΔID (Fig. 3, C, E, and G) after coexpression in HEK293T cells. As shown in Fig. 3, C, E, and G, mutation of all four REs in the re1 re2 re3 re4 quadruple RE mutant allowed each of these cofactors to associate with CARD11, indicating that the REs prevent their association in the absence of antigen receptor engagement. Consistent with these results, the re1 re2 re3 re4 quadruple RE mutant readily associated with the endogenous IKK complex, TRAF6, and caspase-8 in Jurkat T cells under basal conditions after retroviral expression (Fig. 3, D, F, and H), verifying that the REs prevent these cofactors from associating with CARD11 at physiological levels in T cells. The fact that the re1 re2 re3 re4 quadruple RE mutant was capable of binding these four cofactors provided further evidence that the mutations we introduced to
FIGURE 2. RE1, RE2, RE3, and RE4 cooperatively contribute to CARD11 intramolecular interactions. A–C, HEK293T cells were transfected with expression vectors for the indicated Myc-tagged CARD11 variants and for GST-ID or GST as indicated. Glutathione-Sepharose pulldowns were performed as described under “Experimental Procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies. The presence of a mutated RE in a construct is indicated by blue lowercase letters (e.g. re1), and the presence of a wild-type RE is indicated by red capital letters (e.g. RE1).
Functional Properties of Four Repressive Elements in CARD11

Impair RE function do not perturb the folding of CARD11 domains outside of the ID.

**REs Cooperatively Prevent Bcl10 Association with CARD11**—We next assessed which of the four REs are specifically required to prevent the association of CARD11 with Bcl10. We focused our attention on Bcl10 because previous work demonstrated that gain-of-function CARD11 variants with oncogenic potential selectively allow the recruitment of Bcl10, but not other cofactors, to CARD11 in the absence of antigen receptor engagement, thus highlighting Bcl10 recruitment as a key step in CARD11 signaling (26, 27). CARD11 variants containing all combinations of mutated REs were co-expressed with FLAG-Bcl10 after expression in HEK293T cells. Single RE mutants did not appreciably bind Bcl10 (Fig. 4A), indicating that any combination of three wild-type REs was efficient at preventing Bcl10 binding and that a single specific RE is not solely responsible for.

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**FIGURE 3. REs prevent CARD11 association with Bcl10, TRAF6, IKKγ, and caspase-8.** HEK293T cells were transfected with expression vectors for the indicated Myc-tagged CARD11 variants and FLAG-Bcl10 (A), FLAG-IKKγ (C), TRAF6 (E), or FLAG-caspase-8 C360S (G), as indicated. Anti-FLAG immunoprecipitations (IP) were performed as described under “Experimental Procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies. Jurkat T cells were retrovirally transduced to express the indicated Myc-tagged CARD11 variants and stimulated with PMA/ionomycin as indicated. Immunoprecipitations were performed using antibodies to endogenous Bcl10 (B), IKKγ (D), TRAF6 (F), and caspase-8 (H), as described under “Experimental Procedures” and analyzed by Western blotting with the indicated primary antibodies.
limiting Bcl10 association. The mutation of any two REs resulted in Bcl10 binding that was greater than that observed for any single RE mutant (Fig. 4B), and this binding was further enhanced by the mutation of a third RE (Fig. 4C). The binding observed with the triple RE mutants suggested that any single RE is incapable of completely preventing Bcl10
binding. We conclude that all four REs contribute cooperatively to prevent Bc110 association.

We then stably expressed single RE mutants in Jurkat T cells by retroviral transduction and compared their abilities to associate with endogenous Bc110 with that exhibited by the re1 re2 re3 re4 quadruple RE mutant. As shown in Fig. 4D, the mutation of any single RE did not lead to appreciable binding to endogenous Bc110, whereas mutation of all four REs allowed Bc110 to associate with CARD11. These results verified in T cells that no single RE is individually responsible for preventing Bc110 binding in the absence of receptor signaling, and they are consistent with the conclusion that the REs work cooperatively to prevent cofactor association with CARD11.

RE1, RE2, and RE3 Participate in Intramolecular Interactions with Overlapping Targets—To address which REs contribute to ID-mediated intramolecular interactions, we constructed GST fusions of each RE and assayed their abilities to pull down the ΔID variant after coexpression in HEK293T cells. However, while the full-length ID fused to GST, GST-ID, could robustly associate with the ΔID (Fig. 2), none of the single REs when fused to GST could do so (data not shown). We suspected that the binding affinity of a single RE in trans would not be sufficient to elicit a detectable interaction in this assay. Therefore we tested the binding potential of GST fusions containing four copies of each RE. We found that the GST-RE1, GST-RE2, and GST-RE3 multimers could readily pull down the ΔID, whereas the GST-RE4 multimer elicited only a very weak association (Fig. 5, A and B), indicating that RE1, RE2, and RE3 can participate in intramolecular interactions.

We also tested whether each RE multimer could bind to the full-length CARD11 mutant containing mutations in the cognate RE. For example, we probed whether GST-RE1 could bind to the re1 RE2 RE3 RE4 mutant to test whether the mutation of RE1 exposed a binding target for the GST-RE1 in trans. Interestingly, we did not detect this interaction (Fig. 5A), suggesting that in the re1 RE2 RE3 RE4 mutant the binding target for RE1 is occluded such that the GST-RE1 multimer cannot bind. Similarly, we did not detect binding of GST-RE2 to the re1 re2 RE3 RE4 mutant or binding of GST-RE3 to the re1 RE2 re3 RE4 mutant (Fig. 5, A and B). These results suggested that the REs may bind to overlapping targets within CARD11.

Next, we used a panel of double-domain deletion mutants in the ΔID context to map the domain targets for RE multimer binding. The binding of GST-RE1 to the ΔID was most severely reduced by deletion of the coiled coil and the GUK domain and, surprisingly, was enhanced by deletion of the L3 and SH3 domains (Fig. 6A and Table 1). The deletions that had the largest effect on GST-RE2 binding included those in the CARD, LATCH region, coiled coil, and L3 domain (Fig. 6B and Table 1). Finally, deletions of the CARD and coiled coil most severely affected GST-RE3 binding (Fig. 6C and Table 1). The results are consistent with these three REs binding overlapping targets with some RE-specific determinants. For example, the CARD domain appears to be targeted by RE2 and RE3, whereas the coiled coil is targeted by RE1, RE2, and RE3. The LATCH region appears to be uniquely required for RE2 binding, whereas the GUK domain contributes mostly to RE1 binding.

Disruption of RE-mediated Intramolecular Interactions by DLBCL-associated Mutations in the CARD, LATCH, and Coiled-coil Domains—Gain-of-function CARD11 mutations associated with diffuse large B cell lymphoma have been found in the CARD, LATCH, and coiled-coil domains of CARD11. We have previously shown that mutations in each of these domains perturb ID-mediated autoinhibition and intramolecular binding, as assayed by effects on the binding of GST-ID to ΔID variants (26, 27). To determine whether gain-of-function mutations affect the intramolecular binding of one or more REs, we assayed the ability of GST-RE multimers to bind ΔID variants containing gain-of-function mutations C49Y and F97Y in the CARD domain, G123D and F130I in the LATCH domain, and L232LI in the coiled-coil domain. As shown in Fig. 7 and Table 2, each of these five mutations negatively affected the binding of GST-RE1, GST-RE2, and GST-RE3, but to differing degrees. C49Y and F97Y severely affected GST-RE2 binding and also had clear effects on GST-RE1 and GST-RE3 binding. G123D had strong effects on the binding of GST-RE1, GST-RE2, and GST-RE3, whereas F130I had larger effects on GST-RE2 and GST-RE3 than on GST-RE1 binding. The largest effect of the L232LI insertion was on GST-RE1 binding.
Figure 6. Domain requirements for intramolecular binding by RE1, RE2, and RE3. A–C, HEK293T cells were transfected with expression vectors for the indicated Myc-tagged CARD11 variants and for GST-RE14 (A), GST-RE24 (B), GST-RE34 or GST (C), as indicated. Glutathione-Sepharose pulldowns were performed as described under “Experimental Procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies.
but it also had measurable effects on the binding of GST-RE2, and GST-RE3. The results are consistent with the conclusion that potent oncogenic CARD11 mutations perturb intramolecular binding by multiple REs as part of the mechanism by which they induce constitutive hyperactive CARD11 signaling.

**Discussion**

Our results illuminate how the unusual array of REs in the CARD11 ID, defined in our accompanying paper (28), can function cooperatively with redundancy to prevent CARD11 signaling prior to antigen receptor triggering. Each of the four REs contributes to keeping CARD11 in the closed inactive state, as revealed by the cooperative manner in which they prevent the binding of the GST-ID in trans (Fig. 2), and each RE contributes to the prevention of spontaneous Bcl10 binding (Fig. 4). Furthermore, the data begin to explain how single amino acid mutations in the CARD, LATCH, or coiled-coil, associated with DLBCL, can disrupt or bypass repression by multiple REs. They appear to do so in part by perturbing intramolecular interactions involving RE1, RE2, and RE3 (Fig. 7).

Interestingly, RE1, RE2, and RE3 appear to bind to overlapping targets in the other CARD11 domains, which may partly explain their functional redundancy. For example, both RE2 and RE3 require the CARD domain for binding, and the coiled-coil domain is required for optimal binding by RE1, RE2, and RE3. Gain-of-function mutations in the CARD, LATCH, or coiled-coil domains may perturb these intramolecular interactions by altering the protein-protein interface between REs and their targets or by otherwise converting CARD11 into a conformation that is incompatible with maximal repressive RE binding. Our data do not discriminate between these possibilities.

The requirement of the GUK domain for RE1 association reveals a previously unrecognized role for the GUK domain in ID binding and CARD11 basal repression. Interestingly, the GUK domain has been shown recently to bind to the SH3 domain in an intramolecular interaction that may regulate CARD11 signaling potential (30). The fact that the deletion of the SH3 stimulates RE1 intramolecular binding suggests that RE1 and the SH3 domain may bind to overlapping epitopes in the GUK domain. The stimulatory effect of the L3 deletion on RE1 binding may also occur as a result of perturbing SH3/GUK interactions.

RE4 does not appear to participate strongly in intramolecular interactions, despite the fact that our quantitative reporter analysis in the accompanying paper (28) suggests that RE4 is perhaps the most potent RE. RE4 clearly promotes the closed

### Table 1

| Effect of domain deletions on RE binding to ΔID | Relative binding* |
|-----------------------------------------------|-------------------|
|                              | GST-RE1            | GST-RE2            | GST-RE3            |
| ΔID                        | 1.00              | 1.00              | 1.00              |
| ΔID-CARD                   | 0.79              | 1.30              | 1.27              |
| ΔID-LATCH                  | 2.05              | 0.41              | 1.26              |
| ΔID-CC                     | 0.42              | 1.17              | 1.54              |
| ΔID-ΔL3                    | 0.79              | 1.30              | 1.27              |
| ΔID-ΔSH3                   | 0.70              | 1.03              | 0.87              |
| ΔID-ΔL4                    | 0.23              | 0.75              | 1.15              |

* The relative binding in each column was calculated by determining the bound/input ratio for each CARD11 variant and normalizing to that observed with the ΔID after densitometric analysis of the blots in Fig. 6.

### Table 2

| Effect of oncogenic mutations on RE binding to ΔID | Relative binding* |
|-----------------------------------------------|-------------------|
|                              | GST-RE1            | GST-RE2            | GST-RE3            |
| ΔID                        | 1.00              | 1.00              | 1.00              |
| ΔID-C49Y                   | 0.33              | 0.06              | 0.26              |
| ΔID-F97Y                   | 0.46              | 0.05              | 0.63              |
| ΔID-G123D                  | 0.12              | 0.05              | 0.16              |
| ΔID-F130I                  | 0.38              | 0.20              | 0.15              |
| ΔID-L232LI                 | 0.23              | 0.73              | 0.53              |

* The relative binding in each column was calculated by determining the bound/input ratio for each CARD11 variant and normalizing to that observed with the ΔID after densitometric analysis of the blots in Fig. 7.

**FIGURE 7.** DLBCL-associated gain-of-function CARD11 mutations perturb intramolecular binding by RE1, RE2, and RE3. A–C, HEK293T cells were transfected with expression vectors for the indicated Myc-tagged CARD11 ΔID variants and for GST-RE1, GST-RE2, or GST (C) as indicated. Glutathione-Sepharose pulldowns were performed as described under “Experimental Procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies.
in active state of CARD11, judging by the contribution RE4 makes to the prevention of GST-ID binding in trans (Fig. 2). It is possible that poor detection of RE4-mediated intramolecular interactions was due to the configuration of the GST–RE4 multimer used in the association studies, which may not reflect the true potential of RE4 to bind to other CARD11 domains in cis. Alternatively, RE4 may repress by recruiting an unidentified inhibitor to CARD11. Further experiments will be required to resolve how RE4 inhibits CARD11 signaling and whether DLBCL-associated mutations interfere with RE4-mediated repression. It is likely that they do, because the combined mutation of RE1, RE2, and RE3 results in only 28-fold higher activity than wild-type CARD11 (see the accompanying paper (28)), a level surpassed by potent oncogenic mutations.

Previous studies have indicated that gain-of-function DLBCL-associated CARD11 mutations selectively allow the spontaneous recruitment of Bcl10 to CARD11 but not that of other signaling cofactors examined (26, 27). The binding of Bcl10 to these CARD11 variants results in the polyubiquitination of Bcl10 (27), which allows it to associate with the IKK complex in a signaling step that is also required in the normal antigen receptor signaling pathway (31). Our analysis indicates that no single RE is responsible for preventing Bcl10 association in the basal wild-type state; rather, all four REs contribute to blocking Bcl10 binding. Because Bcl10 requires two RE targets, the CARD and coiled coil, for association (11), it is possible that the REs occupy and sterically occlude the binding surface recognized by Bcl10. Alternatively, the REs may promote a CARD11 conformation that is incompatible with Bcl10 binding without directly competing in cis for a binding interface. Although our data indicate that the REs also control the association of TRAF6, IKKγ, and caspase-8 (Fig. 3), it is important to note that the REs must be regulating steps in CARD11 signaling in addition to the recruitment of Bcl10 and these cofactors. This is made most apparent by the fact that although the re1 re2 re3 re4 quadruple RE mutant is 6.7-fold more active than the ΔID, the re1 re2 re3 re4 quadruple RE mutant clearly does not display a higher apparent affinity for the cofactors than does the ΔID. This lack of correlation between signaling output and cofactor binding clearly indicates that one or more signaling steps that remain undefined, but which are also regulated by the REs, determines the signaling output of CARD11.

Clearly, CARD11 has evolved a remarkable strategy for preventing spontaneous signaling to NF-κB, an array of four REs that function cooperatively with redundancy. The REs are not predicted to form stable folded structures independently (28), but it is possible they assume induced structures upon binding to other CARD11 domains in cis or other targets in trans. Elucidation of their bound three-dimensional structures will be required to fully understand the inhibition they mediate and how their inhibitory interactions can be neutralized by antigen receptor engagement in a process that involves the phosphorylation of serines 564, 567, 577, and 657 in the ID (12, 13, 15, 16).

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References

1. Schulze-Luehrmann, J., and Ghosh, S. (2006) Antigen-receptor signaling to nuclear factor κB. *Immunity* 25, 701–715
2. Gaidle, O., Favier, B., Legler, D. F., Bonnet, D., Brissoni, B., Valitutti, S., Bron, C., Tschopp, J., and Thome, M. (2002) CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-κB activation. *Nat. Immunol.* 3, 836–843
3. Wang, D., You, Y., Case, S. M., McAllister-Lucas, L. M., Wang, L., DiStefano, P. S., Nuñez, G., Bertin, J., and Lin, X. (2002) A requirement for CARMA1 in TCR-induced NF-κB activation. *Nat. Immunol.* 3, 830–835
4. Pomerantz, J. L., Denny, E. M., and Baltimore, D. (2002) CARD11 mediates factor-specific activation of NF-κB by the T cell receptor complex. *EMBO J.* 21, 5184–5194
5. Hará, H., Wada, T., Bakal, C., Koziarzadki, L., Suzuki, S., Suzuki, N., Nghiem, M., Griffiths, E. K., Krawczyk, C., Bauer, D., D’Acquisto, F., Ghosh, S., Yeh, W. C., Baier, G., Rottapel, R., and Penninger, J. M. (2003) The MAGUK family protein CARD11 is essential for lymphocyte activation. *Immunity* 18, 763–775
6. Jun, J. E., Wilson, L. E., Vinuesa, C. G., Lesage, S., Bley, M., Miosge, L. A., Cook, M. C., Kucharska, E. M., Hará, H., Penninger, J. M., Domashen, H., Hong, N. A., Glynne, R. J., Nelms, K. A., and Goodnow, C. C. (2003) Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. *Immunity* 18, 751–762
7. Egawa, T., Albrecht, B., Favier, B., Sunshine, M. J., Mirchandani, K., O’Brien, W., Thome, M., and Littman, D. R. (2003) Requirement for CARMA1 in antigen receptor-induced NF-κB activation and lymphocyte proliferation. *Curr. Biol.* 13, 1252–1258
8. Newton, K., and Dixit, V. M. (2003) Mice lacking the CARD of CARMA1 exhibit defective B lymphocyte development and impaired proliferation of their B and T lymphocytes. *Curr. Biol.* 13, 1247–1251
9. Steppensky, P., Keller, B., Buchta, M., Kiemler, A. K., Elpeleg, O., Somech, R., Cohen, S., Shachar, I., Miosge, L. A., Schlesier, M., Fuchs, I., Enders, A., Eibl, H., Grimbacher, B., and Warnatz, K. (2013) Deficiency of caspase recruitment domain family member 11 (CARD11), causes profound combined immunodeficiency in human subjects. *J. Allergy Clin. Immunol.* 131, 477–485 e471
10. Greil, J., Rausch, T., Giese, T., Bandapalli, O. R., Daniel, V., Bekeredjian-Ding, I., Stütz, A. M., Drees, C., Roth, S., Ruland, J., Korbel, J. O., and Kulozik, A. E. (2013) Whole-exome sequencing links caspase recruitment domain 11 (CARD11) inactivation to severe combined immunodeficiency. *J. Allergy Clin. Immunol.* 131, 1376–1383
11. McCully, R. R., and Pomerantz, J. L. (2008) The protein kinase C-responsive inhibitory domain of CARD11 functions in NF-κB activation to regulate the association of multiple signaling cofactors that differentially depend on Bcl10 and MALT1 for association. *Mol. Cell. Biol.* 28, 5668–5686
12. Matsumoto, R., Wang, D., Blonska, M., Li, H., Kobayashi, M., Pappu, B., Chen, Y., Wang, D., and Lin, X. (2005) Phosphorylation of CARMA1 plays a critical role in T cell receptor-mediated NF-κB activation. *Immunity* 23, 575–585
13. Sommer, K., Guo, B., Pomerantz, J. L., Bandaranayake, A. D., Moreno-García, M. E., Ovechkina, Y. L., and Rawlings, D. J. (2005) Phosphorylation of the CARMA1 linker controls NF-κB activation. *Immunity* 23, 561–574
14. Wegener, E., Oeckinghaus, A., Papadopoulou, N., Lavitas, L., Schmidt-Supprian, M., Ferch, U., Mak, T. W., Ruland, J., Heissmeyer, V., and Krappmann, D. (2006) Essential role for IkB kinase β in remodeling Carma1-Bcl10-Malt1 complexes upon T cell activation. *Mol. Cell* 23, 13–23
15. Shinohara, H., Maeda, S., Watarai, H., and Kurosaki, T. (2007) IkB kinase β-induced phosphorylation of CARMA1 contributes to CARMA1 Bcl10 MALT1 complex formation in B cells. *J. Exp. Med.* 204, 3285–3293
16. Thome, M., Charton, J. E., Pelcer, C., and Hallfinger, S. (2010) Antigen
receptor signaling to NF-κB via CARMA1, BCL10, and MALT1. *Cold Spring Harb. Perspect. Biol.* 2, a003004
17. Lim, K. H., Yang, Y., and Staudt, L. M. (2012) Pathogenetic importance and therapeutic implications of NF-κB in lymphoid malignancies. *Immunol. Rev.* 246, 359–378
18. Rui, L., Schmitz, R., Ceribelli, M., and Staudt, L. M. (2011) Malignant pirates of the immune system. *Nat. Immunol.* 12, 933–940
19. Davis, R. E., Brown, K. D., Siebenlist, U., and Staudt, L. M. (2001) Constitutive nuclear factor κB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J. Exp. Med.* 194, 1861–1874
20. Ngo, V. N., Davis, R. E., Lamy, L., Yu, X., Zhao, H., Lenz, G., Lam, L. T., Dave, S., Yang, L., Powell, J., and Staudt, L. M. (2006) A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 441, 106–110
21. Lenz, G., Davis, R. E., Ngo, V. N., Lam, L., George, T. C., Wright, G. W., Dave, S. S., Zhao, H., Xu, W., Rosenwald, A., Ott, G., Muller-Hermelink, H. K., Gascoyne, R. D., Connors, J. M., Rimsza, L. M., et al. (2008) Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science* 319, 1676–1679
22. Compagno, M., Lim, W. K., Grunn, A., Nandula, S. V., Brahmachary, M., Shen, Q., Berton, F., Ponzoni, M., Scardurra, M., Califano, A., Bhat, G., Chadburn, A., Dalla-Favera, R., and Pasqualucci, L. (2009) Mutations of multiple genes cause deregulation of NF-κB in diffuse large B-cell lymphoma. *Nature* 459, 717–721
23. Lohr, J. G., Stojanov, P., Lawrence, M. S., Auclair, D., Chapuy, B., Sougnez, C., Cruz-Gordillo, P., Knoechel, B., Asmann, Y. W., Slager, S. L., Novak, A. J., Dogan, A., Ansell, S. M., Link, B. K., Zou, L., et al. (2012) Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3879–3884
24. Bu, R., Bavi, P., Abubaker, J., Jehan, Z., Al-Haqawi, W., Ajarim, D., Al-Dayef, F., Uddin, S., and Al-Kuraya, K. S. (2012) Role of NF-κB regulators-TNFAIP3 and CARD11 in Middle Eastern diffuse large B cell lymphoma. *Leuk. Lymphoma* 53, 1971–1977
25. Dong, G., Chanudet, E., Zeng, N., Appert, A., Chen, Y. W., Au, W. Y., Hamoudi, R. A., Watkins, A. J., Ye, H., Liu, H., Gao, Z., Chuang, S. S., Srivastava, G., and Du, M. Q. (2011) A20, ABIN-1/2, and CARD11 mutations and their prognostic value in gastrointestinal diffuse large B-cell lymphoma. *Clin. Cancer Res.* 17, 1440–1451
26. Lamason, R. L., McCully, R. R., Lew, S. M., and Pomerantz, J. L. (2010) Oncogenic CARD11 mutations induce hyperactive signaling by disrupting autoinhibition by the PKC-responsive inhibitory domain. *Biochemistry* 49, 8240–8250
27. Chan, W., Schaffer, T. B., and Pomerantz, J. L. (2013) A quantitative signaling screen identifies CARD11 mutations in the CARD and LATCH domains that induce Bcl10 ubiquitination and human lymphoma cell survival. *Mol. Cell. Biol.* 33, 429–443
28. Jattani, R. P., Tritapoe, J. M., and Pomerantz, J. L. (2016) 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
29. Lamason, R. L., Kupfer, A., and Pomerantz, J. L. (2010) The dynamic distribution of CARD11 at the immunological synapse is regulated by the inhibitor kinesin GAKIN. *Mol. Cell. Biol.* 40, 798–809
30. Haru, H., Yokosuka, T., Hikakawa, H., Ishihara, C., Yasukawa, S., Yamaizaki, M., Koseki, H., Yoshida, H., and Saito, T. (2015) Clustering of CARMA1 through SH3-GUK domain interactions is required for its activation of NF-κB signalling. *Nat. Commun.* 6, 5555
31. Wu, C. J., and Ashwell, J. D. (2008) NEMO recognition of ubiquitinated Bcl10 is required for T cell receptor-mediated NF-κB activation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3023–3028