CARMA1 Coiled-coil Domain Is Involved in the Oligomerization and Subcellular Localization of CARMA1 and Is Required for T Cell Receptor-induced NF-κB Activation*

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T lymphocyte (T cell) activation and proliferation is induced by the activation of multiple signal transduction pathways. Earlier studies indicate that CARMA1, a Caspase Recruitment Domain (CARD) and Membrane-associated GUanylate Kinase domain (MAGUK)-containing scaffold protein, plays an essential role in NF-κB activation induced by the costimulation of T cell receptor (TCR) and CD28 molecules. However, the molecular mechanism by which CARMA1 mediates TCR-CD28 costimulation-induced NF-κB activation is not fully understood. Here we show that CARMA1 is constitutively oligomerized. This oligomerization of CARMA1 is through its Coiled-coil domain. Disruption of the predicted structure of the Coiled-coil domain of CARMA1 impaired its oligomerization and, importantly, abrogated CARMA1-mediated NF-κB activation. Interestingly, disruption of the CC1 domain abrogates CARMA1 localization, whereas disruption of the CC2 domain seems to inhibit CARMA1 self-association. Together, our results demonstrate that the oligomerization of CARMA1 is required for TCR-induced NF-κB activation.

phospholipase. These signaling pathways activate several transcription factors, including NF-κB, AP-1, and NF-AT, which ultimately control the production of various cytokines, leading to T cell activation and proliferation.

NF-κB is one of the key transcriptional factors activated by TCR engagement. TCR-induced NF-κB activation plays important roles for the proliferation, differentiation, and survival of T cells (1). Stimulation of T cells by antigen-presenting cells induces the formation of a large multicomponent complex at the contact area between the T cell and the antigen-presenting cell, termed the supramolecular activation complex (SMAC) or immunological synapse (IS) (2). The SMAC/IS of a T cell is highly enriched in cholesterol and glycosphingolipids, also termed lipid rafts. Some signaling molecules are constitutively associated with lipid rafts, whereas others are recruited into lipid rafts following CD3–CD28 costimulation (3). Although it is not fully understood how signaling pathways induced by CD3–CD28 costimulation lead to the activation of NF-κB, recent studies from our group and others have demonstrated that CARMA1, a scaffold molecule, plays an essential role for CD3–CD28 costimulation-induced NF-κB activation (4–6).

CARMA1 is a scaffold molecule containing an N-terminal Caspase Recruitment Domain (CARD), a Coiled-coil (C-C) domain, a PDZ domain, a Src homology 3 domain, and a C-terminal guanylate kinase-like (GUK) domain and is a member of the Membrane-associated GUK (MAGUK) family of proteins (7, 8). Consistent with the roles of MAGUK family proteins, CARMA1 is constitutively associated with the cytoplasmic membrane and is recruited into the immunological synapse following CD3–CD28 costimulation (5, 6). Biochemical and gene-targeting studies have confirmed that CARMA1 is required for TCR-induced IkB kinase (IKK) activation and functions downstream of protein kinase C θ (6, 9, 10). More recent studies suggest that protein kinase C θ and protein kinase B β phosphorylate CARMA1 in T and B cells, respectively (11, 12). This phosphorylation likely induces conformational changes in CARMA1, which recruits downstream signaling components Bcl10 (B Cell Lymphoma-10) and MALT1 (mucosa-associated lymphoid tissue protein-1, also known as human paracaspase) to the immunological synapse (9, 10, 13, 14). Genetic studies also demonstrate that Bcl10 and MALT1 are required for antigen receptor-induced NF-κB activation (15–18). It has been suggested that oligomerization of Bcl10 regulates MALT1 and TRAF6, two potential ubiquitin-protein isopeptide ligase ubiquitination ligases (E3), leading to NEMO.

The abbreviations used are: TCR, T cell receptor; CARD, caspase recruitment domain; Bcl10, B cell lymphoma 10; MALT1, mucosa-associated lymphoid tissue protein-1; CC, Coiled-coil; HA, hemagglutinin; WT, wild type; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay.
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(NF-κB essential modulator) ubiquitination (19, 20). However, how CARMA1 induces Bcl10 oligomerization remains to be determined.

Although it has also been proposed that the stimulation of antigen receptors induces oligomerization of CARMA1 (21, 22), there is no direct evidence showing that the oligomerization of CARMA1 is functionally important. In this study, we demonstrated that CARMA1 could indeed form oligomers and, importantly, that this oligomerization was required for TCR-induced NF-κB activation and disruption of this oligomerization completely inhibited CARMA1-mediated NF-κB activation. Furthermore, domain mapping of the CC domain shows different regions contribute to membrane localization and oligomerization. Thus, our studies provide biochemical evidence that oligomerization and localization of CARMA1 play an essential role in TCR-induced NF-κB activation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Yeast two-hybrid interaction assays were performed in yeast strain AH109 transfected by the standard lithium acetate method (Clontech). A human lymph node cDNA library was used (Clontech). Screening plasmids were generated by inserting the CARD + CC domains of CARMA1 in-frame with the GAL4 DNA-binding domain in the pGBKTK7 vector (Clontech). Screening was performed at high stringency as described in the manufacturer’s instructions. Positive clones were screened for β-galactosidase activity and sequenced. Further confirmatory yeast two-hybrid experiments used high stringency plate agar.

Plasmids and Antibodies—Plasmids encoding Myc- and FLAG epitope-tagged CARMA1 were described previously (14). CARMA1 point mutants were generated using site-directed mutagenesis and confirmed by sequencing analysis. These mutants were inserted into pCMVTag2B (FLAG), pRK6 (Myc or HA), or lentiviral vector pRV (Myc or HA) plasmids. Mouse full-length WT and L298Q CARMA1 expression plasmids were kindly provided by C. C. Goodnow (23). Antibodies for c-Myc, HA, and green fluorescent protein (GFP) were from Santa Cruz Biotechnology; antibodies for p-JNK, JNK, Bcl10, p-ERK, ERK, and MALT1 were from Cell Signaling, Inc.; antibodies for FLAG (M2) were from Sigma Co.; and antibodies for CARMA1 were from Axonra Life Science Inc.

Cell Culture and Stimulation—Jurkat E6.1, JPM50.6 (6), and Raji cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum at 37 °C in 5% CO2. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in 5% CO2. Costimulation of Jurkat cells (1 × 10⁵) was performed in a final volume of 1 ml by addition of anti-CD3 (5 μg/ml) and anti-CD28 (3 μg/ml) antibodies, with goat anti-mouse (3 μg/ml) (Signal Transduction Laboratories). Additional stimulations include phorbol 12-myristate 13-acetate (PMA) (10 ng/ml), ionomycin (100 ng/ml), and 20 μg of expression vectors of CARMA1 and its CC mutants or vector controls. After a 16-h incubation, the cells were stimulated with or without anti-CD3 (5 μg/ml) and anti-CD28 (3 μg/ml) antibodies or PMA (10 ng/ml) plus anti-CD28 (3 μg/ml) antibodies for 6 h. Luciferase activities in the cell lysates were determined by utilizing the Dual-Luciferase kit (Promega). For HEK293T cell luciferase assays, 6-well plates were seeded with 2 × 10⁶ cells and transfected with 100 ng of NF-κB-dependent luciferase (firefly) reporter plasmid and 1 ng of EF1α promoter-dependent Renilla luciferase plasmid together with 2 μg of expression vectors of CARMA1 or CARMA1-CCM.

Stable Cell Lines—Jurkat or JPM50.6 cells were transfected at 250 V (950 μF) with 20 μg of plasmid DNA. Stable cell lines were selected with either neomycin (1500 ng/ml) or hygromycin (250 ng/ml) medium by limited dilution. Clonal populations were assayed for gene expression to verify the presence of an integrated plasmid. Lentiviral transduction was also used to create some stable cell lines with vectors containing different CARMA1 mutants. First, HEK293T cells were seeded in 10-cm dishes and transfected with 5 μg each of pEnv/pHep for virus packaging of 10 μg of Myc-tagged or HA-tagged WT CC1M, CC2M, or CCM CARMA1 by calcium phosphate precipitation. After 16 h, viral supernatant was removed and replaced with complete RPMI1640 medium. After an additional 24 h, virus-containing medium was collected and plated with 1–3 × 10⁶ Jurkat or JPM50.6 cells. Cells were expanded and expression was verified by Western blot with appropriate antibodies.

EMSA Analysis—Nuclear extracts were prepared from different cells after various stimulations. Cells (1 × 10⁶) were resuspended in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40, and 1% protease inhibitor mixture). Cytoplasmic fractions were isolated for biochemical analysis. Nuclear pellets were washed in 500 μl of lysis buffer and then resuspended in 50 μl of EMSA extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture). After vortexing for 15 min at 4 °C, the samples were centrifuged, and the nuclear proteins in the supernatant were collected. Protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin standards. EMSA analysis was performed with 10 μg of nuclear extract and 32P-labeled NF-κB or Oct-1 probes (Promega).

Cell Conjugation Assay—Jurkat T cells expressing GFP-CARMA1 WT, CC1M, CC2M, or CCM were stained with ALEXA594-conjugated cholera toxin B (8 μg/ml (Sigma)) at 4 °C for 20 min. Cells were then washed and resuspended in serum-free RPMI1640 at 10⁶ cells/ml. Raji B cells were incubated with or without 2 μg of Staphylococcus enterotoxin E (SEE) (Toxin Technology, Inc., Sarasota, FL) per ml at 37 °C for 90 min. Raji cells were then washed, mixed with an equal volume of GFP-CARMA1-expressing Jurkat cells, pelleted, fixed in 4% paraformaldehyde, and attached to poly-L-lysine-coated microscope slides by Cytospin® centrifugation. Fluorescence was detected on an Olympus Fluoview FV300 confocal laser scanning biological microscope.
Co-immunoprecipitation—Various FLAG/Myc-tagged constructs were transfected into HEK293 cells by calcium phosphate precipitation. After 16-h of incubation, proteins were immunoprecipitated with various indicated antibodies and Protein A-agarose (Roche Applied Science). Samples were washed four times with lysis buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM NaCl, and 1% Nonidet P-40) and eluted with 2× SDS loading buffer. The eluted samples were subjected to SDS-PAGE and Western blotting.

RESULTS

CARMA1 Oligomerization—To determine the molecular mechanism by which the function of CARMA1 is regulated, we performed a yeast two-hybrid screen using the N terminus of CARMA1 (CARD and CC domains) as the bait against a human lymph node cDNA library. Several positive clones were isolated and sequenced. One of the positive clones encoded an N-terminal truncated CARMA1 (131-residue truncation) in which the N-terminal CARD domain was removed, leaving a portion of the CC domain as well as the entire MAGUK domain. This screening result suggests that CARMA1 can self-associate. To test this possibility, several constructs were introduced into the yeast two-hybrid system and assayed for positive interaction (Table 1). We found that the CARD-CC fragment was able to self-associate. This fragment was able to associate with the CARMA1 CC domain, but not with the CARD domain. Furthermore, when two CC domains were used, positive interaction occurred (Table 1). Together, these results indicate that CARMA1 can form a dimer through its CC domain.

Mouse and human CC domains could associate with each other (data not shown), indicating that the dimerization is a common feature of both mouse and human CARMA1. Although the formation of a homotrimer has been found in other CC domain-containing proteins, the Y2H assay cannot investigate this possibility. In addition, a known functional CC mutation, L298Q, was still capable of dimerization, indicating that the L298Q mutation disrupts another aspect of CARMA1 signaling (23). Together, these results indicate that CARMA1 can form a dimer or oligomer through its coiled-coil domain and that the CARMA1 CC alone is both necessary and sufficient for this oligomerization.

To further investigate whether CARMA1 forms dimers/oligomers in mammalian cells, an immunoprecipitation experiment was performed. Expression vectors encoding Myc- and FLAG-tagged CARMA1 proteins were transfected into HEK293 cells. After transfection, cells were lysed and subjected to anti-FLAG immunoprecipitation. Consistent with the above results, we found that Myc-CARMA1 was able to associate with FLAG-CARMA1, whereas Myc-TRAF2, which also contains a CC domain, was unable to associate with FLAG-CARMA1 (Fig. 1A). Additionally, to determine whether oligomerization is a common structural feature among CARMA family members, CARMA3, a homologue of CARMA1 with different tissue distribution, was assayed for its ability to form oligomers (24). We found that CARMA3 could self-oligomerize as well as associate with CARMA1 (Fig. 1B). These results suggest that CARMA1 and CARMA3 act in a similar fashion in signal transduction pathways. Earlier studies indicate that CARMA3 can functionally reconstitute CARMA1-deficient cells (11). Together, these data indicate that the CARMA1 protein is assembled into a dimeric (or possibly oligomeric) form.

To determine the oligomerization status of CARMA1 in resting T cells and whether this status changes after cell stimulation,
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JPM50.6WT (reconstituted with Myc-CARMA1) cells were transduced with or without a lentivirus encoding an HA-tagged CARMA1 and stimulated with or without CD3-CD28 for 0, 10, or 20 min. Cells were then subjected to immunoprecipitation with anti-HA antibodies and Western blotting for Myc and HA epitope-tagged proteins. As shown in Fig. 1C, the status of CARMA1 oligomerization does not change after stimulation. This indicates that stimulation does not alter CARMA1 oligomerization status and CARMA1 is self-associated regardless of its activation state. Stimulation was confirmed by phospho-ERK immunoblot (Fig. 1C, bottom panel).

Generation of a Coiled-coil Mutant—To determine the functional significance of CARMA1 oligomerization, the structure of the CARMA1 CC domain was analyzed. Using MultiCoil and COILS programs (25), we predicted that CARMA1 residues Leu-149, Leu-152, Leu-225, Leu-229, Ile-421, and Leu-424 might contribute to the interactions between their CC domains (Fig. 2A). The graphs indicate regions of potential Coiled-coil formation at critical residues that can bind two Coiled-coil domains together (Fig. 2B). There are three peaks of predicted coiled coil formation. CARMA1 mutants with pair mutations including LL149/152TT (CC1M) and IL421/424TT (CC2M) were generated by site-directed mutagenesis. Additionally, a CARMA1 mutant, CCM, was constructed with the mutation LL149/152/225/229/421/424TTTT (Fig. 2A). Shown in Fig. 2B are graphs from Multicoil that show regions of predicted Coiled-coil formation (left panel) (25). After threonine mutagenesis, predicted regions of coiled-coil formation (right panel) (25).

FIGURE 2. Generation of a CARMA1 coiled-coil mutation to disrupt self-oligomerization. A, several CC domain point mutants of CARMA1 were generated at critical residues as predicted by using COILS and MultiCOIL programs. Pair mutations include LL149/152TT and IL421/424TT. Predicted regions of coiled-coil formation, named CC1 and CC2, are shown. Also indicated is the XhoI site where the ΔCD CARMA1 construct transcription/translation site is located. B, graphs from MultiCOIL show proposed regions important in Coiled-coil dimer formation (left panel), including predictions after key residues are modified to threonine residues (right panel).

Determination of the Functional Relevance of Impaired CARMA1 Oligomerization—To determine whether mutations in the CC domain altered CARMA1 function, we reconstituted JPM50.6 cells by lentiviral transductions in which either CARMA1-WT, -CC1M, -CC2M, or -CCM were expressed. These cell lines were analyzed by EMSA for their ability to rescue the TCR-induced NF-κB defect. Although wild type CARMA1 could effectively rescue the NF-κB defect in CARMA1-deficient cells, we found that each CC mutation could not rescue CD3−CD28 costimulation- or PMA/IONO/mycin-induced NF-κB activation (Fig. 4A). Comparable levels of CARMA1-WT and its mutants were found in these reconstituted cells (Fig. 4B). The Oct-1 levels show equivalent loading of nuclear extracts. Consistent with previous results (6), CARMA1 deficiency had no effect on TNFα-induced NF-κB activation (Fig. 4A). In addition, we examined ERK activation induced by these stimulations and found that ERK was effectively activated by PMA/IONO/mycin stimulation in all cells (Fig. 4B).

To determine whether the CCM mutations inadvertently disrupted signaling downstream of CARMA1, wild type and CCM mutant of CARMA1 were evaluated for their ability to activate the NF-κB pathway downstream of CARMA1. To determine whether the CCM mutant is defective in activation of downstream signaling components, we transfected expression plasmids encoding Myc-tagged wild type or CCM version of CARMA1 and stimulated with or without CD3–CD28 for 0, 10, or 20 min. Cells were then subjected to immunoprecipitation with anti-HA antibodies and Western blotting for Myc and HA epitope-tagged proteins. As shown in Fig. 1C, the status of CARMA1 oligomerization was confirmed by phospho-ERK immunoblot (Fig. 1C, bottom panel).

Investigation of Coiled-coil Mutant Oligomerization—To determine whether mutation of these residues disrupts the oligomerization of CARMA1, HA-tagged CARMA1-WT and Myc-tagged WT, CC1M, CC2M, or CCM version of CARMA1 were reconstituted into JPM50.6 cells (CARMA1-deficient Jurkat T cells). Therefore, the resultant cells expressed HA-tagged wild type CARMA1 as well as Myc-tagged WT or mutants of CARMA1. We found that CARMA1-WT was effectively co-immunoprecipitated with CARMA1-WT and CARMA1-CC1M, but to a significantly lesser extent with CARMA1-CC2M, and not with CARMA1-CCM (Fig. 3). These results indicate that CARMA1 is oligomerized in lymphocytes and that mutation in the CARMA1 CC domain can abrogate its oligomerization. Furthermore, the CC2 region of CARMA1 appears to be more involved in CARMA1 oligomerization than the CC1 region, although both regions appear to contribute to CARMA1-CARMA1 interaction.

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of CARMA1 with an NF-κB-dependent luciferase reporter into HEK293T cells. We found that overexpressed CARMA1-WT, -CC1M, -CC2M, or -CCM. These cells were either left unstimulated or stimulated with anti-CD3–CD28 (3/28), PMA/ionomycin (P/I), or TNFa (T) as indicated for 30 min. Nuclear and cytoplasmic fractions were prepared, and nuclear protein was incubated with 32P-labeled NF-κB or Oct-1 probes and subjected to electrophoretic mobility shift assays and autoradiography (upper panels). B, cytoplasmic fractions (Lysates) were subjected to SDS-PAGE and analyzed by immunoblotting using the indicated antibodies.

FIGURE 5. CARMA1 oligomerization mutant can transduce a downstream signal to NF-κB. A, expression plasmids encoding wild type or CCM mutant of CARMA1 were transfected into HEK293T cells along with an NF-κB-dependent luciferase reporter and EF1α promoter-dependent Renilla luciferase plasmid. 24 h after the transfection, cells were lysed and cell lysates were examined for luciferase activities. B, JPM50.6 cells were transduced with either CARMA1-WT or CARMA1-CCM and subjected to stimulation with PMA/ionomycin for 15 min. Cell lysates were then immunoprecipitated with anti-Myc antibodies, and eluants were subjected to SDS-PAGE and immunoblot for the presence of Myc, Bcl10, and MALT1 proteins.

CARMA1, which cannot functionally incorporate into the TCR signaling pathway because of a defective Coiled-coil domain.

The CC1 Mutation Impairs CARMA1 Subcellular Localization—To determine whether mutation of the CC domain of CARMA1 affects the subcellular localization of CARMA1, we compared the localization of WT, CC1M, CC2M, and CCM of CARMA1. Jurkat T cells stably expressing GFP-tagged CARMA1 WT and mutants were generated. These cells (Jurkat-WT, Jurkat-CC1M, Jurkat-CC2M, and Jurkat-CCM) were used in a cell conjugation assay to determine the effect of CC domain mutation on proper localization to the immunological synapse upon antigen presentation. Jurkat-CARMA1 cells were labeled with ALEXA594-conjugated cholera toxin B, which associates with and labels lipid rafts. These cells were then mixed with Raji B cells primed with or without Staphylococcus enterotoxin B, which associates with and labels lipid rafts. These cells were then mixed with Raji B cells primed with or without Staphylococcus enterotoxin E. Cell conjugates were examined under confocal microscopy for the colocalization of GFP-CARMA1 and lipid rafts stained by cholera toxin B. In unstimulated cells, CARMA1 was localized throughout cells (Fig. 6). Following stimulation, we found that CARMA1 WT and CC2M were localized at the Jurkat/Raji cell interface upon Staphylococcus enterotoxin E stimulation, whereas stimulation did not redistribute CARMA1-CC1M or CARMA1-CCM to the lipid raft domain (Fig. 6, A–D). These results indicate that mutations in the CC1 domain of CARMA1 alter its subcellular localization, suggesting that the CC1 region is involved in the local-
ization of CARMA1, because the mutant CC1M shows improper localization whereas CARMA1-CC2M shows proper localization.

**Dominant Negative Function of the Coiled-coil Mutant**—Because the mutations in the CC1 and CC2 regions (Fig. 2) significantly blocked PMA/ionomycin-induced NF-κB activation (Fig. 4), it is possible that these regions could function as a dominant negative mutant in TCR signaling. A stable Jurkat cell line that expressed CARMA1-CCM was used to perform an EMSA. As shown in Fig. 7A, CARMA1-CCM expression in Jurkat-CCM cells blocked PMA/ionomycin activation of NF-κB, in contrast to normal Jurkat cells.

In an effort to determine the minimal region of interaction between CARMA1 oligomers, Jurkat cells were transfected with either CARMA1-CC1 or CARMA1-CC2 expression plasmids together with an NF-κB-dependent luciferase reporter, and a luciferase assay was performed with stimulation. We found that expression of these small fragments of CARMA1 effectively inhibited PMA/ionomycin-induced NF-κB activation (Fig. 7B). The CC1 region inhibited NF-κB activation by ~40%, and the CC2 region inhibited NF-κB activation by ~90%.

**DISCUSSION**

Protein-protein interactions enable the flow of signaling information between proteins. A complex array of interactions is necessary for the transduction of appropriate TCR signaling to activate target gene expression for the initiation of an immune response. Earlier studies have demonstrated that CARMA1 plays an essential role in TCR-induced NF-κB activation. In this study, we demonstrate that CARMA1 exists as a dimer/oligomer in T cells. The oligomerization of CARMA1 is mainly through its Coiled-coil domain. Importantly, our results indicate that CC domain mutants significantly impair the function of CARMA1. This supports the idea that CARMA1 oligomerization does not depend on cell stimulation, suggesting that TCR signaling may alter the association of CARMA1 with other proteins but not affect CARMA1 oligomerization. Therefore, our studies provide biochemical and genetic evidence demonstrating that the oligomerization of CARMA1 is required for TCR-induced NF-κB activation.

It has been shown CARMA1 recruits signaling components such as Bcl10, MALT1, and IKK (inhibitor of κB kinase) complex into the immunological synapse (13, 14, 26). Therefore, proper subcellular localization of CARMA1 is required for TCR-induced NF-κB activation. Interestingly, although CARMA1-CCM is able to associate with Bcl10 and MALT1 after activation, these downstream components were not activated. It seems distinct events regulate the formation and the signal transduction through the CARMA1-Bcl10-MALT1 complex.

Our earlier studies suggest that the C-terminal MAGUK domain of CARMA1 contributes to the subcellular localization of CARMA1 (11, 14). In this study, we found that mutation of the CC1 domain of CARMA1 also impaired its subcellular localization, suggesting that the CC domain of CARMA1 may also contribute to its subcellular localization. Although our
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results suggest that both the CC1 and CC2 regions are required for dimerization, it appears that only the CC1 region could serve as an inhibitor for modulating lymphocyte activation.

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