Regulation of NF-κB Activation in T Cells via Association of the Adapter Proteins ADAP and CARMA1

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The adapter protein ADAP regulates T lymphocyte adhesion and activation. We present evidence for a previously unrecognized function for ADAP in regulating T cell receptor (TCR)–mediated activation of the transcription factor NF-κB. Stimulation of ADAP-deficient mouse T cells with antibodies to CD3 and CD28 resulted in impaired nuclear translocation of NF-κB, a reduced DNA binding, and delayed degradation and decreased phosphorylation of IκB (inhibitor of NF-κB). TCR-stimulated assembly of the CARMA1–BCL-10–MALT1 complex was substantially impaired in the absence of ADAP. We further identified a region of ADAP that is required for association with the CARMA1 adapter and NF-κB activation but is not required for ADAP-dependent regulation of NF-κB. These findings provide new insights into ADAP function and the mechanism by which CARMA1 regulates NF-κB activation in T cells.

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der proteins nucleate multimolecular complexes that are essential for effective transmission of intracellular signals during an adaptive immune response (1). In T lymphocytes, the adhesion- and degranulation-promoting adapter protein (ADAP) regulates T cell receptor (TCR)–dependent changes in the function of integrin adhesion receptors (2, 3). ADAP-deficient (ADAP−/−) T cells also exhibit impaired proliferation and cytokine production after stimulation of the TCR and the CD28 costimulatory receptor (2, 3). Stimulation of these receptors leads to activation of the NF-κB family of transcription factors, which are critical for T cell activation and survival (4). A multiprotein complex consisting of the membrane-associated adapter protein CARMA1 (5, 6), the caspase-like protein MALT1 (7, 8), and the adapter protein BCL-10 (9) is critical for TCR-dependent activation of the IκB kinase complex and subsequent NF-κB nuclear translocation (10).

Like ADAP-deficient T cells, protein kinase Cε (PKCε)–deficient T cells exhibit defective TCR-mediated proliferation, even though proximal TCR signaling events, such as extracellular signal–regulated kinase (ERK) activation, are normal (11). Therefore, we examined PKCε-dependent signaling in ADAP−/− T cells (12).

Membrane localization of PKCε was similar in ADAP−/− and ADAP−/+ T cells upon stimulation with antibodies to CD3 and CD28 (Fig. 1, A and B). Stimulated ADAP−/− and ADAP−/+ T cells also showed similar levels of PKCε phosphorylation (Fig. 1C). Thus, ADAP is not required for TCR signaling events leading to and including PKCε activation. Because PKCε regulates NF-κB activation downstream of the TCR (12, 13), we next examined NF-κB signaling in ADAP−/− T cells. Image scanning flow cytometry (14, 15) (fig. S1) revealed a striking defect in p65 nuclear translocation after stimulation of ADAP−/− lymph node T cells (Fig. 2, A and B) or CD4 T cells (fig. S2) by CD3 and CD28 (CD3/CD28). In contrast, no impairment in NF-κB activation was detected after stimulation with tumor necrosis factor–α (TNF-α), which activates NF-κB independently of the TCR. These results were confirmed with electrophoretic mobility shift assays (Fig. 2C). ADAP−/− T cells also displayed defective NF-κB translocation after treatment with phorbol 12-myristate 13-acetate (PMA), which activates PKCε (Fig. 2, A and B).

Fig. 1. TCR-dependent membrane localization and activation of PKCε in ADAP−/− T cells. (A) Localization of PKCε (bottom) in ADAP−/+ and ADAP−/− T cells to the contact site with beads coated with antibodies to CD3 and CD28. Differential interference contrast (DIC) images are shown in top panels. (B) Quantification of PKCε localization. T cell–bead conjugates (minimum 90 per group) were scored for PKCε polarization from two independent experiments. Graph shows the average percent of T cell–bead conjugates with polarized PKCε (±SD). (C) Phosphorylation of PKCε after CD3/CD28 stimulation of ADAP−/− and ADAP−/+ T cells for the indicated time points was assessed by Western blotting of whole-cell lysates with antibody to phosphorylated PKCε (Thr538) (top panels). Blots were also probed with antibody to β-actin (bottom panels).
CD3/CD28 stimulation of ADAP+/− T cells also resulted in reduced induction of intercellular adhesion molecule–1 (ICAM-1), which is encoded by a NF-κB-regulated gene (16) (Fig. 2D).

We also examined signaling events proximal to nuclear translocation of the p65 NF-κB subunit in ADAP−/− T cells. In resting T cells, NF-κB subunits are sequestered in the cytoplasm via interactions with IκBα (4). Relative to ADAP+/− T cells, CD3/CD28 stimulation of ADAP+/− T cells led to a delay in IκBα degradation and decreased IκBα phosphorylation (Fig. 2E and fig. S3). Consistent with previous results (2, 3), the kinetics of ERK phosphorylation after CD3/CD28 stimulation were not affected by loss of ADAP (Fig. 2E). Activation of the IκB kinase (IKK) complex, which phosphorylates IκBα, was also impaired after CD3/CD28 stimulation of ADAP−/− T cells (Fig. S4). Thus, ADAP acts as a positive regulator of TCR-dependent NF-κB activation, downstream of PKCζ yet upstream of IKK activation, IκBα degradation, and NF-κB subunit translocation.

We next examined the role of ADAP in inducible membrane localization of BCL-10, MALT1, and CARMA1 (17, 18). In unstimulated ADAP+/− or ADAP+/− T cells, only low levels of BCL-10 and MALT1 were detected in membrane fractions (Fig. 3A). CD3/CD28 or PMA stimulation of ADAP+/− T cells, but not ADAP−/− T cells, resulted in enhanced membrane localization of both BCL-10 and MALT1 (Fig. 3A). ADAP also localized to the membrane in stimulated ADAP+/− T cells (Fig. 3A). The levels of CARMA1 in membrane fractions were comparable between ADAP+/− and ADAP−/− T cells both before and after stimulation (Fig. 3A), consistent with previous results (17). Thus, ADAP is critical for the activation-dependent membrane localization of BCL-10 and MALT1.

To define potential interactions between ADAP and the CARMA1–BCL-10–MALT1 signaling complex, we performed coimmunoprecipitation experiments. ADAP could be immuno-precipitated only from membrane fractions isolated from activated T cells (Fig. 3B). Coimmunoprecipitation of CARMA1, BCL-10, and MALT1 with ADAP was observed from stimulated membrane fractions of either lymph node T cells (Fig. 3B) or purified CD4 T cells (fig. S5). Similarly, MALT1, BCL-10, and ADAP coimmunoprecipitated with CARMA1 only after T cell stimulation (Fig. 3B). However, in activated ADAP−/− T cells, MALT1 and BCL-10 did not coimmunoprecipitate with CARMA1 (Fig. 3B), and CARMA1 and MALT-1 did not coimmunoprecipitate with BCL-10 (Fig. 3C and fig. S6). To confirm that ADAP is required for inducible complex assembly, we used resting T cells expressing the hCAR adenovirus receptor to permit adenoviral-mediated expression of ADAP (19). ADAP reexpression in ADAP−/− T cells restored coimmunoprecipitation of CARMA1 and MALT1 with BCL-10 after CD3/CD28 or PMA stimulation (Fig. 3C). Thus, ADAP is a component of the CARMA1–BCL-10–MALT1 complex and is required for normal complex formation.

We next examined interactions between ADAP and purified BCL-10, MALT1, and CARMA1. A glutathione S-transferase (GST)–ADAP fusion protein interacted in vitro with purified CARMA1, but not with purified BCL-10 or MALT1 (Fig. 3D). A truncated form of CARMA1 (CARMA1/651–1147) containing just the C-terminal PDZ, SH3, and GUK-like domains typical of membrane-associated guanylate kinase (MAGUK)–family proteins (20) (Fig. 3E) also interacted with GST-ADAP in vitro (Fig. 3D). Thus, the interaction of ADAP with CARMA1 is not dependent on the caspase-recruiting domain that mediates the interaction of CARMA1 with BCL-10. Colocalization of ADAP with CARMA1 was also observed at the contact site between wild-type T cells and beads coated with antibodies to CD3 and CD28 (Fig. 3F).

Truncation and deletion mutants of ADAP were used to define sites within ADAP critical for its interaction with CARMA1 (Fig. 4A). Wild-type and mutant forms of hemagglutinin (HA) epitope–tagged ADAP were immunoprecipitated from transiently transfected Jurkat T cells after PMA stimulation, and these immunoprecipitates were analyzed for the presence of coimmunoprecipitating BCL-10, MALT1, and CARMA1 proteins (Fig. 4B). Wild-type ADAP and an
ADAP mutant lacking the N-terminal 327 amino acids (ADAPA1-327), but not an ADAP mutant containing only the N-terminal 426 amino acids (ADAPA2-426), communoprecipitated the CARMA1–BCL-10–MALT1 complex (Fig. 4B). The CARMA1 binding site in ADAP was mapped to a region of ADAP between amino acids 426 and 541 (Fig. 4A), because a deletion mutant of ADAP lacking this region (ADAPA426-541) was completely unable to communoprecipitate the CARMA1–BCL-10–MALT1 complex (Fig. 4B). This region of ADAP contains the N-terminal helical SH3 (hSH3) domain (21–23) between amino acids 482 and 541, and an adjacent region rich in Glu and Lys residues (E/K-rich region) between amino acids 426 and 481. ADAP deletion mutants lacking either the N-terminal hSH3 domain (ADAPA482–541) or the E/K-rich region (ADAPA426–481) (Fig. 4A) showed weak communoprecipitation of the CARMA1–BCL-10–MALT1 complex relative to wild-type ADAP (Fig. 4B). A GST-ADAP fusion protein expressing both the hSH3 domain and the E/K-rich region of ADAP was able to associate with the truncated CARMA1/651-1147 protein in vitro (fig. S8), which suggests that this region of ADAP is sufficient for CARMA1 association.

Expression of wild-type ADAP in resting ADAP−/− T cells restored the ability of PMA (Fig. 4C) or CD3/CD28 stimulation (Fig. 4D) to induce nuclear translocation of p65. ADAP expression in transduced T cells was verified by intracellular flow cytometry (fig. S9). Expression of the ADAPA1-327 mutant, but not the ADAPA2-426 mutant, in ADAP−/− T cells was also able to fully restore CD3/CD28-mediated NF-κB translocation (Fig. 4D). In contrast, the ADAPA426–541 deletion mutant did not restore CD3/CD28-mediated NF-κB translocation after expression in ADAP−/− T cells (Fig. 4E). Expression of either the ADAPA426–481 or ADAPA482–541 deletion mutants partially restored NF-κB p65 nuclear translocation (Fig. 4E).

ADAP also regulates TCR-mediated integrin activation (2, 3) and thus ADAP−/− T cells exhibit impaired integrin-dependent conjugate formation with antigen-pulsed antigen-presenting cells (24) (Fig. 4F). Expression of either wild-type ADAP or the ADAPA426–541 mutant in ADAP−/− T cells restored TCR-induced conjugate formation to levels observed in control T cells (Fig. 4F). Thus, the region of ADAP between amino acids 426 and 541 is critical for NF-κB activation but is not required for the regulation of integrin-dependent conjugate formation.

We have identified a novel function for ADAP in the regulation of NF-κB activation in T cells. We propose that the association between the C-terminal end of CARMA1 and the region of ADAP between amino acids 426 and 541 is critical for assembly of the CARMA1–BCL-10–MALT1 complex at the membrane. ADAP may provide mechanisms for membrane localization and stabilization of the CARMA1–BCL-10–MALT1 complex, as the C-terminal ADAP hSH3 domain can associate.
Fig. 4. The region of ADAP containing the N-terminal helical SH3 domain and an E/K-rich region is critical for ADAP-CARMA1 association and TCR-dependent activation of NF-κB, but is dispensable for ADAP-dependent regulation of antigen-dependent conjugate formation. (A) Diagram of the HA-tagged ADAP truncation and deletion mutants used in this study. Numbers indicate amino acid position in mouse ADAP. Asterisks indicate Tyr residues (amino acids 547/549, 584, 615, and 687) implicated in ADAP binding to the SLP-76 adapter protein. (B) Jurkat T cells were transiently transfected with the indicated HA-ADAP constructs and then stimulated with PMA before immunoprecipitation with BCL-10 mAb, followed by Western blotting with antibodies specific for the indicated proteins. (C) T cells isolated from ADAP+/+ and ADAP−− hCAR transgenic mice were transduced with control adenovirus encoding Thy1.1 (ctrl) or adenovirus encoding wild-type ADAP and Thy1.1 (ADAPwt). Cells were either unstimulated or stimulated with PMA or TNF-α before analysis of NF-κB p65 nuclear translocation as in Fig. 2. (D and E) T cells isolated from ADAP+/+ and ADAP−− hCAR transgenic mice were transduced as in (C) with either a control adenovirus or adenovirus encoding the indicated ADAP constructs. Cells were either unstimulated or stimulated with antibodies to CD3 and CD28 before analysis of NF-κB p65 nuclear translocation. Graphs show the average increase (±SD) in p65 nuclear translocation in stimulated relative to unstimulated T cells for three (C) and (E) or two (D) independent experiments. (F) T cells isolated from ADAP+/+ and ADAP−− DO11.10/hCAR transgenic mice were transduced as in (E) with control adenovirus or the indicated ADAP constructs. T cells were then analyzed by flow cytometry for their ability to form conjugates with unpulsed (unstim.) or ovalbumin-pulsed (OVA) splenocytes.

with membrane phospholipids (22). The interaction of ADAP with the MAGUK region of CARMA1 may also alter intramolecular interactions within CARMA1 (25, 26), thereby promoting recruitment of BCL-10 and MALT1. The region of ADAP critical for association with CARMA1 is not required for ADAP-dependent regulation of integrins (2, 3), which involves the association of ADAP with the SKAP-55 and SLP-76 adapters (27–29). Two biochemically distinct pools of ADAP can be identified in CD3/CD28-stimulated T cells: one that interacts with the CARMA1–BCL-10–MALT1 complex, and one that interacts with SLP-76 (fig. S10). In contrast, CARMA1 is required for NF-κB activation (2, 3, 30, 31) but is not required for conjugate formation (31). Thus, ADAP serves distinct roles downstream of the TCR that promote functions critical to T cell immune responses.

References and Notes
12. See supporting material on Science Online.
Specialized Inhibitory Synaptic Actions Between Nearby Neocortical Pyramidal Neurons

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We found that, in the mouse visual cortex, action potentials generated in a single layer-2/3 pyramidal (excitatory) neuron can reliably evoke large, constant-latency inhibitory postsynaptic currents in other nearby pyramidal cells. This effect is mediated by axo-axonic ionotropic glutamate receptor–mediated excitation of the nerve terminals of inhibitory interneurons, which connect to the target pyramidal cells. Therefore, individual cortical excitatory neurons can generate inhibition independently from the somatic firing of inhibitory interneurons.

To further characterize these interpyramidal IPSCs (ip IPSCs), we compared them with two kinds of monosynaptic currents: unitary EPSCs (uEPSCs) recorded from pyramidal neuron pairs at the reversal potential (−70 mV) of IPSCs (Fig. 1C) and unitary IPSCs (uIPSCs) from nonpyramidal neurons to pyramidal neurons (Fig. 1D).

In pyramidal neuron pairs, the probability of detecting an ip IPSC [28% (31 out of 110 (31/110))] was slightly higher than that for the detection of an EPSC (22%; 24/110). NBQX blocked ip IPSCs in all of the tested pairs (n = 27). Reciprocal interpyramidal inhibitory connections were never observed. Six pairs had both inhibitory and excitatory connections. The direction was the same for three pairs and opposite for three pairs. In these pyramidal neuron pairs, patch pipettes containing a Cs+-based internal solution were used for recording from both pre- and postsynaptic neurons. Similar ip IPSCs were also recorded with a K+-based internal solution (Fig. S1). Recordings from pairs involving an inhibitory neuron and a pyramidal neuron had a detection probability for uIPSCs of 32% (19/60), which was slightly higher than that for ip IPSCs. The amplitudes of ip IPSCs were significantly larger (P < 0.01) than those of uIPSCs (Fig. 1E), and their time course was similar to that of uIPSCs (Fig. 1, A and D, and fig. S2). Although the average latency of ip IPSCs was significantly (P < 0.02) longer than that of either uIPSCs or uEPSCs, it was distributed in a wide range that included latency values for the two monosynaptic connections (Fig. 1F). If ip IPSCs resulted from conventional polysynaptic activation involving action-potential generation at the somata of inhibitory neurons, the expected variation in latency for each pair should be far larger than that in monosynaptic connections. However, the coefficient of variation of their latency was indistinguishable (P > 0.2) from those for either uEPSCs or uIPSCs (Fig. 1G), suggesting that they were unlikely to be mediated by the generation of somatic action potentials in inhibitory interneurons. Consistent with this supposition, the failure rate of ip IPSCs was not significantly different (P > 0.1) from that of uIPSCs or uEPSCs (fig. S3). This interpretation is also supported by the observation that unitary excitatory inputs alone induce only small postsynaptic responses that are subthreshold for action-potential generation in inhibitory interneurons (4–7). Thus, we hypothesized that ip IPSCs are generated by direct excitation of the presynaptic terminals of inhibitory neurons, which in turn connect to the target pyramidal neuron (Fig. 2A). This mechanism implies that the axo-axonic synaptic transmission must be strong enough to release GABA immediately from the inhibitory terminals. If this synaptic transmission is very strong, extraordinarily quick depolarization would occur at the terminals because of their small volume and lack of strong filtering effects on input signals seen in dendrites. This may, at least in part, explain the short latency of ip IPSCs, together with the absence of conduction time in interneurons. We tested this hypothesis, as described below.

If such excitatory axo-axonic synapses are present, the frequency of miniature IPSCs (mIPSCs) recorded from pyramidal cells in the presence of 1 μM tetrodotoxin (TTX), a sodium channel blocker, may be affected by glutamatergic agents. Bath application of glutamate (10 μM) significantly (P < 0.02) increased the frequency of mIPSCs without any significant (P > 0.6) changes in their amplitude (Fig. 2, B, D, and E). Similar facilitative effects were produced by the selective activation of AMPA receptors with AMPA (1 μM) and kainate receptors with (RS)-2-amino-3-(3-hydroxy-5-(tert-butylisoxazol-4-yl) propanoic acid (ATPA) (1 μM) (8) or a low dose (200 nM) of domoic acid (9), suggesting that both AMPA and kainate receptors contribute to the facilitation of mIPSC frequency (Fig. 2, D and E). We confirmed this supposition with a pharmacological blockade of these receptors (fig. S4). The effect of these receptors may be mediated by the depolarization of nerve terminals, because the facilitation of mIPSC frequency was not found in the presence of Co2+, which blocks voltage-gated Ca2+ channels, and because the metabotropic action of kainate receptors was not involved in this process (fig. S5).

The application of NBQX significantly (P < 0.02) reduced the frequency of mIPSCs without any significant (P > 0.9) changes in their ampli-