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TRAF-dependent association of protein kinase Tpl2/COT1 (MAP3K8) with CD40

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Abstract

Signaling by TNF-family receptor CD40 involves TRAF-family adaptor proteins, leading to activation of protein kinases that induce NFκB-family transcription factors. We report here that mitogen activated protein kinase kinase kinase-8 (MAP3K8), Tpl2/COT1, is recruited to the CD40 complex via a mechanism dependent on TRAF-binding sites in CD40. Tpl2/COT1 was shown to participate in CD40 signaling based on the ability of a catalytically inactive mutant to suppress CD40-mediated IκB kinase activation and induction of NFκB-responsive promoters, without affecting signaling by TNF. Tpl2 (−/−) fibroblasts were also deficient in CD40 but not TNF signaling, further supporting a unique role for Tpl2 in CD40 signaling. Experiments using dominant-negative Tpl2 suggest this kinase functions distal to TRAFs but proximal to the TAK1/TAB1 signaling complex, within the IKK/NFκB activation pathway. These results indicate a distinction between TNF Receptor family members CD40 and TNFR1 in their utilization of MAP3Ks, and demonstrate TRAF-dependence of Tpl2 association with the CD40 receptor complex.

Keywords: CD40; TRAF; Tpl2; COT1; MAP3K8

CD40 is a member of the TNF-receptor (TNFR) superfamily, which has emerged as a promising drug-target because of the critical role this receptor plays in autoimmunity, allograft rejection, and atherosclerosis [1–4]. Thus, a need exists to understand the downstream signaling events triggered by CD40, so that points for therapeutic intervention can be identified. CD40, as well as the type I and type II TNF-receptors (TNFRI; TNFR2), activates similar kinase cascades, including mitogen-activated protein kinases (MAPKs) such as Jun N-terminal kinase (JNK) and the IκB kinase (IKK) [5,6]. Upon ligand binding, these TNF-family receptors inducibly associate with members of the TRAF family of adaptor proteins, which then recruit downstream protein kinases. Several MAP3Ks have been implicated as downstream targets of TRAFs, including NIK, ASK1, and MEKK 1, 2, and 3 [7,8].

MAP3K8 is a MAP3K family member also known as COT1 in humans and Tpl2 in mice and rats [9,10], which has recently been implicated in CD40 [11,12]. When over-expressed, this serine/threonine kinase activates ERK, JNK, NFAT, and NFκB signal cascades [13–17]. The recent generation of Tpl2 knockout mice has further clarified the roles of this kinase in CD40 signaling in various types of cells, showing that Tpl2 plays redundant as well as unique roles in CD40 signaling in B-lymphocytes and macrophages [12,18]. However, the upstream mechanisms responsible for Tpl2 activation following CD40-stimulation and the downstream consequences remain poorly understood. Here, we compared the role of Tpl2/COT1 in mediating CD40 and TNFR induction of NFκB, and examined the role of

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TRAF-family proteins in linking CD40 to Tpl2/COT1 signaling.

Experimental procedures

Reagents. TNF was obtained from Alexis Biochemicals (San Diego, CA) and CD40L (CD154) from R&D Systems (Minneapolis, MN). Rabbit antibodies specific for human CD40 (N17, C20), COT1/Tpl2, TRAF6, IKKγ, TNF-RI, and mouse anti-myc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 affinity gel and peroxidase-labeled anti-FLAG M2 antibodies were purchased from Sigma (St. Louis, MO). High-affinity rat anti-HA (unconjugated or peroxidase conjugated) was obtained from Roche Diagnostics (Indianapolis, IN). Antibodies recognizing IκBα, JNK (SAPK), and phosphorylated JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA), while anti-T7 antibody was purchased from Novagen (Madison, WI). For in vitro kinase assays, purified GST-IκB (1–317) protein was purchased from Santa Cruz Biotechnology.

Cell culture, transfections, and reporter assays. HEK293 and HeLa cells were cultured in DMEM supplemented with 5–10% fetal bovine serum (FBS), 1–2 mM L-glutamine, and antibiotics (penicillin/streptomycin). Follicular lymphoma cell line RS11846 was passaged in RPMI with 10% FBS. Tpl2 knockout and corresponding wild-type mouse embryo fibroblasts (MEFs) were cultured in DMEM containing 10–20% FBS [18]. Transfections of HEK293 cells were performed using Superfect reagent (Qiagen; Valencia, CA) in either 60 mm dishes (co-immunoprecipitation and in vitro kinase assays) or as triplicates in 96-well plates (luciferase reporter gene assays). Luciferase activity was assayed using the Dual-Luciferase Reagent Kit from Promega (Madison, WI). Transfections of MEFs were performed using the Lipofectamine-Plus reagent (Invitrogen; Carlsbad, CA).

Plasmids. Expression plasmids encoding TRAFs, CD40, and various mutants have been described previously [19]. For co-immunoprecipitation studies, *Bam*HI/*Xho*I fragments containing either wild-type or mutant CD40 were subcloned into pcDNA3-myc. FLAG-tagged versions of wild-type (WT) or the kinase-deficient (K167M) Tpl2 were generated from pCMV5-Tpl2 or pCMV5-Tpl-2 (K167M) [20] by PCR and subcloned into pcDNA3-FLAG. pCMV-HA-TAK1, the kinase inactive pCMV-HA-TAK1K63W, pCMV-TAB1, and pCMV-T7-TAB2 expression plasmids were generously provided by Dr. Kuni Matsumoto (Nagoya University, Japan). The luciferase reporter plasmid pNFκB-Luc was purchased from Stratagene (La Jolla, CA), while the *Renilla* luciferase plasmid was used for normalizing data. The pRLtk-Luc plasmid was obtained from Promega (Madison, WI). The c-IAP2-Luc (−1400) promoter reporter was kindly provided by Dr. Tae H. Lee [21].

Immunoprecipitation and kinase assays. HEK293 cells were transfected with in 60 mm dishes and cultured for 24–36 h before lysis in co-IP buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 2 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, 0.4% Triton, and complete protease inhibitor tablet) for 0.5 h. Clarified extracts were pre-cleared with the appropriate isotype-matched antibody and BSA-blocked protein G–Sepharose beads (Zymed Laboratory; South San Francisco, CA) for 1 h at 4 °C. Myc-tagged proteins were immunoprecipitated with anti-myc (9E10)-conjugated agarose beads for 4 h at 4 °C with rotation. For FLAG-tagged proteins, lysates were adjusted to 0.4 M NaCl, followed by incubating with anti-FLAG M2-agarose beads for 3 h. T7-tagged TAB2 was immunoprecipitated overnight with 2 μg of the anti-T7 antibody, followed by addition of BSA-blocked protein G–Sepharose beads for an additional 2 h. For immunoprecipitation of endogenous proteins, 2 μg anti-CD40–agarose (N-17) or anti-IKKγ–agarose (FL-419) antibodies was used. Immunoprecipitates were then washed extensively in co-IP buffer before boiling in SDS–sample buffer and analyzing by SDS–PAGE/immunoblotting. For kinase assays, immunoprecipitates were washed 3 times in co-IP buffer followed by two subsequent washes in kinase buffer (20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM Na_3VO_4, 1 mM DTT, 2 mM NaF, and 10 mM β-glycerophosphate). Immunoprecipitates were then incubated for 0.5 h at 30 °C with kinase buffer containing 5 μM ATP, 5 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 1 μg GST-IκB (1–317) before protein fractionation by SDS–PAGE and transfer to nitrocellulose membranes.

In vivo radiolabeling. HeLa cells transfected with HA-Tpl2KM and either myc-TRAF2 or myc-TRAF6 expression plasmids were washed once with phosphate-free DMEM prior to incubation with the same media supplemented with 10% dialyzed FBS for 2 h. [^{32}P]Orthophosphoric acid (0.5 mCi) was then added for an additional 2 h before the cells were washed and lysed in co-IP buffer, and immunoprecipitated with anti-HA–agarose beads. Immunoprecipitates were washed extensively in co-IP buffer before analysis by SDS–PAGE and transferred onto nitrocellulose membranes and exposing to X-ray film. To determine the amount of expressed TRAF2 and TRAF6, membranes were further immunoblotted with anti-myc antibodies.

Results and discussion

Involvement of Tpl2 in signaling by CD40 but not TNF

We used a catalytically inactive mutant of Tpl2 containing a lysine 167 methionine substitution in the ATP-binding pocket [13] to explore the role of this kinase in CD40 signaling. The ability of Tpl2 (K167M) to block IKK activation induced by CD40 was demonstrated by gene transfer experiments using HEK293 cells. Transfecting increasing amounts of CD40-encoding plasmid triggered dose-dependent increases in IKKα kinase activity and IκBα phosphorylation, while co-expression of Tpl2 (K167M) significantly inhibited this induction (Fig. 1A).

To corroborate the kinase assay results, CD40-dependent NFκB activity was also evaluated via luciferase-reporter assays (Fig. 1B). Both a synthetic NFκB-reporter and a NFκB-responsive natural promoter (derived from the *cIAP2* gene) displayed increased activity in HEK293 cells transfected with CD40-encoding plasmid, while co-transfection of Tpl2 (K167M) decreased this CD40-mediated NFκB induction.

In contrast to its effects on CD40, Tpl2 (K167M) had little or no effect on IκBα phosphorylation or NFκB activity induced by treatment of HEK293 cells with TNF or transfection with TNFR1-encoding plasmid (Fig. 1C). Tpl2 dominant-negative also did not prevent TNF-mediated induction of NFκB-responsive reporter genes (Fig. 1D).

Because experiments using dominant-negative versions of kinases can produce non-physiological results, we employed Tpl2 knockout cells to further address the role of this kinase in CD40 signaling. Using early-passage MEFs derived from Tpl2 (−/−) and Tpl2 (−/+) embryos [18], we transfected plasmids encoding human CD40 (hCD40) along with NFκB-reporter gene plasmids, and then treated cells with recombinant human

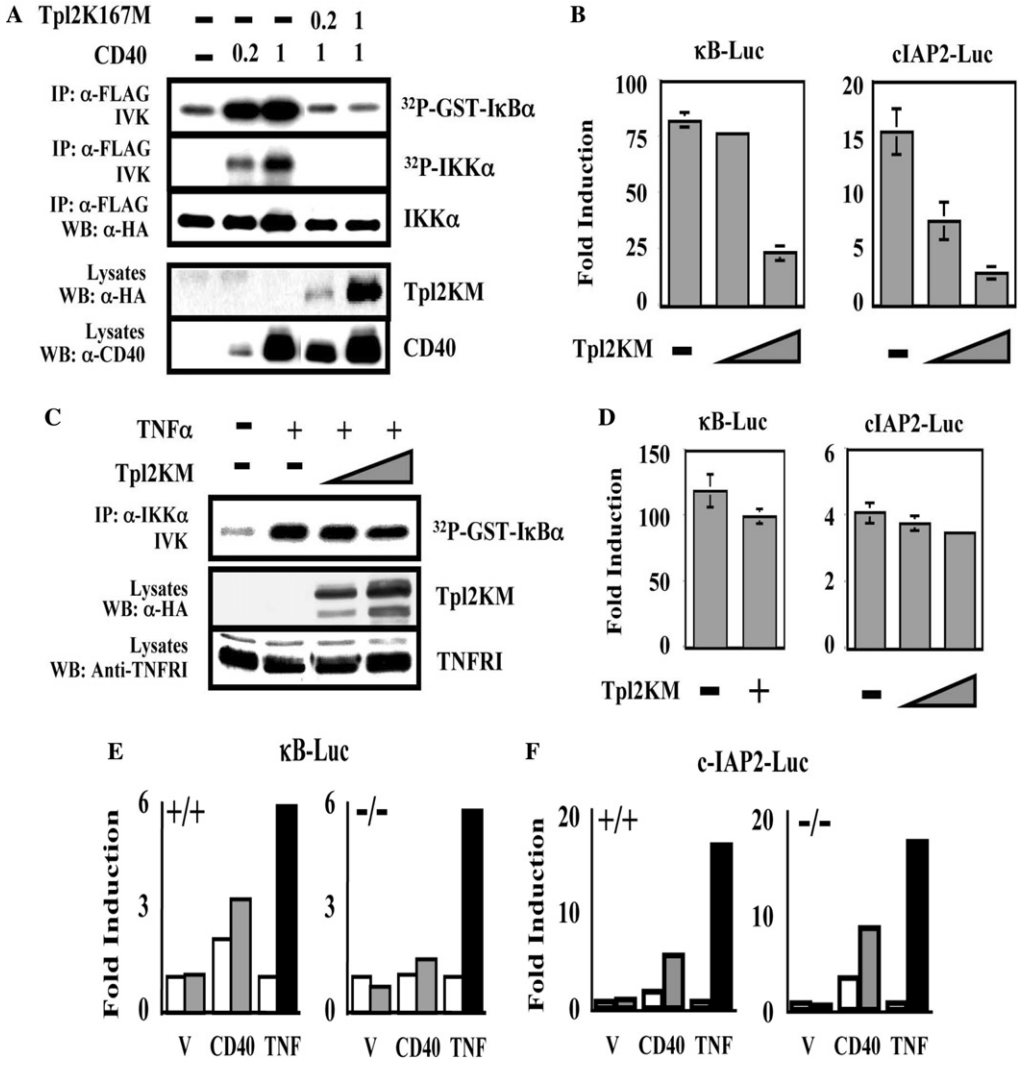

Fig. 1. Tpl2 regulates IKK activation and NFκB induction by CD40. (A) Kinase assays: HEK293 cells were transfected with vector or plasmids encoding CD40 (200 ng, 1 μg), or CD40 (1 μg) and Tpl2K167M (200 ng; 1 μg). As a sensor for IKK activity, FLAG-IKKα (100 ng) was co-transfected in each sample. After 24 h, anti-CD40 antibody (G28-5; 10 μg/ml) was added for 15 min and then cells were lysed and immunoprecipitation were performed using anti-FLAG–agarose beads. Immune-complexes were incubated with GST-IκBα (1–317) and [γ-^{32}P]ATP before SDS–PAGE, transfer, and autoradiography. Lysates were loaded directly into gels to determine the amounts of Tpl2KM and CD40 expressed in each sample. (B) Reporter assays: HEK293 cells were transfected with either κB-Luc (left) or cIAP2-Luc (right) reporters and CD40 (50 ng) with increasing amounts of Tpl2KM (10 ng; 50 ng). To normalize for transfection efficiency, pRLtk-Luc was co-transfected. After 24 h, cells were processed for their luciferase activity using the dual-luciferase reporter assay system. Normalized luciferase units in triplicates were averaged and fold induction was calculated for each sample relative to vector control-transfected cells. (C) In vitro kinase assays: HEK293 cells were transfected with control plasmid or Tpl2K167M (200 ng, 1 μg). The next day, rhTNFα (20 ng/ml) was added for 15 min and cells were lysed for immunoprecipitation with anti-IKKα antibody and agarose beads. Precipitates were washed and used for in vitro kinase assays with GST-IκBα, as above. (D) Reporter assays: HEK293 cells were transfected with TNFRI (50 ng) with either κB-Luc (left) or cIAP2-Luc (right). After 24 h, cells were processed for dual-luciferase measurements. (E,F) Equally passaged wild-type (+/+) or Tpl2 knockout (−/−) MEFs were transfected with vector (V) or huCD40 (0.5 μg) along with κB-Luc (E) or cIAP2-Luc (F) reporter constructs. At 3 days post-transfection, PBS (white bars), 1 μg/ml rhCD40L (gray bars), or 20 ng/mL TNF (black bars) was added for 36 h before lysates were collected for dual-luciferase measurements as described. Results shown are representative of three individual experiments.

CD40L or murine TNF, and measured luciferase activity. NFκB activity induced by TNF was similar in Tpl2-expressing and knockout MEFs, when tested on two different reporter genes (Figs. 1E and F), confirming that TNF-signaling is largely Tpl2-independent. When control-transfected MEFs were stimulated with hCD40L, NFκB activity did not change, presumably because these cells express little of any CD40 endogenously. In contrast, transfection of CD40 into wild-type MEFs induced a significant rise in NFκB activity, which was further augmented by addition of CD40L (Fig. 1E). Comparisons of Tpl2-expressing and Tpl2-deficient MEFs revealed that CD40-mediated induction of NFκB activity was markedly blunted in cells lacking Tpl2,

confirming a role for Tpl2 in CD40-mediated induction of NFκB. However, when measured with a natural promoter (*cIAP2*, −1400 to −1 bp), reporter activity was not significantly diminished by Tpl2-deficiency (Fig. 1F). Overall, these data imply that although Tpl2 contributes to CD40-directed NFκB activation, Tpl2-independent pathways also exist for inducing activation of the *cIAP2* promoter.

Tpl2/COT1 participates in CD40 signaling via interactions with TRAFs

Having observed a functional role for Tpl2/COT1 in CD40 signaling, we next asked whether this kinase is recruited to the CD40 receptor complex upon ligand binding, as would be expected if this kinase plays a proximal role in the signal transduction mechanism. To this end, the RS11846 human B-cell follicular lymphoma line was treated with CD154 (CD40L) for up to 20 min, then lysed for immunoprecipitation with anti-CD40 antibody. To ensure that proper signaling occurred, portions of the cell lysates were also used for immunoblotting analysis of IκBα, phospho-SAPK/JNK1, and total SAPK/JNK1 protein. Endogenous COT1/Tpl2 associated with endogenous CD40 as early as 5 min after treatment with CD40L (Fig. 2A). IKK kinase activity increased with similar kinetics in RS11846 cells after CD40L stimulation, as determined by in vitro kinase assays using anti-IKKγ immunoprecipitates. Within 10 min after CD40L addition, levels of endogenous IκBα had dramatically diminished, consistent with CD40-mediated activation of IKK, while levels of phospho-SAPK/JNK significantly increased. Thus, COT1/Tpl2 is rapidly recruited to the CD40 receptor complex upon ligand binding, confirming the data obtained using another CD40-responsive B-cell lymphoma line BJAB [12].

Since the cytosolic domain of CD40 directly binds several TRAF-family proteins (TRAF2, TRAF3, and TRAF6) [19], we explored whether Tpl2 indirectly associates with CD40 through interactions with these adaptor proteins. Accordingly, the ability of Tpl2 to co-immunoprecipitate with CD40 mutants that are defective in binding certain TRAF-family proteins was tested [19]. For these experiments, Myc-tagged CD40 or CD40 mutants defective either in binding TRAF2 and TRAF3, or in binding TRAF6 were co-transfected with HA-tagged wild-type Tpl2 into HEK293 cells. Lysates were immunoprecipitated with anti-myc-conjugated beads followed by immunoblot analysis with anti-HA antibodies (Fig. 2B). Wild-type CD40 and single-site mutants of CD40 deficient in binding TRAF2/3 or TRAF6 retained the ability to co-immunoprecipitate Tpl2. In contrast, little Tpl2 co-immunoprecipitated with CD40 mutants lacking all TRAF-binding sites, including: (a) a two-site mutant (ET235/254AA: ETAA) in which both the TRAF2/3-binding (T254A) and TRAF6-binding (E235A) sites were mutated and (b) a truncation mutant of CD40 (Δ32E235A) that lacks the C-terminal 32 amino-acids required for TRAF2/3 binding and that also contains a E235A mutation which abolishes TRAF6 binding [7]. Immunoblot analysis of lysates from the transfected cells confirmed the production of all plasmid-encoded proteins (Fig. 2B). Thus, the TRAF-binding sites of CD40 are important for association with Tpl2.

Since TRAF-binding sites on CD40 are required for Tpl2 association with CD40, we next asked whether TRAFs modulate the activity of Tpl2. Because a suitable in vitro kinase assay for Tpl2 does not exist, we instead used phosphorylation of Tpl2 as a surrogate indicator of its activation status. To avoid *trans*-autophosphorylation, kinase-dead Tpl2 (K167M) [13] was employed and co-expressed with TRAF2 or TRAF6. In [^{32}P]orthophosphoric acid-labeled HeLa cells, over-expression of either TRAF2 or TRAF6 induced increases in Tpl2 phosphorylation. Immunoblot analysis of immunoprecipitated Tpl2 complexes demonstrated equivalent recovery of Tpl2 protein from all samples and also showed the presence of Tpl2-associated TRAF2 and TRAF6 (Fig. 2C), indicating that TRAFs associate directly or indirectly with Tpl2. Thus, TRAF proteins induce Tpl2 phosphorylation. It is unclear, however, whether the phosphorylation of Tpl2 is caused by TRAF-mediated association of endogenous (active) Tpl2 with the transfected (inactive) Tpl2 versus by another TRAF-associated kinase.

Having observed that TRAF over-expression induces Tpl2 phosphorylation, we next investigated if Tpl2 modulates TRAF-dependent activation of IKK and subsequent induction of NFκB activity. For these experiments, HEK293 cells were transfected with plasmids encoding TRAF2 or TRAF6, alone or in combination with Tpl2K167M, then either IKK activity was measured via in vitro kinase assays (Fig. 2D) or NFκB activity was measured by reporter gene assays (Figs. 2E and F). Similar to the results obtained with CD40, over-expression of either TRAF2 or TRAF6 induced striking increases in IKK and NFκB activity, which were severely diminished by co-expression of Tpl2K167M. Thus, Tpl2 modulates the ability of TRAF2 and 6 to activate IKK and to induce NFκB activity, suggesting that Tpl2 is a downstream effector of these TRAFs.

It should be noted however that these inhibitory effects of Tpl2 (K/M) could be due to its competition with other kinases for binding to TRAFs, rather than a reflection of a requirement for Tpl2 for TRAF signaling. In this regard, several kinases are reportedly involved in TRAF-mediated signaling into the IKK/NFκB pathway, including NIK, MEKK1, MEKK3, GSK3β, TBK, ζPKC, and ASK1 (reviewed in [22]). Thus, our

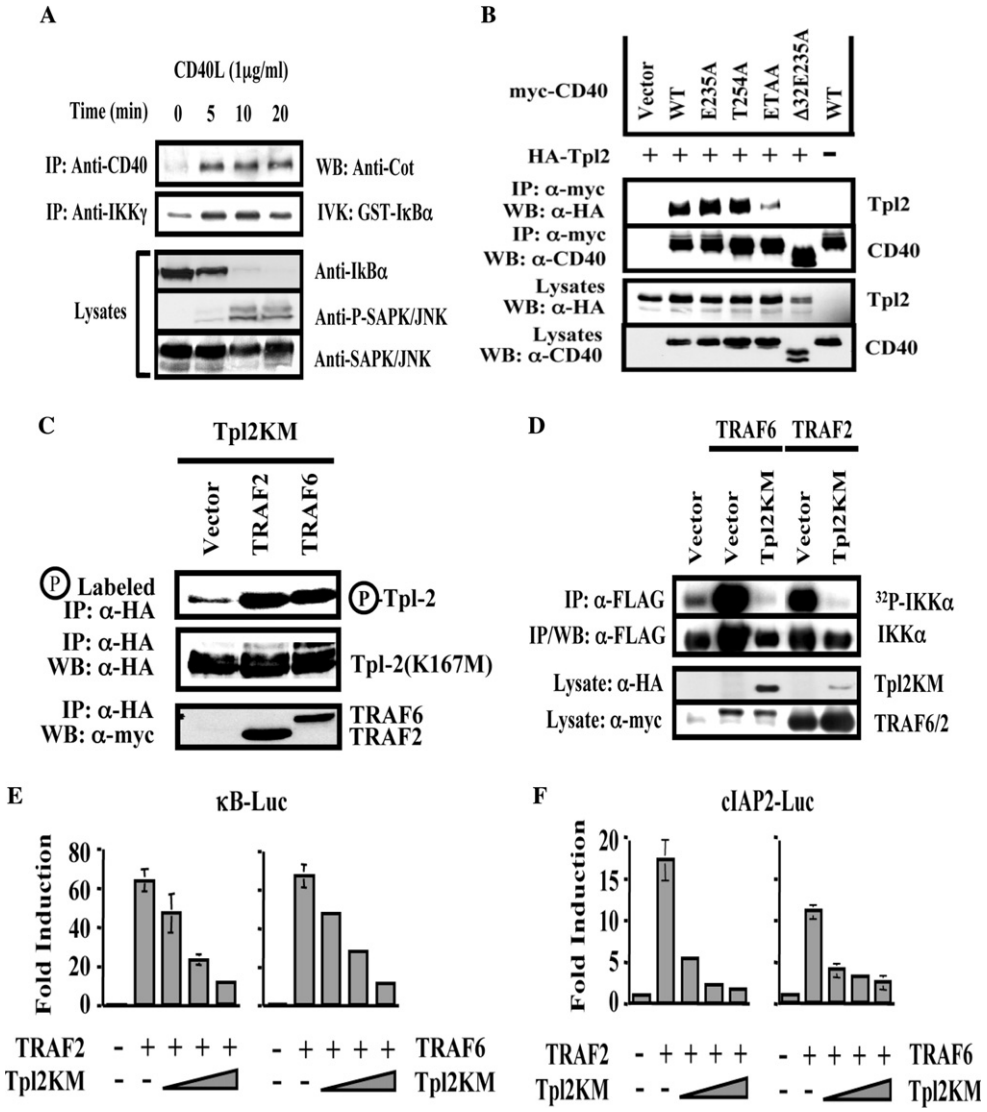

Fig. 2. COT1/Tpl2 interacts with and modulates signaling by TRAFs. (A) Tpl2/COT1 is recruited to CD40 complex. RS11846 cells ($4–5 \times 10^7$) were activated with hrCD40L (1 μg/ml) for the indicated times before washing and lysis. Pre-cleared lysates were immunoprecipitated with anti-CD40–agarose or anti-IKKγ–agarose for 4 h. Anti-CD40 immunoprecipitates were washed extensively in lysis buffer and analyzed by immunoblotting with anti-COT1. Anti-IKKγ immunoprecipitates were washed extensively in lysis buffer followed by kinase buffer before using an in in vitro kinase assay with GST-IκBα. As controls for loading and activation, lysates normalized for total protein were blotted using anti-IκB, anti-phospho-SAPK/JNK, or anti-SAPK/JNK antibodies. (B) TRAF-binding sites are required for Tpl2 association with CD40. HEK293 cells were transfected with HA-Tpl2 (1 μg) along with vector, wild-type (WT), E235A, T254A, ETAA, or Δ32E235A CD40 expression encoding plasmids (1.5 μg). After 24 h, cells were lysed, and immunoprecipitations were performed using anti-myc–agarose. Bound Tpl2 was analyzed by immunoblotting with anti-HA-HRP antibodies. Lysates were also blotted with anti-CD40 and anti-HA-HRP to confirm equivalent sample loading. (C) TRAF2 and 6 induce Tpl2 phosphorylation. HeLa cells were transfected with HA-Tpl2KM (1 μg) and either vector, TRAF2, or TRAF6 (2 μg) encoding plasmids. After 36 h, cells were cultured in phosphate-free medium, and then grown in [^{32}P]orthophosphate labeling (2 h) before lysis and immunoprecipitation with anti-HA–agarose. Tpl2 immune-complexes were washed extensively before analysis by SDS–PAGE and autoradiography. (D) Tpl2 modulates TRAF-induced IKK activation. Vector, TRAF6, or TRAF2 (1 μg) encoding plasmids were cotransfected with FLAG-IKKα (100 ng) and either vector or HA-Tpl2KM (2.5 μg) into HEK293 cells. After 24 h, cells were lysed and immunoprecipitations were preformed for in vitro kinase assays using anti-FLAG–agarose. Immune complexes were washed and incubated with [γ-^{32}P]ATP and GST-IκBα, followed by SDS–PAGE and autoradiography. Lysates were immunoblotted against anti-myc, anti-HA, or anti-FLAG antibodies for expression control. (E,F) Tpl2 modulates TRAF induction of NFκB activity. HEK293 cells were transfected with TRAF2 or TRAF6 and Tpl2KM (10 ng, 50 ng) along with pRLtk-Luc and κB-Luc (E) or cIAP2-Luc (F) reporters. After 24 h, cells were processed for dual-luciferase assay determination.

data demonstrate the potential of Tpl2 to modulate TRAF-dependent signaling into the IKK/NFκB pathway but do not imply a requirement for Tpl2, particularly given the known redundancy of kinases downstream of TRAFs. Nevertheless, the experiments using CD40 mutants provide evidence that TRAFs are critically important for association of Tpl2 with the CD40 receptor complex.

Tpl2 engages the TAK1 signaling complex

TAK1 is a MAP3K previously reported to initiate IKK and MAPK activation mediated by IL-1, LPS, RANKL, and TRAF6-dependent signaling pathways [23–25]. Partnered with its adaptor TAB1, TAK1 is recruited to TRAF6 via a second adaptor TAB2, which releases TRAF6 from MyD88 and IRAK after activation [26]. Functional TAK1 is then thought to engage and activate the IKK signaling complex. Since Tpl2 interacts with TRAF6, and previous reports have shown that Tpl2/COT1 can engage and activate the IKK complex [17,27], we explored whether the ability of Tpl2 to induce IKK activity involves the TAK1 signaling complex as an intermediary.

For these experiments, combinations of wild-type and dominant-negative Tpl2 and TAK1 proteins were co-expressed in HEK293 cells to determine their hierarchical positions in pathways leading to IKK activation (measured by in vitro kinase assays) and NFκB induction (measured by reporter gene assays). Co-expressing TAK1 dominant-negative with Tpl2 suppressed Tpl2-mediated IKK activation (Fig. 3A). In contrast, co-expressing Tpl2 dominant-negative with TAK1 did not inhibit TAK-mediated IKK activation. Similarly, in NFκB reporter gene assays, expression of TAK1 dominant-negative inhibited Tpl2-induced NFκB activity, while expression of Tpl2 dominant-negative failed to inhibit TAK1-induced NFκB activity (Fig. 3B). Taken together, these observations suggest that Tpl2-dependent IKK and NFκB activation either requires downstream participation of the TAK/TAB signaling complex or that the TAK/TAB complex can at least compete for other downstream effectors linked to Tpl2-induced IKK/NFκB signaling.

Next, we asked whether Tpl2 can physically associate with components of the TAK1/TAB complex, as would be expected if TAK1/TAB complex proteins are direct substrates of Tpl2. Since recent studies suggest that IL-1 activation mediates the phosphorylation and translocation of TAB2 as a prerequisite for TAK1 activation [26], we focused on TAB2. Control, wild-type, and kinase dead Tpl2 were co-transfected with T7-TAB2 into HEK293 cells followed by immunoprecipitation of T7-tagged TAB2. Immune-complexes were subjected to in vitro kinase assays to monitor [γ-^{32}P]ATP incorporation into TAB2. As shown in Fig. 3C, TAB2 immune-complexes obtained from cells expressing active Tpl2 demonstrated robust phosphorylation of TAB2 in vitro. In contrast, TAB2 phosphorylation was not detected with immune-complexes prepared from cells expressing Tpl2 (K167M).

We further explored whether Tpl2 associates with TAB2. To this end, HEK293 cells were transfected with

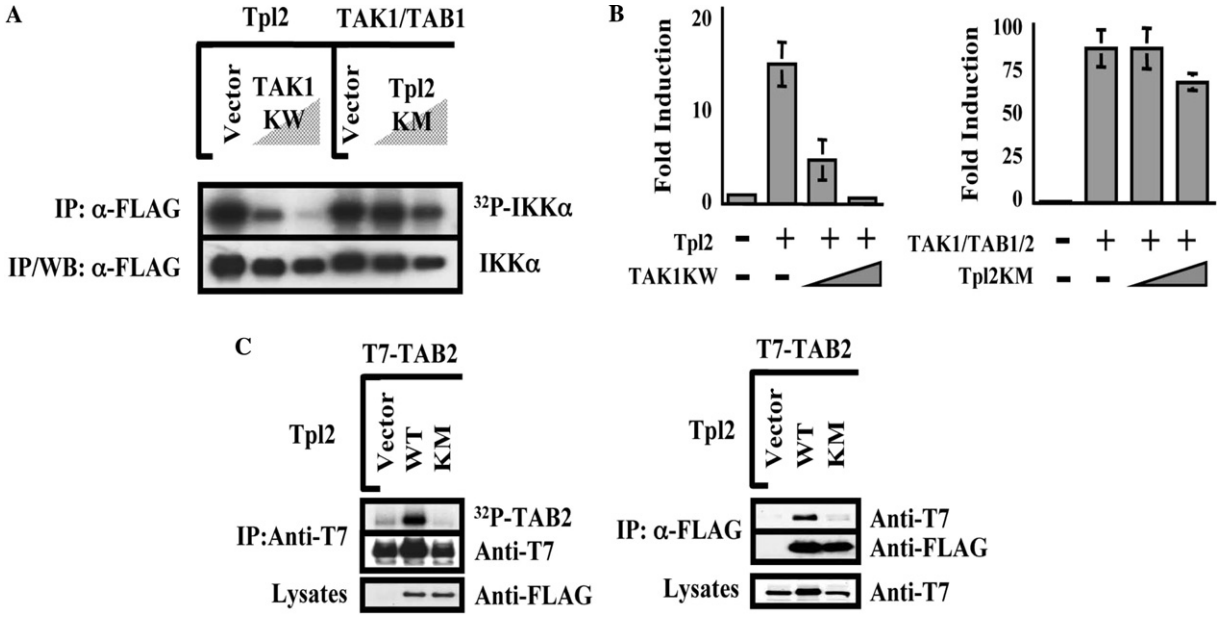

Fig. 3. Hierarchy of Tpl2–TAK/TAB interactions. (A) In vitro kinase assays: wild-type HA-Tpl2 (1.5 μg) plasmid was co-transfected with plasmids encoding FLAG-IKKα (200 ng) and HA-TAK1K63W (500 ng, 1.5 μg) into HEK293 cells. Conversely, wild-type TAK1 (1 μg) and TAB1 (1 μg) encoding plasmids were co-expressed with either vector or Tpl2KM (500 ng, 1.5 μg). FLAG-IKKα was immunoprecipitated, washed and incubated with [γ-^{32}P]ATP for in vitro kinase assay, and then analyzed by SDS–PAGE/autoradiography. (B) Reporter gene assays: vector or wild-type Tpl2 plasmids were cotransfected with κB-Luc, pRLtk, and TAK1K63W plasmids into HEK293 cells. Conversely, TAK/TAB1/2 plasmids were cotransfected with vector or Tpl2KM. After 24 h, luciferase activity was measured, as described. (C) TAB2 phosphorylation and interaction with Tpl2: vector, wild-type (1.5 μg), or Tpl2KM (1.5 μg) plasmids were cotransfected with T7-TAB2 (2 μg) into HEK293 cells for 24 h before lysis and immunoprecipitation with either anti-T7 or anti-FLAG antibodies. Anti-T7 complexes were washed and incubated with [γ-^{32}P]ATP before SDS–PAGE and autoradiography (right). Anti-FLAG complexes were washed and analyzed by immunoblotting with anti-T7 antibodies (left).

plasmids encoding T7-tagged TAB2 in combination with either active or inactive Tpl2. As shown in Fig. 3C, only wild-type Tpl2 co-immunoprecipitated TAB2, suggesting that catalytically competent Tpl2 is required for TAB2 association.

Taken together, these data lend further support for a downstream role for TAK/TAB proteins in the IKK/NFκB signal transduction pathway induced by Tpl2/COT1. It should be noted however that these observations are based on the use of over-expression and dominant-negative mutants, and thus the caveat exists that gene ablation methods will be required to formally demonstrate the hierarchical relation of Tpl2 relative to TAK1, as well as to ascertain how critical TAK1 is to the ability of Tpl2 to induce NFκB activity in cells. (Attempts to knock-down expression of Tpl2/COT1 and TAK1 in B-cells using siRNA or antisense were unsuccessful.) Nevertheless, Tpl2 is capable of associating with and modulating phosphorylation of components of the TAK1/TAB complex, demonstrating the potentiality of Tpl2/COT1 to function as an upstream activator of TAK1.

Conclusions

Here, we demonstrate a role for Tpl2 in selectively transducing signals from CD40 (but not TNF) to NFκB, based on dominant-negative and gene ablation studies, and show for the first time that Tpl2 association with CD40 is dependent on TRAF-family adaptor proteins. The extent to which CD40-mediated signaling requires Tpl2/COT1 however is likely to vary among cell types due to redundancy among the MAP3Ks that can mediate transmission of CD40 signals to downstream kinases involved in NFκB activation. Recent data from B-cells and macrophages isolated from *tpl2* (−/−) mice indicate that this kinase is not essential for CD40-mediated IKK activation and induction of NFκB activity [12], implying that other kinases may substitute for Tpl2 in those cell-types. In contrast, we observed that kinase-dead Tpl2 significantly reduced CD40-mediated activation of IKK and NFκB in transfected epithelial cells, and also found that *tpl2*-gene ablation greatly reduces CD40-stimulated NFκB signaling in fibroblasts. In this regard, CD40 is expressed on fibroblasts and many types of epithelial cells, in addition to B-cells and macrophages [2,3]. Thus, in addition to its role in communication among immune cells, presumably CD40 also allows CD40L-expressing cells to invoke host-defense pathways at body surfaces (epithelial cells) or to modulate wound-healing responses (fibroblasts). Cell-type specific differences in expression of MAP3Ks may play an important role in dictating which of these kinases is preferentially utilized for signaling by TNF-family receptors. The data provided here argue that Tpl2/COT1 is among the MAP3Ks capable of participating in CD40 signal-transduction leading to NFκB activation.

Although the TNFRI and CD40 receptor complexes both recruit TRAFs, the specific TRAF-family members involved only partially overlap. Upon TNF activation, the death domain of TNFRI interacts with adaptor proteins TRADD and RIP, which in turn bind to TRAF2, also indirectly recruiting TRAF1 in some instances (reviewed in [28]). In contrast to TNF, CD40 directly binds TRAF-2, 3, and 6. Thus, different adaptor proteins are recruited to these receptor complexes, suggesting differences in the relative requirements for Tpl2/COT1 among TRAF-family members. In addition, the specific combinations of TRAFs brought to receptor complexes may play a significant role in dictating the kinase activation mechanism, with different TRAFs perhaps recruiting complementary kinases or kinase-activators to the complex. Thus, the differences between CD40 and TNFR1 with respect to the TRAF-family members they recruit may explain the preferential ability of Tpl2(K167M) dominant-negative and of *tpl2* gene ablation to interfere with NFκB activation induced by CD40 compared to TNF in epithelial cells and fibroblasts, respectively.

Altogether, the data presented here suggest a pathway linking CD40 to NFκB induction, whereby CD40 binds TRAFs, which in turn bind Tpl2/COT1, recruiting this kinase to the CD40 receptor complex. Tpl2/COT1 then associates with and activates the TAK1/TAB complex, resulting in downstream activation of IKK and subsequent induction of NFκB. While alternative pathways and other molecules for connecting CD40 to NFκB signaling exist, our findings nevertheless reveal one of the possible signal transduction pathways available, and thus provide insights into the signaling mechanisms of this TNF-receptor family member that has been implicated in multiple human diseases.

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