Physical and Functional Interaction of Filamin (Actin-binding Protein-280) and Tumor Necrosis Factor Receptor-associated Factor 2*

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Tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) is an intracellular protein involved in signal transduction from TNF receptor I and II and related receptors. TRAF2 is required for TNF-induced activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and TRAF2 can also mediate activation of NF-κB. Here we have identified the actin-binding protein Filamin (actin-binding protein-280) as a TRAF2-interacting protein. Filamin binds to the Ring zinc finger domain of TRAF2. Overexpressed Filamin inhibits TRAF2-induced activation of JNK/SAPK and of NF-κB. Furthermore, ectopically expressed Filamin inhibits NF-κB activation induced via TNF, interleukin-1, Toll receptors, and TRAF6 but not activation induced via overexpression of NIK, a downstream effector in these pathways. Importantly, TNF fails to activate SAPK or NF-κB in a human melanoma cell line deficient in Filamin. Reintroduction of Filamin into these cells restores the TNF response. The data imply a role for Filamin in inflammatory signal transduction pathways.

Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 are among the most potent physiological activators of NF-κB transcription factors as well as of SAPK/JNK MAP kinases (1–3). The latter kinases regulate the activity of activated protein 1 transcription factors via phosphorylation (4). NF-κB activity is regulated by nuclear translocation in response to signals. In the absence of signals, IκB inhibitory proteins bind to NF-κB factors and hold them in an inactive state in the cytoplasm by shielding their nuclear localization sequences (5). Following an appropriate signal, IκB inhibitors are phosphorylated and subsequently degraded by proteasomes in a ubiquitin-dependent manner (6–8). The transcription factors NF-κB and activated protein 1 are critical to induced transcription of many genes during inflammatory responses.

Much is known about signaling via members of the TNF receptor superfamily, via IL-1 receptors (IL-1R) and via IL-1R-related Toll receptors. Although these transmembrane proteins have distinct structures, they employ apparently similar strategies during signaling. Ultimately, they all recruit members of the TRAF family of adaptors (9–13). Of the known TRAFs, TRAF2, TRAF5 and TRAF6 have all been linked to NF-κB activation in various studies (10, 14, 15), and TRAF2 in particular has been linked to activation of JNK/SAPK (16). Among these three TRAFs, TRAF2 appears to be central to signaling via TNF receptors I and II, whereas TRAF2, TRAF5, and sometimes even TRAF6 may be engaged simultaneously by other members of the TNF receptor superfamily, including CD40 (17–18), RANK (19), CD30 (20), and CD27 (21). Finally, TRAF6 seems to be the primary TRAF protein utilized in IL-1 and Toll receptor signaling (10, 12–13, 22). The critical importance of TRAF2 in TNF-initiated activation of SAPK has been confirmed by the failure of TNF to activate this MAP kinase in cells of mice lacking TRAF2 or expressing a dominant negative acting TRAF2 (23, 24). Somewhat unexpectedly, NF-κB activation was only partially blocked in these same cells. It was suggested that other TRAF proteins, such as TRAF5 or -6, may have compensated for the lack of TRAF2 in TNF-mediated activation of NF-κB. It is also possible that parallel, TRAF-independent mechanisms exist in addition, by which the TNF receptors may activate NF-κB.

TRAF proteins consist of a conserved C-terminal TRAF domain and an N-terminal region containing Ring and zinc finger motifs (25). The TRAF domain is involved in receptor association as well as homo- and heterotypic associations. The N-terminal Ring and zinc finger domains of TRAF2, -5, and -6 are reported to be the effector domains responsible for activation of NF-κB (15, 26). In the case of TRAF2 these two domains are also important for activation of SAPKs (16, 27). The immediate target of TRAF2 in TNF-mediated SAPK activation may be the MAP3K ASK1 or members of the GCK families of kinases (28–30). At least one immediate target of TRAF2 leading to NF-κB activation appears to be the NIK kinase. Since this kinase is not involved in SAPK activation, the signaling pathways leading to activation of NF-κB and of SAPK/JNK appear to diverge at the level of TRAF2 (31–32). NIK is a member of the MAP3K family and was first identified as a TRAF2-interacting protein but is now known to bind multiple members of the TRAF family (31, 33). Binding to NIK depends primarily on the TRAF domain, although the Ring zinc fingers may play a role in this as well (33). Bound TRAF appears to induce NIK activ-

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§ The abbreviations used are: TNF, tumor necrosis factor; IL-1, interleukin-1; IL-1R, IL-1 receptors; NF-κB, nuclear factor κB; IκB, inhibitor κB; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MAP3K, mitogen-activated protein 3-kinase; TRAF, TNF receptor associated factor; NIK, NF-κB inducing kinase; IKK, IκB inducing kinase; GST, glutathione S-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; PCR, polymerase chain reaction; PMCA, phorbol 12-myristate 13-acetate.
ity by an as yet unknown mechanism, possibly involving oligomerization of NIK, since NIK overexpression alone can also induce this kinase to activate NF-κB (34). Kinase-inactive mutants of NIK act as dominant inhibitors of NF-κB activation mediated by members of the TNF receptor superfamily, IL-1 and Toll receptors. These NIK mutants also inhibit TRAF2-, TRAF5-, and TRAF6-mediated NF-κB activation, indicating that NIK is a common mediator in the NF-κB signaling cascades triggered by inflammatory stimuli such as TNF and IL-1 (16, 31, 33). It is important to note, however, that such experiments involving a kinase dead mutant do not establish NIK as an essential component of these pathways although they do suggest some contribution by NIK.

NIK physically associates with and activates IκB kinase α (IKKα) and IκB kinase β (IKKβ) heterodimers (35, 36). IKKs are responsible for phosphorylating two critical serine residues in IκBs, triggering events that then lead to proteolytic degradation of these inhibitors (35–39). With the exception of UV, all other signals activating NF-κB appear to flow through the IKKs (40). Recently, IKKα and IKKβ knockout mice have been generated (41–46). Analyses of these mutant mice suggest that IKKβ is critical for activation of NF-κB by inflammatory cytokines, whereas IKKα is largely dispensable for this but is important instead during development of epidermal skin.

In addition to NIK, only one other kinase, the MAP3K MEKK1, is known to be able to activate directly IKKs (47–49). Although the role of MEKK1 in TNF- and IL-1-mediated activation of NF-κB remains obscure, some evidence suggests a role in TNF-initiated activation of SAPK (27, 50).

Despite these insights, many aspects of the molecular signaling pathways initiated by the TNF receptor superfamily, by the IL-1, and by the Toll receptors are not yet understood, including the functions of the TRAF proteins in this process. In an effort to understand better how TRAF2 signals downstream responses, we utilized a yeast two-hybrid system to isolate proteins interacting with the zinc and Ring finger domains of TRAF2. We present evidence that Filamin (actin-binding protein-280) interacts with these domains of TRAF2 in vitro and in vivo and that Filamin functions in activation of SAPK and NF-κB by inflammatory stimuli.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—**A full-length TRAF2 cDNA construct was isolated from a human-activated T cell cDNA library, using a PCR fragment as template. This TRAF2 clone was inserted into the mammalian expression vector pmT2T (51). A construct encoding the Ring finger and zinc finger domains of TRAF2 (amino acids 1–225) was generated by inserting an NheI-XhoI fragment of TRAF2 into pmT2T; the sequence upstream of the NheI site was replaced with a linker encoding the first 4 amino acids of TRAF2. A construct encoding the Ring finger domain of TRAF2 (amino acids 1–105) was generated by inserting an EcoRI-EcoRI fragment of TRAF2 into pmT2T. A construct containing the zinc finger domain of TRAF2 (amino acids 76–282) was generated by PCR and cloned into pmT2TXSE. PMT2TXSE is a modified pmT2T vector that supplies an N-terminal in-frame methionine in the context of a Kozak sequence. The N-terminal deletion mutant of TRAF2 (TRAF2Δ105), was obtained by deletion of a BamHI-EcoRI fragment encoding amino acids 1–105 from TRAF2. Bluescript SK plasmid containing full-length Filamin was provided by J. H. Hartwig (52). To enable its expression in eukaryotic cells, the full-length Filamin sequence was excised from BSK-Filamin as a HindIII/Xhol fragment and ligated into the HindIII/Xhol site of the vector pCDNA3 (Invitrogen, Inc.). TRAF6 was cloned from a Jurkat cell cDNA library (CLONTECH) using a PCR-generated DNA fragment as a probe. pCDNA3-TRAF6 was constructed in conjunction of the EcoRI/EcoRI fragment of TRAF6 into the BamHI/EcoRI sites of pCDNA3 (Invitrogen). Full-length human NIK was PCR-amplified from HeLa cell cDNA by using primers based on the published sequence. The PCR product was digested with BamHI/EcoRI and cloned into the BamHI/EcoRI sites of the vector pCDNA3-HA (Invitrogen). A constitutively active form of human Toll receptor, composed of the transmembrane and the cytoplasmic domains of human Toll fused with the extracellular domain of mouse CD4 (CD4/Toll), was a gift of Drs. Janeway and Medzhitov (53).

**Cell Culture, Transfection, and Immunoprecipitation—**The human embryonic kidney cell line 293 was obtained from the American Type Culture Collection. These cells were maintained in Dulbeco’s modified Eagle’s medium supplemented with 100 units/ml penicillin and streptomycin, and 1% glucose. Human melanoma cell lines (parental line M2 and stably transfected with full-length filamin M2T7A7) (54, 55) were a gift from Dr. O. Cantiello and Dr. P. Janmey (Harvard Medical School, Boston). These cells were grown in minimal Eagle’s medium supplemented with 8% newborn calf serum, 2% fetal bovine serum, and 1% transfected human fibroblast conditioned medium (Amersham Pharmacia Biotech). Expression and purification of the derived GST-TRAF2 fusion proteins were performed essentially as described (56). Binding of Filamin to the GST-TRAF2 fusion proteins was performed by incubating 5 μl of a slurry of glutathione-Sepharose beads bound to the GST-TRAF2 fusion protein with 1 ml of 293 cell extract for 2 h at 4 °C. The beads were extensively washed with lysis buffer, and the bound material was resolved by 10% SDSPAGE and transferred to nitrocellulose filters (Schleicher & Schuell). Western blots were performed, and the HA-tagged proteins were detected with the anti-HA antibody 12CA5 (Roche Molecular Biochemicals). The antigen-antibody complexes were washed with lysis buffer, reduced with β-mercaptoethanol, which reverses the cross-linker, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), followed by transfer to polyvinylidene difluoride membrane (Immobilon) and immunoblotting with anti-Filamin antibodies. The anti-Filamin antibody was a polyclonal rabbit antipeptide antibody directed against amino acids 21–34 of Filamin.

**GST Protein Binding Assay—**TRAF2 inserts were excised out of the constructs and used as bait in the two-hybrid system (see below) and then subcloned in-frame into the GST fusion protein vector pGEX-1 (Amersham Pharmacia Biotech). Expression and purification of the derived GST-TRAF2 fusion proteins were performed essentially as described (56). Binding of Filamin to the GST-TRAF2 fusion proteins was performed by incubating 5 μl of a slurry of glutathione-Sepharose beads bound to the GST-TRAF2 fusion protein with 1 ml of 293 cell extract for 2 h at 4 °C. The beads were extensively washed with lysis buffer, and the bound material was resolved by 10% SDS-PAGE and transferred to nitrocellulose filters (Schleicher & Schuell). Western blots were performed, and the HA-tagged proteins were detected with the anti-HA antibody 12CA5 (Roche Molecular Biochemicals). The antigen-antibody complexes were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).
Expression of the pRSV-βGal vector (0.2 μg) was used to normalize transfection efficiencies. For the IKK kinase assay, M2 and M2TA7 cells were stimulated with TNF (2000 units/ml) for 10 min and lysed in 1% Triton lysis buffer. Endogenous IKK complex was immunoprecipitated by using the anti-IKK antibody H744 (Santa Cruz Biotechnology), and the kinase activity was assayed using an HA-tagged TRAF2 (lane 2), HA-tagged TRAF2Δ105 (lane 3), or empty plasmid (lane 1). Lysates from 293 cells were incubated with anti-HA antibodies. Coprecipitated endogenous Filamin was detected by Western blotting with anti-Filamin antibodies. The lower panel shows the relative amounts of HA-TRAF2 and HA-TRAF2Δ105 in the 293 cell extract. Positions of molecular mass standard (in kilodaltons) are indicated. NS, nonspecific; Ig HC, immunoglobulin heavy chain.

RESULTS

Previous studies have demonstrated that the N-terminal zinc binding domains of TRAF2 (Ring and zinc fingers) are involved in mediating downstream signaling events (see Introduction). Seeking proteins that interact with the zinc binding domains of TRAF2, we used a TRAF2 fragment (amino acids 1–225) as bait to screen a mouse embryo cDNA expression library constructed in the two-hybrid system described by Hollenberg et al. (57). About one hundred His+ and LacZ+ colonies were analyzed. Among these we identified multiple inserts containing overlapping fragments derived from the C-terminal segment of actin-binding protein-280 (actin-binding protein-280 or Filamin) (52). Re-transformation assays performed with three overlapping Filamin clones confirmed that the interaction between Filamin and TRAF2 was specific. We then performed two-hybrid deletion mapping analysis of TRAF2 to delineate the domain required for interaction with Filamin. Both the N-terminal Ring finger and the zinc finger domains were required for interaction with Filamin. A TRAF2 fragment (residues 1–225) containing both the Ring finger domain and the

FIG. 1. Interaction of TRAF2 and Filamin. A, the binding of the C-terminal Filamin fragment aa 1644–2118 to various TRAF2 segments was tested by yeast two-hybrid analysis. Diagrams of full-length TRAF2 and of the different constructs of TRAF2 used for mapping interaction with Filamin in yeast are shown. Numbering is based on the sequence of the full-length protein. B, interaction of Filamin fragment aa 1644–2118 with different GST-TRAF2 fusion proteins. An expression vector encoding the HA-tagged C-terminal Filamin fragment aa 1644–2118 was transfected into 293 cells. After 36 h cell lysates were prepared and incubated with a GST fusion protein containing the TRAF2 Ring zinc finger (lane 1), or the TRAF2 Ring finger (lane 2), or the TRAF2 zinc fingers (lane 3), or the GST alone (lane 4). Proteins coprecipitated with GST fusion proteins were analyzed by Western blot with anti-HA antibodies. The same amounts of GST fusion protein were used (data not shown). C, interaction between TRAF2 and endogenous Filamin. 293 cells were transfected with an expression vector encoding an HA-tagged TRAF2 (lane 2), HA-tagged TRAF2Δ105 (lane 3), or empty plasmid. (lane 1). Lysates from 293 cells were incubated with anti-HA antibodies. Coprecipitated endogenous Filamin was detected by Western blotting with anti-Filamin antibodies. The lower panel shows the relative amounts of HA-TRAF2 and HA-TRAF2Δ105 in the 293 cell extract. Positions of molecular mass standard (in kilodaltons) are indicated. NS, nonspecific; Ig HC, immunoglobulin heavy chain.
zinc finger domain interacted strongly with Filamin, whereas the Ring finger domain of TRAF2-(1–105) or the zinc finger domain of TRAF2-(76–282) alone failed to interact (Fig. 1A).

Filamin is a flexible actin-binding protein present in the cortical cytoplasm, responsible for the formation of orthogonal actin networks. Filamin contains an N-terminal actin binding domain, followed by an extended, rod-like structure created by 24 repeats. Each repeat is about 96 amino acids in length and contains eight short β-sheet structures separated by turns. The C-terminal repeat (number 24) comprises a homodimerization domain. Several proteins have been demonstrated to interact with Filamin including the cytoplasmic tail of β2-integrin subunit CD18 (58), the von Willebrand factor receptor (59), the SEK-1 protein kinase (61), the small GTPase RalA (62), and presenilin 1 (63).

To confirm the interaction of TRAF2 with Filamin, an expression vector encoding the HA-tagged Filamin fragment, aa 1644–2118, was transiently transfected into human 293 cells; this fragment interacted with TRAF2 in the two-hybrid system. Extract from transfected cells was incubated with different purified prokaryotic recombinant GST-TRAF2 fusion proteins bound to glutathione-agarose beads. Bound protein was analyzed by SDS-PAGE and immunoblotted using anti-HA monoclonal antibodies. The Filamin fragment bound to the TRAF2 fragment containing both Ring and zinc finger domains (Fig. 1B, lane 1). Deletion of the Ring finger domain or of the zinc finger domain abolished binding (Fig. 1B, lanes 2 and 3), in agreement with the yeast two-hybrid data. As a control, anti-HA antibodies detected the appropriately sized HA-tagged Filamin fragment from transfected cells but not in the untransfected cell (Fig. 1B, lanes 5 and 6).

We further tested the association of endogenous Filamin and ectopically expressed TRAF2. 293 cells were transfected with plasmids encoding HA-tagged TRAF2 (Fig. 1C, lane 2), or HA-tagged TRAF2 lacking the first 105 amino acids (TRAF2 Δ105) (Fig. 1C, lane 3) or empty vector (Fig. 1C, lane 1). 24 h after transfection cells were lysed and treated with the thiol-cleavable cross-linker diithiobis(succinimidylpropionate) as described under “Experimental Procedures.” Lysates from 293 cells were incubated with anti-HA antibodies to precipitate ectopic TRAF2. Subsequent Western blotting with anti-Filamin antibody showed that a 280-kDa band corresponding to endogenous Filamin was co-purified with TRAF2 but not with TRAF2 Δ105. This result indicated an association of TRAF2 with endogenous Filamin. Probing the same filter with Filamin preimmune serum failed to show the band at 280 kDa (data not shown). We were not able to detect the interaction between ectopically expressed TRAF2 and Filamin in the absence of cross-linker or with endogenous TRAF2, presumably because it does not readily withstand lysis procedure. Although our yeast two-hybrid and immunoprecipitation data strongly suggest a direct interaction between TRAF2 and Filamin, we do not discount the possibility that other as yet unidentified proteins may mediate this interaction, but if so, such proteins would have to exist in both yeast and mammalian cells.

TNF treatment did not affect the strength of the interaction between Filamin and ectopically expressed TRAF2 (data not shown). This result, however, does not exclude the possibility that the interaction between Filamin and TRAF2 is modulated by TNF. Under our experimental conditions, ectopically expressed TRAF2 is already activating, possibly due to concentration-dependent oligomerization.

Overexpressed TRAF2 potently activates both NF-κB and SAPK in transfected 293 cells. The association between Filamin and TRAF2 suggested that Filamin may be involved in TRAF2-mediated effector functions. We tested the effect of Filamin overexpression on TRAF2-induced reporter expression dependent on NF-κB activation using a transient transfection assay in 293 cells. Both the Filamin fragment containing aa 1644–2118 and full-length Filamin blocked TRAF2-mediated NF-κB activation in a dose-dependent manner (Fig. 2A). Since TRAF2 appears to also mediate NF-κB activation triggered by the TNF receptors I and II (11, 14), we tested the effect of Filamin overexpression on TNF-induced NF-κB activation. Overexpression of Filamin fragment containing aa 1644–2118 and of full-length Filamin in 293 cells inhibited TNF-induced NF-κB activation in a dose-dependent manner comparable in strength to the inhibition observed with a dominant negative
form of TRAF2 (ΔN105) (Fig. 2B). The negative effect of overexpressed Filamin on TRAF2- and TNF-induced activation does not necessarily imply an inhibitory role for endogenous Filamin since the negative effect could also be due to Filamin titrating essential components (such as TRAF2) out of endogenous signaling complexes, thereby rendering them nonfunctional. Therefore, Filamin could have a positive role during normal signal transduction in cells. This point will be addressed below.

Because TRAF2 activates not only NF-κB but also JNK/SAPK, we examined whether Filamin could block TRAF2-dependent JNK/SAPK activation. Filamin fragment encoding aa 1644–2118 or full-length Filamin were cotransfected into 293 cells together with TRAF2 and a hemagglutinin (HA)-tagged SAPK expression vector, and the activity of HA-SAPK was measured 36 h after transfection. Both full-length Filamin (Fig. 3A) and Filamin fragment encoding aa 1644–2118 (Fig. 3B) blocked TRAF2-dependent SAPK activation.

We next examined the effect of Filamin on IL-1-induced NF-κB activation. Filamin also blocked the IL-1-induced NF-κB activation (Fig. 4A). IL-1 induction of NF-κB is mediated by TRAF6 (10). Ectopic expression of TRAF6 expression in 293 cells activated NF-κB, and this activation was blocked by Filamin (Fig. 4B). To exclude the possibility that Filamin nonspecifically repressed reporter gene activation, and also to investigate the hierarchical relationship of Filamin to downstream elements of the NF-κB activation pathway, we examined the effect of Filamin overexpression on NIK-induced NF-κB activation. In this case, Filamin was not able to block NF-κB activation by NIK (Fig. 4C).

It is possible that any protein that has the ability to associate with TRAF2 may titrate TRAF2 out of endogenous signaling complexes, thus causing an inhibition of the signaling events, regardless of whether such a protein is normally part of the particular signaling complex in question or not. To have more insight into the physiological role of Filamin in TNF signaling, we utilized the human melanoma cell line M2 that has spontaneously lost expression of Filamin (54, 55). These cells are characterized by extensive membrane blebbing immediately after plating and by the absence of translocational cell movement. Stable expression of transfected Filamin in these cells (M2TA7) resulting in a normal molar ratio of Filamin to actin (1:160) corrected these defects (see Fig. 5B, lower panel) for Filamin expression. The Filamin-repleted M2TA7 cells stop blebbing 12 h after plating, whereas the Filamin-depleted M2 cells stop blebbing 3 days after plating, following a compensatory increase in their content of actin. We therefore analyzed cells at least 3 days after plating. M2 and M2TA7 cells were transfected with a κB-reporter and 3 days later were stimulated for 5 h with TNF. NF-κB was not activated by TNF in M2 cells but was activated by PMA/ionomycin, suggesting that the inability of M2 cells to respond to TNF was not due to a general inhibition of NF-κB. On the other hand, Filamin-repleted M2TA7 cells did respond to TNF stimulation (Fig. 5A). Defective NF-κB activation in M2 was further demonstrated by the lack of TNF-induced IκBα activation. M2 and M2TA7 cells were stimulated with TNF for 10 min, and the activity of the immunoprecipitated endogenous IκBα complex was measured by its ability to phosphorylate its substrate, IκBα (Fig. 5B, upper panel). Despite the presence of comparable amounts of IκKα in the extract of M2 and M2TA7 (Fig. 5B, lower panel), there was no increase in IKK activity in M2 extract following TNF treatment, whereas M2TA7 showed an approximately 3-fold increase in IKK activity over background in response to TNF. In agreement with previously published data (63), the importance of Filamin in TNF signaling was further demonstrated by the inability of the M2 cell line to activate SAPK in response to TNF (Fig. 5C). The response was restored in M2TA7 cells expressing Filamin. The ability of the M2 cell line to activate SAPK in response to anisomycin demonstrated that the M2 cell
Fig. 5. TNF fails to activate NF-κB and SAPK in the Filamin-deficient cell line M2. Ectopic expression of Filamin restores the TNF response. A, M2 and M2TA7 cells (4 × 10⁴) were transfected in triplicate with 0.5 μg of IκB-Luc reporter plasmid and 0.2 μg of a pRSV-βgal plasmid. 3 days after transfection cells were stimulated with TNF (2000 units/ml) or PMA (100 ng/ml) for 5 h, and the activity of the reporter plasmid was measured. B, M2 and M2TA7 all were stimulated with TNF (2000 units/ml) for 10 min. Cytoplasmic extracts were immunoprecipitated with anti-IκBα antibodies, and IκBα kinase activity was determined by an in vitro immune complex kinase assay with GST-IκBα as substrate. Lower panel, aliquots containing equal amounts of M2 and M2TA7 cell extract were subjected to immunoblot using anti-Filamin or anti-IκBα antibodies. C, M2 and M2TA7 (4 × 10⁶) cells were transfected with a vector encoding HA-tagged SAPK (2 μg). 3 days after transfection cells were stimulated with TNF (2000 units/ml) for 20 min or anisomycin (50 μg/ml) for 40 min. SAPK activity was assayed as described under “Experimental Procedures.” HA immunoblot for SAPK is shown in the lower panel.

line was not intrinsically unable to activate SAPK.

Recently, Filamin has been shown to interact with the Toll receptor in Drosophila, although the functional significance was not addressed (64). The Drosophila Toll receptor has a mammalian homolog, which can mediate NF-κB activation (53) via recruitment of TRAF6, similar to IL-1 receptor-mediated NF-κB activation (12, 13). These similarities prompted us to investigate whether Filamin was also able to interfere with Toll signaling. Ectopic expression of a constitutively active form of Toll receptor in the Filamin-lacking M2 cell line did not result in NF-κB activation, whereas Filamin-expressing M2TA7 cells did activate the transcription factor (Fig. 6A). Similar to results presented in Figs. 2 and 4, overexpression of Filamin in 293 cells also blocked NF-κB activation caused by introduction of the constitutively active Toll receptor (Fig. 6B).

These results implicate Filamin in TNF, IL-1, and Toll-initiated signaling cascades and suggest that Filamin exerts its function by interacting with TRAF2 and possibly other TRAF family members.

Discussion

The present data provide evidence for a role of Filamin (actin-binding protein-280) in TRAF protein-mediated activation of NF-κB and SAPK. We demonstrate by yeast two-hybrid analyses, communoprecipitations from cell extracts, and in vitro binding assays that the Ring plus zinc finger domains of TRAF2 associate with a C-terminal segment of Filamin. This association is likely to be direct, but final proof remains to be established. In addition to a physical association, we also provide evidence for a functional role of Filamin in signaling via the TNF, IL-1, and Toll receptor pathways. Specifically, we show that overexpression of full-length Filamin or of its TRAF2-binding C-terminal segment inhibits TRAF2-mediated activation of SAPK as well as TNF- and TRAF2-mediated activation of NF-κB in a dose-dependent manner. Filamin overexpression also inhibits TRAF6, IL-1, and Toll receptor-mediated activation of NF-κB. Finally, a human melanoma cell line (M2) lacking expression of Filamin is not able to activate NF-κB or SAPK in response to TNF stimulation, whereas an M2 cell line expressing a permanently transfected Filamin (M2TA7) is able to do so. This result suggests a positive role for Filamin in signaling for SAPK and NF-κB, and it further suggests that the inhibitory effect of overexpressed Filamin may be due to its ability to titrate out essential components needed for endogenous signaling complexes to function.

Filamin is an actin cross-linking protein, possessing an actin binding domain and a homodimerization domain. As such, Filamin is a determinant of the submembranous cytoskeletal architecture of cells, and consistent with this role, Filamin appears to be involved in cell adhesion and migration. The structure of Filamin suggests complex roles, given that most of the protein consists of 23, approximately 96-amino acid-long repeats, regularly spaced between the actin binding and homodimerization domains (65). To date several of the repeats have been shown to associate with receptors or signaling proteins. Most of the proteins known to bind Filamin presumably do so to functionally link them to changes in cytoskeletal architecture. For example, clustering of the β₂-integrin receptors will lead to clustering of the associated Filamin proteins, contributing to extensive actin cross-linking at focal adhesions, for example (66–67). The platelet von Willebrand factor receptor, glycoprotein Ib-IX (GPIb-IX), is constitutively associated with Filamin, and upon thrombin activation, the Filamin-GPIb-IX complex moves from the resting to the activated cytoskeleton (59). Other proteins associate with Filamin in a signal-dependent manner. The FcγRI receptor binds to Filamin only in the absence of ligand (60), whereas tissue factor, the protease receptor initiating the coagulation cascade, binds to Filamin only in the presence of ligand (68). In this latter case Filamin is reported to mediate the cell adhesion and migration effects of activated tissue factor. The involvement of Filamin in cell adhesion and cell motility is further supported by the recent finding that Filamin is recruited into filopodia upon GTP-dependent association with the small GTPase RalA (62). In addition Filamin binds to and colocalizes with the integral membrane protein presenilin-1 to lamellipodia (63).

Unlike these Filamin-binding proteins, the binding of the MAP kinase kinase SEK-1 to Filamin does not appear to be obviously related to Filamin’s actin cross-linking activity.
SEK-1 is the upstream regulator of the MAP kinase SAPK, and the presence of Filamin has been reported to be necessary for TNF to rapidly activate SAPK, at least in a melanoma cell line (61). Because this function of Filamin does not require the dimerization domain, actin cross-linking activity is not required.

Recently, Filamin has been shown to interact with the Toll receptor in Drosophila, although the functional significance of this association is not known (64). Filamin was also shown to bind Tube, a protein necessary for the Toll-mediated activation of the Rel/NF-κB protein Dorsal in developing Drosophila embryos (64). A mammalian homolog of the Drosophila Toll receptor has been shown to mediate activation of NF-κB during innate immune responses (53). Although a mammalian homolog of Tube has not been reported, MyD88 may carry out partially analogous functions. MyD88 is an adaptor protein that links the mammalian Toll receptor to the IRAK kinase to facilitate its activation (12–13). It remains to be determined if Filamin binds to mammalian Toll and MyD88. Signaling to activate NF-κB via the IL-1 receptor mirrors that initiated via the Toll receptor. Both receptors share structural homologies in their intracytoplasmic domains. Upon binding of IL-1, the IL-1 receptor heterodimerizes with the IL-1 receptor-associated protein, and this in turn leads to recruitment of MyD88 and then IRAK (69) (and probably also IRAK-2 (70)). The recruitment of IRAK is only transient, however, and once phosphorylated, IRAK then associates with TRAF6 off the receptor. The TRAF6 adaptor is also implicated in Toll signaling.

Signaling via TNF receptors appears to follow a path similar to that of Toll and IL-1 receptors. TNF-induced trimerization of TNF receptors recruits the adaptor TRADD, which in turn attracts the RIP kinase and TRAF2. Since TRADD and MyD88 are both adaptors that recruit a serine/threonine kinase (RIP and IRAK, respectively) and a TRAF adaptor (TRAF2 and TRAF6, respectively), the pathways appear to function analogously. Furthermore, TRAF2 and TRAF6 both associate with and in some unknown way activate NIK, one of the kinases reported to phosphorylate directly and activate IκB kinases. Consistent with the inherent similarities in signal transduction via the TNF, IL-1, and Toll receptor pathways, overexpression of Filamin inhibits activation of NF-κB initiated by any one of these pathways. Overexpressed Filamin not only inhibited activation mediated by these receptors, but it also inhibited the activation mediated by the overexpression of TRAF2 and TRAF6 adaptors used in these pathways. The inhibition is not nonspecific, however, since NF-κB activation mediated by overexpressed NIK is completely unaffected by ectopically expressed Filamin. It is therefore likely that overexpressed Filamin titrates TRAF proteins out of endogenous signaling complexes, thereby impairing normal signal transduction. This may also be the reason why overexpressed Filamin inhibits the TNF-mediated activation of SAPK/JNK, since this activation is mediated via TRAF2 as well.

In light of these results, what role might Filamin play in TNF, IL-1, and Toll receptor-mediated activation of NF-κB and TNF receptor-mediated activation of SAPK? Filamin may provide a scaffold upon which TRAF-dependent signaling cascades can take place. In the case of SAPK activation there is evidence for this role now, since at least two components of the pathway have been shown to bind Filamin, namely TRAF2, as demonstrated here, and one of the immediate upstream activators of SAPK, SEK-1 (61). On the other hand, no component of NF-κB-activating pathways other than TRAF2 has been shown to bind Filamin to date. Thus it is possible that overexpressed Filamin merely titrated endogenous TRAF proteins out of signaling complexes and that these complexes do not normally involve Filamin.

However, contrary to the view that endogenous Filamin might not have a role in NF-κB activation, experiments with the Filamin-deficient melanoma cell line M2 support a positive role for Filamin in these events. The Filamin-lacking cell line M2 is severely impaired in activation not only of SAPK but also of NF-κB. Neither TNF nor ectopically expressed, constitutively active Toll receptors significantly activate this transcription factor nor is the IKK complex responsive to TNF. On the other hand, PMA/ionomycin-initiated activation proceeds unimpeded in M2 cells. Reintroduction of Filamin into these cells (M2T7A) completely restores their ability to respond to TNF and Toll receptor stimulation with activation of both NF-κB and of SAPK. Therefore, at least in these melanoma cells, signaling to activate NF-κB or SAPK downstream of TNF appears to depend on Filamin, which may function as a scaffold or at least help in the assembly of proper signaling complexes. It remains to be determined if TRAF2 engages Filamin prior to or only after activation of the TNF receptor. Although TNF treatment did not appear to change the binding of transfected TRAF2 to Filamin, this experiment does not rule out a signal-dependent association, since overexpressed TRAF2 is already in an activated state without TNF, presumably due to homotypic associations. Finally, the importance of Filamin in trans-
ducing inflammatory signals in cells other than the melanoma cell line remains to be determined. Filamin-facilitated signaling may represent only one of many possible alternative pathways leading to activation of NF-κB in other cells. In summary, we have demonstrated an association of Filamin with TRAF2 (and possibly other TRAF proteins), and we have provided evidence for a functional involvement of Filamin in TNF receptor and TRAF2-mediated activation of SAPK and in TRAF2, TRAF6, TNF, IL-1, and Toll receptor-mediated activation of NF-κB.

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