Membrane Localization of TRAF 3 Enables JNK Activation*

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Members of the tumor necrosis factor receptor family as well as other receptors achieve their diverse biological effects through the activation of intracellular signals including the c-Jun N-terminal kinase (JNK) pathway. Such signals are believed to be delivered through mediators known as TNF receptor-associated factors (TRAFs). Although the N-terminal zinc finger region of TRAFs has been shown to be essential for downstream signaling, there is no indication yet as to the nature of its role or of the factors that distinguish the N terminus of TRAF 3, which does not activate JNK in the systems examined thus far, from those of other TRAFs, which do activate this pathway. In the present study, it is shown that, among the known TRAFs, localization to the insoluble cell pellet fraction consistently correlates with JNK activation and that both characteristics map to the TRAF N terminus. Furthermore, it is demonstrated that forced localization of TRAF 3 to the cell membrane is sufficient to convert this molecule into an activator of JNK. This suggests that one of the roles of the TRAF N terminus may be to participate in interactions that promote the recruitment of TRAFs to the membrane and that this localization effect plays an important role in TRAF-mediated JNK activation.

The tumor necrosis factor receptor (TNFR) superfamily of type I cell surface glycoproteins includes more than 20 members, with roles in a wide variety of biological processes such as inflammatory and immune responses, neural growth, and programmed cell death (1). These receptors are believed to mediate at least part of their effects by influencing the transcriptional regulation of downstream target genes through the activation of specific intracellular signaling pathways including the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and NF-κB pathways (2, 3). Both of these pathways consist of serine/threonine kinase cascades, leading to the dual-specificity phosphorylation of the MAP kinase, which in turn phosphorylates and activates specific transcription factors. The extracellular signal-regulated kinases are traditionally believed to respond to growth-promoting stimuli such as growth factors, whereas the JNKs and p38 kinases are components of stress response pathways activated by conditions such as ultraviolet irradiation and osmotic stress. The activation of JNK by members of the TNFR family is thought to be mediated by a family of intracellular signaling molecules known as TNFR-associated factors (TRAFs) (5). The TRAF family currently consists of six members, and studies in knockout and transgenic mice have demonstrated that at least one member of this family, TRAF 2, is required for TNF-mediated JNK activation (6, 7). Different members of this family display distinct receptor binding specificities, and one member, TRAF 6, is believed to mediate NF-κB and JNK activation by the interleukin 1 receptor (8, 9), which is not a member of the TNFR family. Thus, TRAF-mediated activation of these pathways may be a common mechanism shared by many cell-surface receptors.

Since TRAFs have no enzymatic activity, the biochemical mechanism by which they activate JNK is not presently clear; however, certain TRAF-interacting kinases may play a role in this process. Apoptosis signal-regulating kinase (ASK1), a MAP 3-kinase that is believed to activate JNK and p38 kinases, has been shown to interact with and become activated by TRAFs (10). In addition, it has been suggested that members of the germlinal center kinase family link TRAFs to the JNK pathway through MEKK1, another MAP 3-kinase (11–13). Structurally, TRAFs are defined by a unique C-terminal domain, the TRAF domain, that mediates interactions with a large number of other signaling molecules, including apoptosis signal-regulating kinase NF-κB-inducing kinase (NIK), receptor-interacting protein (RIP), and TNF receptor-associated death domain protein (TRADD) (5, 10), as well as with the intracellular domains of receptors. In addition, all TRAFs, except TRAF 1, contain a predicted N-terminal zinc finger region containing a ring finger and five tandem zinc fingers. Although the biochemical function of this region is not yet clear, it has been shown to be necessary for the activation of downstream signaling (14). We have recently dissected the contribution of this region to the activation of each of two downstream signaling pathways by demonstrating that the ring finger is specifically required for NF-κB but not JNK activation (15).

Among members of the TRAF family, TRAFs 2, 3, 5, and 6 have been studied most extensively as mediators of receptor-activated signal transduction. It is interesting that despite a lack of any obvious structural differences within the N-terminal zinc finger region, TRAFs 2, 5, and 6 can activate downstream signaling pathways such as the JNK pathway, whereas TRAF 3 cannot (16, 17). Through domain exchanges between homologous regions of different TRAF molecules, this functional difference between TRAF 3 and the other TRAFs has
been mapped within the zinc finger region (14, 15). Since all known interactions between TRAFs and other proteins are mediated by the TRAF C terminus, the nature of the zinc finger region’s contribution to signaling is not clear.

In the present study, we examined the significance of the N-terminal sequence difference that functionally distinguishes TRAF 3 from other TRAFs in the activation of JNK signaling. We found that those TRAFs that activate JNK localize to the insoluble pellet fraction after cell lysis, whereas TRAF 3 distributes preferentially to the soluble cytoplasmic fraction, and that this distribution preference maps precisely to the same zinc finger region that is important for signaling. Furthermore, we suggest that localization to the cell membrane distinguishes TRAF 3 from other TRAFs, since forced membrane localization of TRAF 3 is sufficient to convert it to a potent activator of JNK.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—All full-length TRAFs were cloned between the BamHI and NotI sites in pEVB, a mammalian expression vector driven by the elongation factor-1α promoter. TRAF 3/TRAF 5 hybrids were generated by polymerase chain reaction mutagenesis, as described previously (16), and cloned between the BamHI and NotI sites in pEVB. The Ikaros construct, expressed from the pCMV expression vector, was kindly provided by Dr. Stephen Smale (University of California, Los Angeles, CA), and for CD40ct-Ikaros-C, the CD40 cytoplasmic tail (amino acids 216–277) was cloned between KpnI and EcoRI in-frame with an N-terminal Flag tag and a C-terminal Ikaros-C zinc finger domain (amino acids 223–286 of Ikaros 1). For myr-CD40ct-Ikaros-C, the CD40ct-Ikaros-C fusion was polymerase chain reaction-amplified and cloned between the NotI and XhoI sites in pcDNA3-myr, in-frame with a 16-amino acid N-terminal src myristoylation signal (MGSSKSKPGDPSQRER). GST-CD40ct was generated by cloning the CD40 cytoplasmic tail into the BamHI and NotI sites of the pEBG expression vector, in-frame with an N-terminal GST tag. Murine TRAF 3 and TRAF 3 N terminus amino acids 1–294 were polymerase chain reaction-amplified and cloned between EcoRI and BamHI in pcDNA3-myr. For myr-TF3N-Ikaros-C, the Ikaros-C domain was cloned into an XhoI site just C-terminal to and in-frame with TRAF 3-N. The HA-JNK expression vector used for measuring in vitro kinase activity was described previously (15).

**Cell Culture and Transfection**—293T cells derived from human embryonic kidney were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin. About 2 × 10⁶ cells were transiently transfected with 4 µg of TRAF, CD40ct, or control vector (except as indicated) and 2 µg of HA-JNK expression vector using a standard calcium phosphate method, and extracts were collected about 36 h after transfection.

**Lysis and Fractionation**—Lysis of transiently transfected cells was performed on ice using a modified radioimmune precipitation buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, and the cytoplasmic supernatant was removed. Cell pellets were washed in the same buffer and completely dissolved in standard protein sample buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM EGTA, 5 mM NaF, 1 mM Na3VO4, 1 mM NaF, pepstatin, and aprotinin, and 1 mM Na3VO4 and phenylmethylsulfonyl fluoride. After lysis, pellets were washed, dissolved, and sonicated to break up membranes and insoluble particles. Matching dilutions of cytoplasmic and pellet extracts were prepared for Western blot analysis. As shown in Fig. 1, there is a clear difference in solubility between the various TRAFs, with TRAF 3 being the most soluble, whereas the other TRAFs are, to varying extents, localized preferentially to the insoluble pellet fraction. When JNK activation by these TRAFs was measured through an in vitro kinase assay, we observe an inverse correlation between the activation of this pathway and the level of TRAF solubility. That is, TRAFs that activate JNK signaling tend to distribute to the insoluble pellet during cytoplasmic extraction, thus explaining the lower levels detected in cytoplasmic samples. The above analyses of transiently transfected cells demonstrate that the solubility of TRAF 3 upon cell lysis differs from that of other TRAFs. We wished to determine if this was also true for endogenous expression levels of TRAF molecules. For this purpose, we prepared cytoplasmic and pellet extracts from three distinct cell lines representing different tissues: Jurkat cells (a

**RESULTS**

The Ability of Different TRAFs to Activate JNK Correlates with a Preferential Localization to the Lysed Cell Pellet Fraction—Early in the course of studies on the mechanisms of downstream signaling by the various TRAF family members, we observed that when cells were transfected with equal amounts of vectors encoding the different TRAFs, there were great differences in the levels of overexpressed protein detectable in cytoplasmic extracts. Moreover, the relative differences in protein level detectable by Western blot were consistent for each TRAF family member across many experiments and under different lysis buffer conditions. Since all of these proteins were being expressed from the same promoter on the same expression vector and detected using the same antibody and epitope tag, there were two potential explanations for the observed results that were considered most reasonable. The first was that the different TRAFs have different intracellular half-lives, and the second was that the differences in protein levels in cytoplasmic extracts actually reflected differences in solubility between the TRAFs.

To test the second hypothesis, we transiently transfected 293T cells with equal amounts of vectors encoding HA-tagged TRAFs 2, 2A, 3, 4, 5, and 6 (Fig. 1). Cytoplasmic extracts were prepared using standard protocols, and a sample was taken for Western blot analysis. In addition, the pellet fractions were washed, dissolved, and sonicated to break up membranes and insoluble particles. Matching dilutions of cytoplasmic and pellet extracts (representing roughly the same number of cells) were prepared and analyzed for TRAF protein content by Western blotting. As shown in Fig. 1, there is a clear difference in solubility between the various TRAFs, with TRAF 3 being the most soluble, whereas the other TRAFs are, to varying extents, localized preferentially to the insoluble pellet fraction. When JNK activation by these TRAFs was measured through an in vitro kinase assay, we observe an inverse correlation between the activation of this pathway and the level of TRAF solubility. That is, TRAFs that activate JNK signaling tend to distribute to the insoluble pellet during cytoplasmic extraction, thus explaining the lower levels detected in cytoplasmic samples.
human T cell line), HeLa cells (a human cervical carcinoma line), and HaCaT cells (a transformed human keratinocyte line). Western blot analysis was performed to determine the levels of TRAF 2 and TRAF 3 in each fraction. As shown in Fig. 2, in all three cell lines, a significant fraction of endogenous TRAF 2 was found in the insoluble pellet fraction after removal of nuclear proteins and extensive washing. TRAF 3, on the other hand, is almost entirely localized to the soluble cytoplasmic fraction and scarcely detectable in the pellet. Thus, in multiple cell types, TRAF 2 can be fractionated with the pellet, as demonstrated above in transiently transfected 293T cells, whereas TRAF 3 was found almost entirely in the cytoplasmic extract.

Pellet Localization Maps to the TRAF N-terminal Zinc Finger Region—We decided to map the region that accounts for the difference in solubility between TRAFs 3 and 5 through domain substitutions. For this purpose, a panel of TRAF 3/5 hybrids were constructed in which different regions were contributed by TRAFs 3 and 5 (Fig. 3A). These hybrid molecules were expressed in 293T cells, and pellet and cytoplasmic extracts were prepared as described above. As shown in Fig. 3B, there was a shift in localization from the cytoplasm to the pellet as an increasing contribution to the hybrid was made by TRAF 5 sequence. Specifically, TF 35/250, which contained the entire TRAF 3 N-terminal zinc binding region and the isoleucine zipper and TRAF domains of TRAF 5, showed no preferential distribution to the pellet fraction; however, substituting the last three zinc fingers with those of TRAF 5 so that only the ring finger and first two zinc fingers were contributed by TRAF 3 (TF 35/162), resulted in a clearly higher level of protein in the pellet than in the cytoplasm. This preferential localization to the pellet fraction again

Fig. 2. Distinct solubility profiles of endogenous TRAF 2 and TRAF 3. Jurkat, HeLa, and HaCaT cells were lysed, and cytoplasmic extracts and solubilized pellets were prepared as described under “Experimental Procedures.” Equal dilutions of both fractions were analyzed by Western blot, and TRAFs were detected using anti-TRAF 2 (C-20) and anti-TRAF 3 (M-51) antibodies (Santa Cruz Biotechnology, Inc.).

Fig. 3. JNK activation and cell pellet localization map to the TRAF N terminus. A, diagrammatic representation of chimeric TRAF molecules generated through domain substitutions between TRAF 3 and TRAF 5. Dark areas represent regions of TRAF 3, and light areas represent TRAF 5 sequences. The numbering in the name of each construct represents the junction points between TRAF 3 and TRAF 5 sequences using the amino acid sequence of full-length TRAF 5 as a reference. All constructs were C-terminally HA-tagged. B, top panel, JNK activation in 293T cells transiently transfected with different TRAF hybrids was determined through an in vitro immune-complex kinase assay. Lower panels, cytoplasmic extracts and solubilized pellets were prepared from the same cells. Equal dilutions of both fractions as well as whole-cell lysates prepared from 10% of the transfected cells were analyzed by Western blot (using an anti-HA monoclonal antibody) for TRAF distribution and HA-JNK expression, respectively.

Fig. 4. JNK activation and cell pellet localization map to the TRAF N terminus. A, diagrammatic representation of chimeric TRAF molecules generated through domain substitutions between TRAF 3 and TRAF 5. Dark areas represent regions of TRAF 3, and light areas represent TRAF 5 sequences. The numbering in the name of each construct represents the junction points between TRAF 3 and TRAF 5 sequences using the amino acid sequence of full-length TRAF 5 as a reference. All constructs were C-terminally HA-tagged. B, top panel, JNK activation in 293T cells transiently transfected with different TRAF hybrids was determined through an in vitro immune-complex kinase assay. Lower panels, cytoplasmic extracts, solubilized pellets, and whole-cell lysates were prepared from the same cells and were analyzed by Western blot for TRAF distribution and HA-JNK expression as described above.
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**Membrane localization of TRAF 3 is sufficient for JNK activation.** Top panel, JNK activation was determined through an in vitro immune-complex kinase assay. Lower panels, cytoplasmic extracts, solubilized pellets, and whole-cell lysates were prepared from the same cells and were analyzed by Western blot for TRAF distribution (using an anti-TRAF 3 polyclonal antibody: M-51, Santa Cruz) and HA-JNK expression (using an anti-HA monoclonal antibody) as described above. For comparable protein expression levels, cells were transfected with different amounts of each construct with the differences made up by empty vector. Fusion constructs included TRAF 3-N (the TRAF 3 zinc finger region (amino acids 1–294)) and Ikaros-C (the C-terminal self-associating zinc finger domain of Ikaros (amino acids 223–286 of Ikaros I)).

Correlates well with the ability of these proteins to activate JNK signaling, suggesting that the TRAF N-terminal zinc binding region plays an important role in both of these effects.

We had previously used domain swapping between TRAF 3 and TRAF 5 to generate a series of chimeric molecules designed to identify the minimum region that distinguishes TRAF3's 3 and 5 in terms of downstream signaling activity (15). In Fig. 4, we examined the partitioning of these molecules between the cytoplasmic and pellet fractions and found once again that those constructs which distribute preferentially to the pellet are the ones which activate JNK. The first zinc finger and the first 10 amino acids of the second zinc finger of TRAF 5 are able to convert TRAF 3 into a JNK activator (TF 353/110–148), and this same region was sufficient to direct this construct to the pellet fraction. Interestingly, TF 353/162 did not contain this region of TRAF 5 and yet still strongly activated JNK; however, as mentioned above, the last three zinc fingers of this construct were contributed by TRAF 5 (Fig. 3A). Thus, it appears that multiple zinc fingers in the TRAF N terminus may contribute to both JNK and pellet localization, and only when all five zinc fingers are contributed by TRAF 3 is there a dramatic reduction in both of these effects.

**Forced Membrane Localization Converts TRAF 3 into an Activator of JNK—Based on the association between pellet fractionation and JNK activity as well as the fact that both features map to the TRAF N-terminal zinc finger region, we suspected that both effects may be a result of TRAF subcellular localization. Since TRAFs are believed to act immediately downstream of TNF family receptors, it seemed likely that localization to the plasma membrane may be a key component in TRAF signal transduction. To directly test the role of membrane localization in TRAF-mediated JNK activation, we decided to determine if it would be possible to produce a gain of function in a TRAF that normally does not activate JNK (TRAF 3; Fig. 1) by forcing it to the membrane.

This was accomplished by attaching an N-terminal myristoylation signal to TRAF 3. As shown in Fig. 5, myristoylated TRAF 3 localized to the pellet fraction and was capable of activating JNK signaling. If the TRAF 3 N-terminal zinc finger region was forced to the membrane, it too was capable of activating JNK; however, this activity was far weaker than that observed with full-length myr-TRAF 3, suggesting that even in the context of the membrane, the TRAF C terminus plays a key role in signaling.

Since the C-terminal half of TRAFs has been shown to be important for TRAF-TRAF interaction as well as interactions between TRAFs and TNF receptors (5), which are believed to trimerize upon activation, we sought to determine if the function of the TRAF C terminus could be replaced by another self-interacting domain. For this purpose, we used a construct in which the C-terminal domain of myr-TRAF 3 was replaced by the two C-terminal zinc fingers of the Ikaros transcription factor. The multiple splice variants of this transcription factor differ in their N-terminal zinc finger domains, which are important for nuclear localization and DNA binding; however, the invariable C-terminal domain consists of two zinc fingers, which mediate self-association between subunits of this transcription factor (20). As shown in Fig. 5, myr-TF3N-Ikaros-C was a strong activator of JNK, suggesting that oligomerization as well as membrane localization of the TRAF N terminus was important for TRAF signaling.

**Oligomerization and Membrane Localization Contribute to Signaling by the CD40 Cytoplasmic Tail—The CD40 antigen as well as many other members of the TNFR superfamily are believed to signal through the direct binding of TRAFs to their intracellular domains. Furthermore, many of these receptors are believed to bind to trimeric ligands, which activate the receptors by trimerizing them (1). To determine if the same factors affecting signaling by the TRAF N terminus are also important for signaling by the CD40 cytoplasmic tail, we generated various constructs containing this domain and tested JNK activation.

As shown in Fig. 6, intracellular expression of the CD40 cytoplasmic domain coupled to a GST epitope tag was, by itself, not sufficient to activate JNK signaling. If, however, this domain was linked to the Ikaros oligomerization domain described above (CD40ct-Ikaros-C; Fig. 6), it was capable of activating JNK. Finally, if the oligomerized CD40 cytoplasmic tail was also forced to the membrane using an N-terminal myristoylation signal, its signaling ability was greatly enhanced.

**DISCUSSION**

Since their discovery as mediators of TNFR and CD40 signaling, the TRAFs have grown both in number and in recognition as key signaling molecules activating pathways down-
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stream of many members of the TNFR superfamily. Recent studies on knockout and transgenic models highlight the prominent role of the TRAF family in the activation of multiple pathways by many receptors. For instance, in TRAF 2-deficient mice as well as mice expressing a dominant-negative TRAF 2 mutant in lymphoid tissues, TNFR-mediated JNK signaling is lost, suggesting an essential role for TRAF 2 in this pathway (6, 7). More recently, TRAF 6-deficient mice were shown to have defects in interleukin 1-mediated JNK signaling as well as interleukin 1-, anti-CD40-, and lipopolysaccharide-mediated NF-κB signaling (9). In addition to demonstrating the importance of TRAFs in signaling, such studies also point to some of the nonredundant biological functions of different members of this family in different tissues. For instance, both TRAF 2- and TRAF 3-deficient mice undergo severe wasting and early postnatal death, and TRAF 3-deficient lymphocytes show defects in T-dependent immune responses (21), whereas TRAF 6-deficient mice survive but develop severe osteopetrosis.

Despite their different biological functions, the predicted structural similarities between these molecules and the fact that they activate similar downstream signaling pathways such as the NF-κB and JNK pathways suggest that they may function similarly at the biochemical level. The nature of their specific biochemical role in signaling is, however, still poorly understood. Like the receptors with which they interact, TRAFs have no enzymatic activity through which to modify downstream molecules, and thus, they are believed to act as adapters that bring key molecules together at the appropriate time. Within the context of such a role, however, there is not yet any clear indication of why TRAF 3 differs from other TRAFs such as TRAFs 2, 5, and 6 in its ability to activate downstream pathways such as the JNK pathway. Although an increasingly large number of signaling molecules are known to interact with TRAFs, none of these interactions consistently distinguishes TRAF 3 from those family members that activate downstream signaling. Similarly, no obvious structural differences can account for this difference in activity, in contrast to the case of TRAF 1, which lacks the N-terminal ring and zinc finger region necessary for signaling activity.

By studying factors that can result in a gain of signaling function for TRAF 3, we learn about the features that distinguish TRAF 3 from other TRAFs and also gain insight into the overall mechanism of TRAF signaling. Previous work identified the first zinc finger and nearby sequences as the key structural feature accounting for the difference in function between TRAF 3 and its close homologue, TRAF 5, and also demonstrated distinct structural requirements for NF-κB and JNK signaling (15). In the present study, a similar domain substitution approach is used to show that the ability to activate JNK correlates with preferential localization to the insoluble pellet fraction during cell lysis (Figs. 3 and 4). Indeed, for all members of the TRAF family that have such an N-terminal zinc finger region, the ability to activate JNK correlates consistently with the tendency to localize to the pellet fraction (Fig. 1). Even expression of TRAF 4 leads to modest activation of JNK activity above base line; however, this molecule is typically not compared with other TRAFs because, unlike other family members, TRAF 4 has a tendency to localize to the nucleus (22). Although the significance of this nuclear distribution is not fully appreciated at this time, it is likely that this distribution pattern contributes to the abundant levels of TRAF 4 that are detected in the pellet fraction (Fig. 1).

A similar trend was observed when we compare the fractionation of endogenous TRAF 2 and TRAF 3 in three distinct cell types (Fig. 2). Although a large amount of both proteins was detected in the cytoplasmic fraction, a significant amount of TRAF 2 was associated with the pellet, whereas in the same sample, TRAF 3 was nearly undetectable, suggesting a solubility difference between these proteins at physiological expression levels.

Pellet localization maps to the N-terminal zinc finger region (Figs. 3 and 4), which is required for JNK activation. This finding is surprising in light of the fact that the C-terminal TRAF domain is the region responsible for interaction with receptor cytoplasmic tails (5); however, it is consistent with the observation that both TRAF 2 and TRAF 3 interact strongly with the intracellular domain of CD40, but only TRAF 2 can activate JNK signaling (23). One might speculate that among other uncharacterized molecular interactions, the N termini of some TRAFs may contribute to their localization to the membrane and help to recruit signaling molecules important for JNK activation to the receptor, with which the TRAFs interact through their C-terminal domains.

Since the initial characterization of TRAFs, the function of the zinc finger region has remained a mystery. Zinc fingers are classically mediators of DNA binding, and some evidence indicates a potential transcriptional role for the TRAF 2 N terminus under some conditions (24). Zinc fingers have, however, also been shown to mediate protein/protein interactions (25), and soon after their discovery, most TRAFs were shown to be located outside the nucleus (5), consistent with roles as adapter molecules. The present work suggests that localization of TRAFs to the cell membrane would account for at least part of the well-documented essential signaling role of the zinc finger region.

In the present study, we show that those TRAFs that activated JNK localized to the insoluble pellet fraction during cytoplasmic extraction, whereas TRAF 3 was predominantly in the cytoplasmic fraction (Fig. 1). To test if membrane localization is important for TRAF signaling, we forced TRAF 3 to the plasma membrane through the addition of a myristoylation signal, and we found that, like the other TRAFs, it became localized predominantly to the pellet and gained the ability to activate JNK (Fig. 5). This suggests that membrane localization is one factor that accounts for the difference between TRAF 3 and other TRAFs and also indicates that when TRAF 3 is localized to the membrane, it can behave like other TRAFs in JNK activation; potentially through similar molecular interactions. Although membrane localization through myristoylation certainly changes the activity of TRAF 3, it is not certain at this point if membrane localization per se is truly responsible for the observed change in solubility. For instance, the low solubility of certain TRAFs may be a result of their association with cytoskeletal scaffolding components with roles in signaling, and forced membrane localization of TRAF 3 may bring it into proximity with such proteins near the cell membrane, thus accounting for both its signaling activity and its localization to the insoluble pellet fraction.

Although current evidence suggests that TRAF 3 is incapable of activating JNK, it is too early to rule out this possibility altogether, since this conclusion is based primarily on overexpression studies. Here, we demonstrate that the cytoplasm-to-pellet distribution ratio of overexpressed TRAF 3 is higher than that of other TRAFs and that this ratio varies inversely with the ability to activate JNK. Perhaps the large amount of cytoplasmic TRAF 3 in these studies interferes with signaling by the small amount that localizes to the membrane (possibly by competing for important signaling molecules that must get recruited to the membrane), and this makes it appear that TRAF 3 is incapable of JNK activation.

TRAF 3 has been reported to interact with a number of receptors including CD40, RANK, lymphotoxin-β receptor (LT-βR), and the Epstein-Barr virus-encoded latent membrane pro-
tein-1 (LMP-1) (26–29), and it is possible that upon recruitment to the membrane by an activated receptor, TRAF 3 may be capable of activating JNK as well as other pathways. In fact it has been shown that overexpression of the TRAF domain of TRAF 3 can act as a dominant negative inhibitor of CD40-mediated JNK activation (30); however, this possibility must be investigated further since this effect may be the result of an inhibition of binding of TRAF 2 or 5 to the receptor.

Members of the TNFR superfamily are believed to interact with trimeric ligands and thus undergo oligomerization during the activation process (1). We confirm the importance of receptor oligomerization for CD40 by showing that oligomerization of the CD40 cytoplasmic tail through fusion to the Ikaros self-association domain leads to JNK activation (Fig. 6). Since the CD40 cytoplasmic domain is believed to act immediately upstream of TRAFs, we also showed, in agreement with the above findings, that localization of this oligomerized cytoplasmic tail to the membrane, presumably mimicking its natural activation state, made it far more efficient at signaling. Cytoplasmic expression of a GST-tagged CD40 cytoplasmic tail, on the other hand, failed to activate JNK to any appreciable extent. This is interesting since GST has been claimed to form dimers (31), but also that this dimerization is too weak to activate signaling or that higher-order oligomers are required for signaling. In support of the latter possibility, the Ikaros self-association domain has been suggested to form higher-order oligomers (20). Interestingly, GST-CD40ct is also unable to significantly inhibit signaling by activated wild-type CD40 when coexpressed (data not shown), suggesting that a nonoligomerized, intracytoplasmic CD40 cytoplasmic domain cannot effectively compete for important signaling molecules with a membrane-bound receptor that has been oligomerized by its ligand. Again, this points to the cell membrane as a key location for molecular interactions that contribute to receptor-mediated JNK activation.

Recent crystallographic analysis of TRAF 2 reveals that TRAFs form trimers through interactions between their TRAF domains and that each monomer can interact with a single TNFR cytoplasmic domain (32). Thus a single TNFR trimer may recruit as many as nine TRAF molecules, six of which can concentrate, downstream kinases such as GCKR and apoptosis molecules, TRAF aggregates may then recruit, and thus locally inhibit of binding of TRAF 2 or 5 to the receptor.

Interestingly, this study also demonstrated the sedimentation of MEKK1, a MAP 3-kinase with a reported role in JNK activation (34), with the oligomerized CD40 cytoplasmic tail. Since TRAF 2 N terminus in the insoluble fraction, suggesting that MEKK1 interacts directly with the TRAF N terminus, or it is recruited to a signaling complex that is aggregated by TRAF oligomerization.

Given the prominent role of TRAFs in signaling by members of the TNFR superfamily, any basic appreciation of the mechanisms by which the TNF receptors, CD40 or any of their relatives mediate their biological functions will require an understanding of the ways in which TRAFs link these receptors to the signaling pathways that they activate. In the present work, we suggest that membrane localization may play an important role in TRAF-mediated JNK activation. It will be important to further establish the importance of membrane localization in this pathway and to identify other molecules in the TRAF signaling complex, particularly those recruited by the TRAF N terminus.

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