A *Drosophila* TNF-receptor-associated factor (TRAF) binds the Ste20 kinase Misshapen and activates Jun kinase

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Two families of protein kinases that are closely related to Ste20 in their kinase domain have been identified – the p21-activated protein kinase (Pak) and SPS1 families [1–3]. In contrast to Pak family members, SPS1 family members do not bind and are not activated by GTP-bound p21Rac and Cdc42. We recently placed a member of the SPS1 family, called Misshapen (Msn), genetically upstream of the c-Jun amino-terminal (JNK) mitogen-activated protein (MAP) kinase module in *Drosophila* [4]. The failure to activate JNK in *Drosophila* leads to embryonic lethality due to the failure of these embryos to stimulate dorsal closure [5–8]. Msn probably functions as a MAP kinase kinase kinase kinase in *Drosophila*, activating the JNK pathway via an, as yet, undefined MAP kinase kinase kinase. We have identified a *Drosophila* TNF-receptor-associated factor, DTRAF1, by screening for Msn-interacting proteins using the yeast two-hybrid system. In contrast to the mammalian TRAFs that have been shown to activate JNK, DTRAF1 lacks an amino-terminal 'Ring-finger' domain, and overexpression of a truncated DTRAF1, consisting of only its TRAF domain, activates JNK. We also identified another DTRAF, DTRAF2, that contains an amino-terminal Ring-finger domain. Msn specifically binds the TRAF domain of DTRAF1 but not that of DTRAF2. In *Drosophila,* DTRAF1 is thus a good candidate for an upstream molecule that regulates the JNK pathway by interacting with, and activating, Msn. Consistent with this idea, expression of a dominant-negative Msn mutant protein blocks the activation of JNK by DTRAF1. Furthermore, coexpression of Msn with DTRAF1 leads to the synergistic activation of JNK. We have extended some of these observations to the mammalian homolog of Msn, Nck-interacting kinase (NIK), suggesting that TRAFs also play a critical role in regulating Ste20 kinases in mammals.

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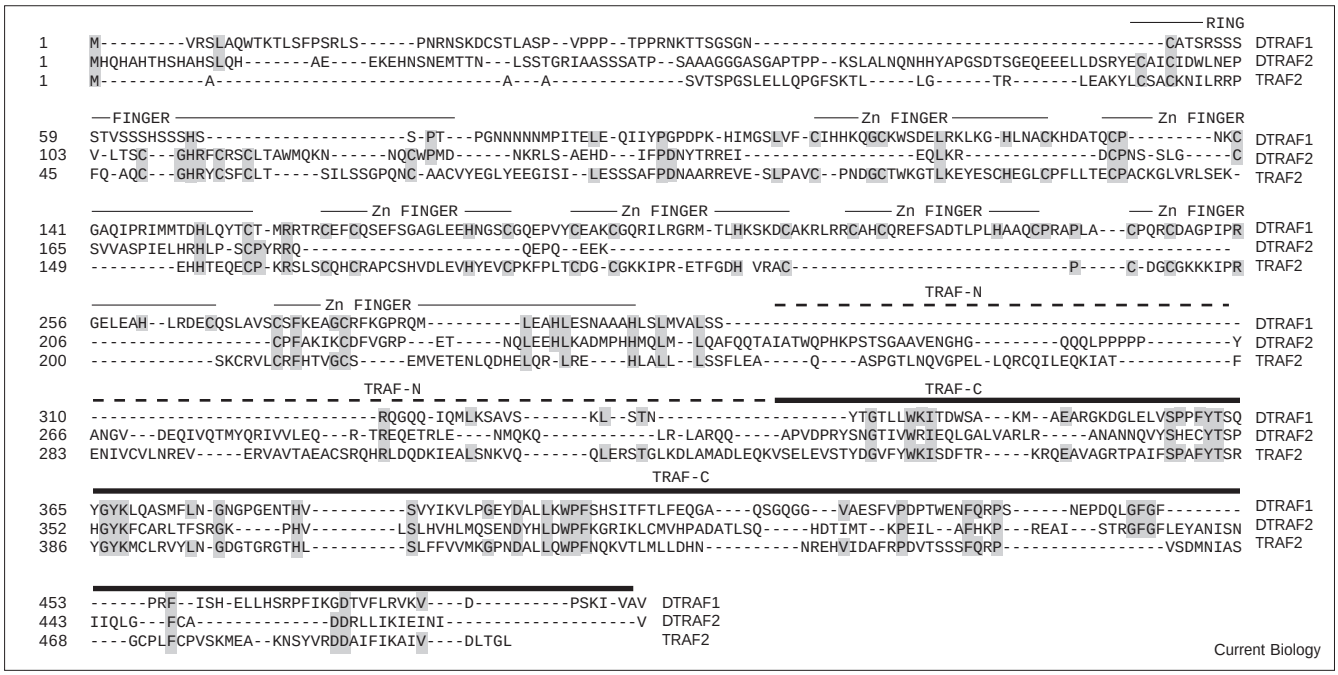
Results and discussion

Despite the finding that SPS1 Ste20 kinases are potent activators of JNK, little is known about the upstream signals that regulate Ste20 kinases or the specific upstream signal that couples Ste20 kinases directly to JNK activation. By screening a *Drosophila* embryo library for Msn-interacting proteins, using the yeast two-hybrid system, we identified a cDNA that encodes a *Drosophila* TRAF, which we refer to as DTRAF1 (Figure 1). The cDNA identified in the yeast two-hybrid screen contained only the TRAF domain of DTRAF1, but a full-length DTRAF1 cDNA was subsequently isolated. Analysis of the predicted protein sequence of full-length DTRAF1 indicated that it contains a string of five zinc-finger motifs at its amino terminus in addition to the TRAF domain located at the carboxyl terminus (Figure 1). A similar string of between four and five zinc-finger motifs has previously been shown to be conserved in all mammalian TRAFs [9,10]. In contrast to mammalian TRAFs, DTRAF1 did not contain an amino-terminal Ring-finger domain; mammalian TRAFs 2–6 contain a Ring-finger domain at their amino terminus and this domain has been shown to be critical for the activation of JNK and the transcription factor NF-κB by TRAF2 and TRAF5 [10–12]. We also identified a *Drosophila* expressed sequence tag (EST) that contained homology to the Ring-finger domains in mammalian TRAFs (Figure 1). Sequencing of this EST showed that it encoded a full-length cDNA, the product of which, designated DTRAF2, shares homology with other TRAF proteins. DTRAF2 showed most similarity to mammalian TRAF6, whereas DTRAF1 showed most similarity to TRAF4 (data not shown).

The clone identified in the yeast two-hybrid system contained only the carboxy-terminal TRAF domain of DTRAF1, indicating that this domain is sufficient to mediate the association of DTRAF1 with Msn. This finding is consistent with previous studies showing that the TRAF domain of mammalian TRAFs mediates their binding to a number of different proteins [10]. Msn and related SPS1 family members are composed of an amino-terminal kinase domain, a carboxy-terminal regulatory domain that probably couples the protein to downstream Ste11 kinases and, between these two domains, a polyproline-rich region that binds Src-homology-3 (SH3) domains [4,13]. We found that the region between the kinase and carboxy-terminal regulatory domains of Msn was sufficient to bind DTRAF1, whereas neither the kinase domain nor the carboxy-terminal domain alone could bind the protein (Figure 2). Moreover, we found

Figure 1

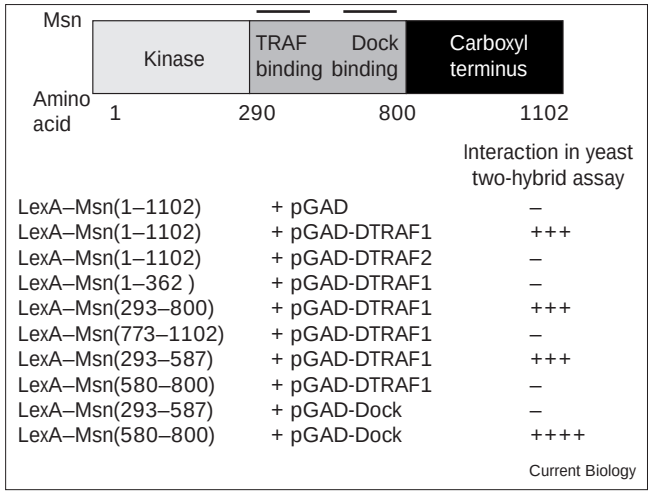


Amino-acid sequence of DTRAF1 and DTRAF2 and their alignment with mammalian TRAF2. In contrast to DTRAF1, DTRAF2 contains an amino-terminal Ring-finger domain. Identical amino acids are shaded. TRAF-N and TRAF-C indicate amino-terminal and carboxy-terminal regions of the TRAF domain, respectively. The accession numbers for DTRAF1 and DTRAF2 are AF119794 and AF119793, respectively.

that a stretch of about 250 amino acids that lies in the amino-terminal portion of the interdomain region was sufficient for Msn to bind DTRAF1 (Figure 2). The carboxy-terminal portion of this region did not interact with DTRAF1 but did interact with the SH3 domains of Dreadlocks (Dock) [14] (Figure 2), supporting the idea that the central region couples Msn and related Ste20 kinases to multiple upstream targets [14]. Mammalian TRAFs have been shown to form homodimers and heterodimers with other TRAFs [10], and it has been proposed that homodimerization of TRAF proteins may be important for their function to activate downstream targets [10,11]. We found that although DTRAF1 and DTRAF2 formed homodimers in the yeast two-hybrid system, they did not interact with each other, indicating that they do not heterodimerize (data not shown).

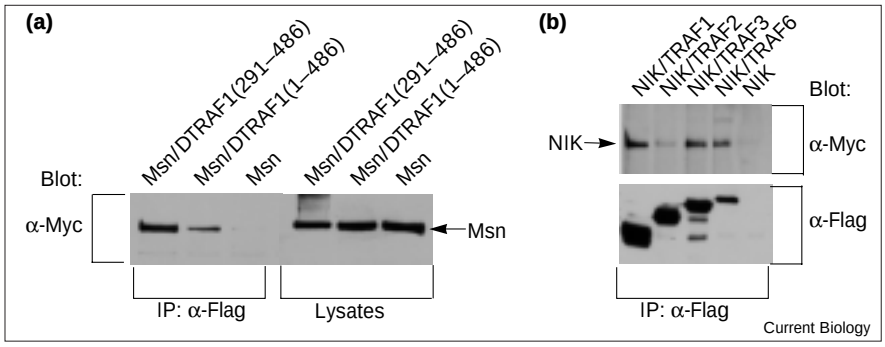
To determine whether Msn also binds DTRAF1 *in vivo*, Myc-epitope-tagged Msn was cotransfected into 293 cells with FLAG-epitope-tagged DTRAF1 and a truncated DTRAF1 containing only the TRAF domain of DTRAF1. The ability of the two proteins to associate was determined by coimmunoprecipitation experiments. We found that Msn and DTRAF1 formed a complex in cells and that the TRAF domain of DTRAF1 was sufficient to mediate binding to Msn (Figure 3a). The Ste20 kinase NIK is the structural and functional homolog of Msn [13]. We therefore predicted, due to the evolutionary conservation of NIK and Msn, that NIK would interact with mammalian TRAFs. Indeed, we found that NIK coimmunoprecipitated with TRAF1, TRAF2, TRAF3 or

Figure 2



Mapping of the interaction between DTRAF1 and Msn using the yeast two-hybrid system. Interaction was determined by selecting for growth on plates lacking histidine and containing 5 mM 3-aminotriazole [4].

Figure 3



Msn and NIK associate with TRAFs in cells. (a) 293 cells were transfected with a construct encoding Myc-epitope-tagged Msn either alone or together with FLAG-epitope-tagged DTRAF1, as indicated, using the vector pRK5 [13]. To assess whether Msn interacts with DTRAF1 in cells, the DTRAF1 constructs were immunoprecipitated (IP) with anti-FLAG antibodies (α-FLAG) and the immunoprecipitates were washed and separated by SDS–PAGE (10%). Msn bound to DTRAF1 was identified by immunoblotting with anti-Myc (α-Myc) antibodies [13]. (b) 293 cells were transfected with Myc-epitope-tagged NIK20 alone or together with FLAG-epitope-tagged TRAF1, TRAF2, TRAF3 or TRAF6. The ability of NIK to associate with the various TRAFs was determined by co-immunoprecipitation experiments, as described in (a).

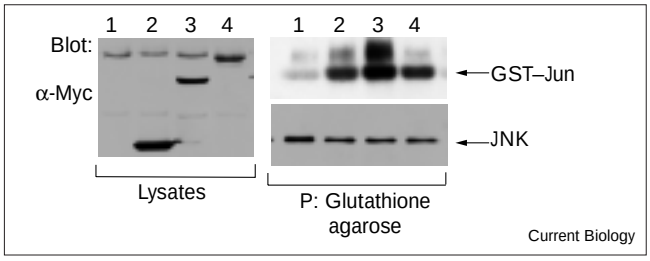
TRAF6 in 293 cells (Figure 3b). Thus NIK, like Msn, associates with TRAFs in cells.

To determine whether DTRAF1 activates JNK, constructs expressing full-length and truncated DTRAF1 proteins were expressed in 293 cells together with glutathione-S-transferase (GST)-tagged JNK. To assess JNK activation, activity assays were performed on JNK precipitates, as previously described [13]. Overexpression of both full-length and a truncated DTRAF1 containing the zinc-finger and TRAF domains led to a 4–5-fold increase in JNK activity when compared with cells transfected with a control vector (Figure 4). Surprisingly, expression of only the TRAF domain of DTRAF1 was sufficient to activate JNK to almost the same extent as the full-length DTRAF1 (Figure 4). These findings indicate that DTRAF1 activates JNK, but — in contrast to the activation of JNK by mammalian TRAFs 2, 5 and 6 — this activation does not require either the Ring-finger or zinc-finger domains [10,11].

To explore the possibility that Msn functions downstream of DTRAF1, we determined whether a truncated Msn lacking the kinase domain functions as a dominant-negative inhibitor by blocking the activation of JNK by DTRAF1. DTRAF1 and GST–JNK were transfected into 293 cells either alone or together with Msn (amino acids 293–1102). Whereas transfection of DTRAF1 led to a roughly fivefold increase in JNK activity, cotransfection of Msn(293–1102) and DTRAF1 markedly inhibited JNK activation (Figure 5a). To further demonstrate that the interaction of DTRAF1 with Msn is important for the activation of JNK by DTRAF1, we transfected suboptimal concentrations of DTRAF1 and Msn that alone barely stimulated an ATF2-dependent luciferase reporter construct; JNK has been shown to phosphorylate and activate several transcription factors including ATF2 [13]. We reasoned that if an interaction between DTRAF1 and Msn was important for DTRAF1 activation of JNK, cotransfection of the two constructs should synergistically activate a JNK-dependent luciferase reporter construct. Cotransfection of suboptimal amounts of Msn and DTRAF1 led to a synergistic stimulation of ATF2-stimulated luciferase activity (Figure 5b). Taken together, these findings suggest that DTRAF1 functions upstream of Msn to activate JNK.

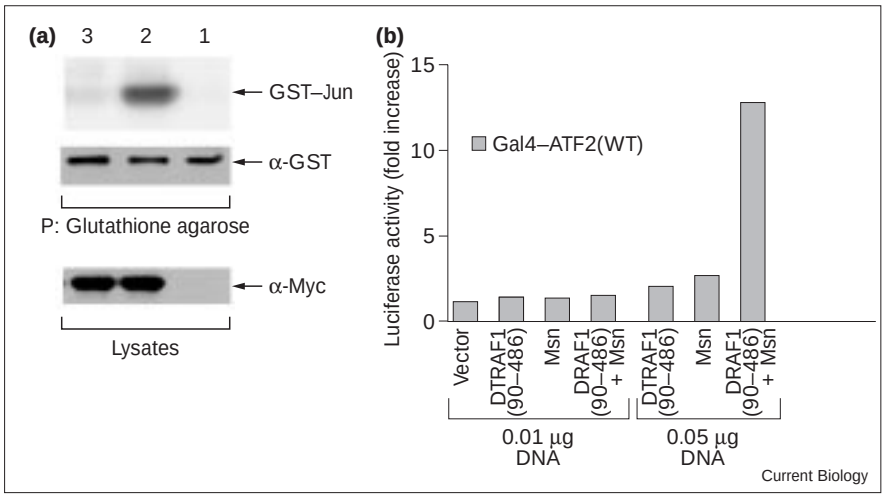
The mammalian TRAF family plays a central role in mediating signals from several TNF receptor superfamily members [11,12,15–17]. A critical role for TRAF2 in coupling the TNF receptor to JNK activation has been substantiated in a study of cells from TRAF2 knockout mice: TRAF2-deficient cells do not activate JNK in response to TNF [16]. Recent evidence has shown that two other SPS1 family members, germinal-center (GC) kinase and GC-related kinase, also interact with mammalian TRAFs and may play a role in coupling TRAFs, as well as TNF,

Figure 4



TRAF1 activates JNK. Constructs encoding full-length or truncated versions of Myc-epitope-tagged DTRAF1 (2 μg) were transfected into 293 cells together with 1 μg GST–JNK. To demonstrate expression of the various DTRAF constructs, lysates were probed with anti-Myc antibodies (left panel). Lane 1, control; lane 2, DTRAF(291–486); lane 3, DTRAF(93–486); lane 4, DTRAF(1–486). To assess JNK activation, GST–JNK was precipitated (P) from 500 μg of lysates using glutathione–agarose and subjected to an *in vitro* kinase reaction using GST–Jun as a substrate, as described previously [13]. Reaction products were separated by SDS–PAGE (12.5%) and visualized by autoradiography (upper right panel). Half the precipitate was immuoblotted with anti-GST antibodies to check that equal GST–JNK levels were precipitated (lower right panel).

Figure 5



Biochemical evidence that DTRAF1 functions upstream of Msn. (a) A dominant-negative Msn mutant protein blocks JNK activation by DTRAF1. Myc-epitope-tagged DTRAF1 (2 μg) was transfected into 293 cells together with 1 μg of GST–JNK, either alone or together with Msn(293–1102). JNK activation was determined as described in Figure 4. Lane 1, control; lane 2, DTRAF1; lane 3, DTRAF1 + Msn(293–1102). (b) Msn and DTRAF1 cooperate to stimulate activation of JNK. To further test whether Msn and DTRAF1 interact in cells, decreased amounts of plasmids encoding Msn and DTRAF1 that barely stimulated activation of an ATF2 GAL4–luciferase reporter system were transfected either alone or together. Luciferase activity is expressed in arbitrary units after being standardized to β-galactosidase activity.

to JNK activation [15,18]. Moreover, a Ste11 kinase, apoptosis-inducing kinase (ASK1), has been shown to bind TRAFs directly and possibly to couple TRAF2 to JNK activation in TNF-stimulated cells, thus bypassing the requirement for a Ste20 kinase [19]. At present, it is difficult to determine which downstream kinase couples TRAFs to JNK activation under specific situations. We now have the tools in hand to assess genetically whether DTRAF1 functions upstream of Msn. Previous studies have indicated that components of the JNK pathway leading to JNK activation and dorsal closure in *Drosophila* are probably conserved between flies, nematodes and mammals [4]. Thus, if the hypothesis that DTRAF1 functions upstream of Msn proves to be correct, the identification of components functioning upstream and downstream of DTRAF1 in *Drosophila* will provide valuable insights into signaling pathways regulated by mammalian TRAFs.

Acknowledgements

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