CHAPTER 5

TRAF4, the Unique Family Member

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Abstract

The fourth member of the TRAF protein family (TRAF4) presents several characteristics that distinguish it from the other members of the family. These characteristics concern the primary sequence of the protein, a strong evolutionary conservation, and a tightly regulated physiological expression during development. The subcellular localization of TRAF4 is controversial as it has been detected at the cell membrane, in the cytoplasm and in the nucleus. Using mouse and fly models, it has been established that TRAF4 is a key molecule in diverse ontogenic processes, particularly in the nervous system. However, the molecular mechanisms of action of TRAF4 remain evasive as it was found to interact with diverse types of proteins, leading either to pro-apoptotic or anti-apoptotic functions. Finally, few studies implicated TRAF4 in human diseases.

The Fourth Member of the TRAF Protein Family

The Tumor Necrosis Factor Receptor-Associated Factor 4 (TRAF4) belongs to the canonical TRAF protein family that contains six members. They are defined by the presence of a carboxy- (C-) terminal TRAF domain composed of two parts, N-TRAF and C-TRAF, the second exhibiting a higher level of conservation. A seventh member, TRAF7, has recently been added although it is devoid of TRAF domains (for general reviews see refs. 1-4).

TRAF4 is unique in several aspects (Table 1, Fig. 1). Although all TRAFs (with the exception of TRAF1) contain an N-terminal RING finger motif, TRAF4 (as well as TRAF5 and TRAF6) contains the C3HC3D motif instead of the classical C3HC4 RING motif, and it is the only one that contains a nuclear localization signal (NLS).[5] The core of TRAF4 is composed of 3 HC3HC3 cysteine-rich domains, defined by Regnier et al as CART domains (Cystein-Rich domain Associated with RING and TRAF domain); each CART domain contains 2 putative zinc fingers.[5] While several groups have interpreted the numerous C and H residues present in this region to suggest the presence of seven zinc fingers instead of six in TRAF4,[1,6] the fact that each CART domain is encoded by distinct exons (exons 4, 5, and 6 for TRAF4) in all TRAFs strongly supports a HC3HC3 structure, and suggests that each is derived from an ancestral exon. Indeed, TRAF4 was first named CART1 because of this domain.[5] TRAF4 is the only member to possess three CART domains; the other TRAFs have two. Furthermore, the first TRAF4 CART domain exhibits a second putative NLS. In the N-TRAF domain, the coiled-coil domain of TRAF4 is short compared with the other TRAFs, with only three heptad repeats while others have more than ten. This might explain the low capacity of TRAF4 to form heterotypic associations. Finally, The three residues R, Y and S, present in TRAF1, 2, 3 and 5, that are involved in the recognition of the cytoplasmic TRAF member interacting motif (TIM) of the TNF-receptors (TNF-R),[7] are not conserved in TRAF4 but replaced by S, F and F, respectively. These substitutions could explain the reduced interaction of TRAF4 with

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Table 1. Summary of TRAF4 characteristics

Gene	
Chromosomal localization	17q11-12 (human) ; 11B5-11C (mouse)
organization	7 exons
regulation	weak kozak sequence; no TATA box; p53; PMA; CD40 ligand; TNF
Protein	
primary sequence	RING C3HC3D; 3 CARTs; 2 NLS; TIM: S, F, F
structure	Short coiled-coil N-TRAF domain; 3 heptads
Expression pattern	
develoment	early and widespread; CNS, PNS, postmitotic undifferenciated neurons
adult	ubiquitous basal expression; regulated in some tissues
Subcellular localization	membrane; cytoplasm; nucleus
KO phenotype	high in utero lethality; CNS, PNS and skeletal alterations
Human diseases	
malignant	breast cancer, Hodgkin
benign	schizophrenia
Putative function	nervous system; pro-apoptosis; anti-apoptosis; cell cycle progression; oxidant production

the members of TNF-R family and suggest that TRAF4 might interact with other types of transmembrane proteins.

Thus, the primary sequence of TRAF4 suggests that it is a particular TRAF member that might be implicated in particular function(s).

TRAF4 Is Highly Conserved during Evolution

TRAF4 protein orthologues have been reported for several species (Fig. 2). The mouse TRAF4 primary protein sequence shows 97% identity with its human counterpart. Databases also contain a rat TRAF4 sequence that shows 97% identity with human TRAF4. The Drosophila genome contains three TRAFs, DTRAF1 corresponds to TRAF4 (45% identity with the human protein), DTRAF2 corresponds to TRAF6, and DTRAF3 corresponds to TRAF1, 2, 3 and 5.[6] Two zebrafish orthologues, TRAF4a (77% identity with the human protein), and TRAF4b (68% identity with the human protein), have also been identified.[8] To date, they are the only TRAFs described in zebrafish. Since the Caenorhabditis elegans genome contains only one TRAF (37% identity with the human TRAF4), the question of the existence of other TRAFs in fish remains open. Searches in the Public Dictybase reveal the existence of a related TRAF gene, zfaA, in Dictyostelium discoideum. This gene

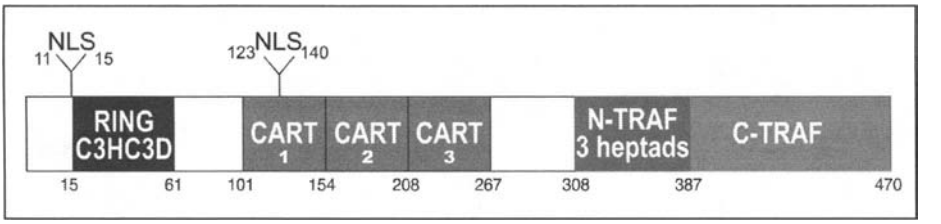

Figure 1. Schematic representation of TRAF4 protein.

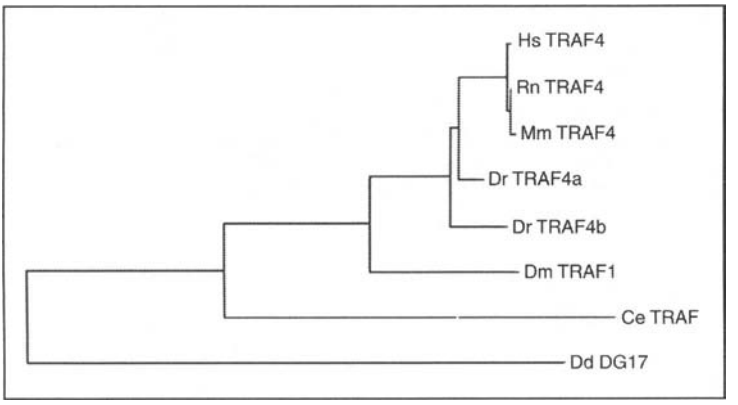

Figure 2. Phylogenic tree built from the multi-alignment comparing the human (Hs), Rat (Rn), mouse (Mm), zebrafish (Dr), and fly (Dm) amino acid sequence of the TRAF4 protein, the TRAF protein present in worm (Ce) and DG17 protein of Dictyostelium discoideum (Dd).

encodes the protein DG17, a presumed zinc ion binding protein expressed during Dictyostelium discoideum aggregation.[5]

At the molecular level, regardless of species, the promoter region of TRAF4 does not have a consensus TATA box and contains a relatively weak Kozak sequence, two characteristics often observed in ubiquitously expressed genes. The various TRAF4 genes also share a similar gene organization; each gene is composed of 7 exons, exons 1 and 2 encode the RING domain, exons 4, 5 and 6 encode the three CART domains, and exon 7 encodes the TRAF domain. Moreover, a syntenic linkage conservation has been reported between mouse and man; the human TRAF4 gene localizes to chromosome 17q11-q12 and the mouse gene lies in the corresponding 11B5-11C region.[9]

The strong evolutionary conservation reinforces the idea that TRAF4 exerts an important biological function. Accordingly, it has been shown that, as TRAF6, TRAF4 precursor gene has arisen early during evolution whereas the other TRAFs have diverged more recently.[6]

Physiological Expression

In all species studied (human, mouse, zebrafish and drosophila), TRAF4 expression during embryogenesis is highly dynamic and complex (Fig. 3). In human fetal tissues at 12-18 weeks of gestation, immunohistochemistry experiments show a strong cytosolic TRAF4 staining that is mostly restricted to the basal epithelial cells.[10] In the mouse embryo, TRAF4 is widely expressed. TRAF4 mRNA is observed in 3.5 day post coitum (dpc) embryonic stem (ES) cells, and reaches maximum expression by 8.5 to 13.5 dpc.[9] Depending on the developmental stage, TRAF4 expression is observed in various organs including neural crest cells, the first, second and third branchial arches, intestine, thymus, salivary gland and the epithelium of the trachea.[9,11] During mouse odontogenesis, TRAF4 is detected in the dental papilla mesenchyme and in both the internal and external enamel epithelium.[12] During zebrafish embryogenesis, TRAF4b is weakly expressed in a ubiquitous manner, but TRAF4a is strongly expressed in a specific and regulated fashion in the sensorial and neural cells, the somites and the blood vessels, suggesting that TRAF4a is responsible for all TRAF4 function.[8] Similarly, the highest levels of expression in the mouse[9] are observed during the ontogenesis of the central (CNS) and peripheral (PNS) nervous systems, and in the nervous tissues of sensory organs. TRAF4 is preferentially expressed by post-mitotic undifferentiated neurons and in oligodendrocytes. Moreover, TRAF4 is developmentally regulated in the mouse CNS, as it is down-regulated between neonates and recently weaned 4-week-old mice.[13] In drosophila, DTRAF1 accumulates in mesodermal cells and neural precursors and is correlated with the onset of morphogenetic and cellular movements. It is largely absent in terminally differentiated cells.[14]

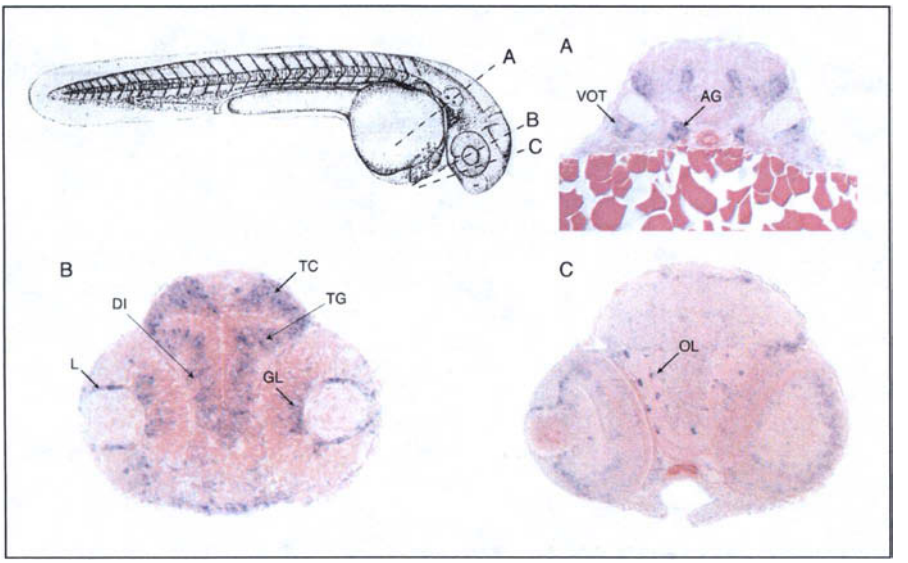

Figure 3. Histological sections highlighting the expression pattern of TRAF4a in sensorial and nervous system during zebrafish development. A) At 36 hours post fecondation (hpf), TRAF4a is detected in ventral otic vesicle (VOT) and statoacoustic ganglia (AG). B) At 48 hpf, TRAF4a is expressed in the tectum (TC), the tegmentum (TG) and the diencephalon (DI). In the eye, expression of TRAF4a is present in the lens (L), and in the ganglion cell layer (GL). C) At 60 hpf, TRAF4a is expressed in the oligodendrocytes (OL).

At the RNA level, no expression was detected in human breast, heart, brain, skin, lung, stomach, colon, liver, kidney and placenta.[5] However, TRAF4 EST have been reported in 27/31 human adult tissues (NCBI Unigene database). This near ubiquitous expression was confirmed at the protein level in a survey of normal adult human tissues that showed strong TRAF4 positivity in the basal cell layer lining the basement membrane of complex epithelia throughout much of the body.[10] Accordingly, in situ hybridization[9] indicates basal levels of TRAF4 expression in most adult mouse tissues. Interestingly, in addition to this constitutive expression, strong TRAF4 expression is observed in some tissues such as adult CNS where TRAF4 is highly expressed in the hippocampus and the olfactive bulb, and in the Purkinje cells of the cerebellum.[9]

Widespread TRAF4 expression at the basal level suggests a generic function in shared biological processes. However, in distinct tissues, high TRAF4 expression is tightly regulated, indicating that it might exert additional tissue-specific function(s). In this context, whereas the other TRAF functions are mostly related to the immune system, that of TRAF4 is related to the nervous system.

Subcellular Localization: A Matter of Debate

Since its discovery, the subcellular localization of TRAF4 has been controversial. Indeed, TRAF4 has been detected at the cell membrane, in the cytoplasm and in the nucleus.

Several studies have shown that TRAF4 preferentially associates with the insoluble fractions of cell extracts. TRAF4 is abundant in the insoluble pellet fraction of human embryonic kidney epithelial cells (HEK293T) transfected with HA-tagged-TRAF4, whereas little is seen in the soluble fraction.[15] In addition, Xu and colleagues recovered TRAF4 largely from the cytoskeleton/membrane fraction that also contains p47phox.[16] In immunofluorescence experiments, Glauner et al noticed a significant local increase of TRAF4 at points of cell-cell contact that is dependent on the C-TRAF domain of the protein.[17] Moreover, a recent study reported a perinuclear distribution of TRAF4 in unstimulated HMEC-1 cells and clear cell surface membrane labeling after exposure to TNFα.[18]

On the other hand, TRAF4 has been detected within cytosolic vesicles or organelles of TRAF4-tranfected HEK293T cells.[10] These authors also reported a cytoplasmic localization in vivo in human breast cancer sections. Furthermore, Sax and El-Deiry showed TRAF4 cytoplasmic localization, even after induced cell damage at the DNA level.[19] In the same way TRAF4-GFP has mainly been found in the cytoplasm and excluded from the nucleus in HeLa cells.[20]

In human breast cancer sections, TRAF4 has been seen in the nuclei of cancer cells by immunohistochemistry.[5] Consistent with a nuclear localization, Glauner et al has shown that full-length TRAF4-GFP chimeric proteins localize to the cytoplasm of HeLa cells while C-terminal TRAF4(259-470)-GFP proteins localize predominantly to the nucleus. Moreover, TRAF4(259-470) can translocate a full-length TRAF4 molecule to the nucleus by forming TRAF4-TRAF4(259-470) heteromeric complexes. A truncated form of TRAF4 lacking the C-terminal end but containing the 2 NLS also goes to the nucleus.[17] However, it remains to be seen if such truncated TRAF4 forms exist in vivo.

Collectively, these data are consistent with the characteristics of TRAF4. Thus, it can be hypothesized that TRAF4 shuttles between different cellular compartments. TRAF4 can be present in the cytoplasm and recruited to the membrane via its association with transmembrane or membrane-related proteins. It can also translocate to the nucleus since it contains 2 putative NLS. However, since nuclear localization has only been observed under pathological conditions, this purported function may also be nonphysiological.

In Vivo Evidence That TRAF4 Is Biologically Relevant

TRAF4-deficient mice, on a mixed 129/Svj X C57BL/6 genetic background, have a localized developmental defect in the upper respiratory tract, with a constricted upper trachea at the site of the tracheal junction with the larynx, showing that TRAF4 is required for anastomosis of the upper and lower respiratory systems during development.[11] This restricted phenotype was strange since TRAF4 is widely expressed during embryogenesis. However, on a pure 129/Svj genetic background, TRAF4 deficiency is embryonic lethal in approximately one third of the homozygote mutants, suggesting that TRAF4 is crucial for early embryogenesis. Surviving animals manifest numerous alterations. Tracheal disruption and respiratory disorders affect 100% of the survivors although other alterations are not fully penetrant. The most frequent and important malformations concern the axial skeleton (ribs, sternum, tail), and defect of the neural tube closure giving rise to spina bifida phenotypes.[21] The phenotypic discrepancies between the two strains of TRAF4-null mice point to the impact of genetic background on gene deficiency studies.

In drosophila, homozygous mutants with a P-element insertion (EP(2)578) in the first exon of DTRAF1, which leads to markedly reduced expression of this gene, show a higher number of adult dorsal bristles, a typical structure of the drosophila peripheral nervous system.[22] Moreover, a null allele for DTRAF1 ($DTRAF1^{ex1}$) is lethal and these mutants fail to develop into the pupal stage.[23] Mutant larvae contain small-sized imaginal discs, especially eye discs, and photoreceptor axons form few axonal bundles and fail to defasciculate in the brain hemisphere. Thus, DTRAF1 is indispensable for the development of imaginal eye discs and the formation of a correct photosensory neuronal array in the brain hemisphere. Heterozygous $DTRAF1^{ex1}$ mutants also exhibit defects of the thorax closure, a phenomenon tightly controlled by the drosophila c-Jun amino terminal kinase (JNK) signaling pathway.

Accordingly, depletion of TRAF4a expression in zebrafish using antisense morpholino oligonucleotides also leads to dramatic abnormalities during embryonic development, with particular defects in the sensory organs of the ear and the eye (our unpublished results).

Thus, TRAF4 is a key molecule in diverse ontogenic processes, particularly in the nervous system. This is a particular role for a member of the TRAF protein family. In fact, the other TRAF-deficient mice with the exception of TRAF6 that also presents nervous alterations, show alteration of their immune system.

Table 2. TRAF4 interacting-proteins, TRAF4 domain involved and function

Protein	Domain	Signaling*	Function*	Reference
TNF-receptor signaling				
p75-NGFR	TRAF	NFκB ↓	cell apoptosis ↑	24
LT-βR	TRAF	ND	ND	10
GITR	ND	NFκB ↑	cell survival ↑	27
Msn	TRAF	JNK ↑	cell apoptosis ↑	28
Pelle	TRAF	NFκB ↑	ND	26
Membrane-related proteins				
p70S6K	ND	S6 phosphorylation ↑	cell cycle ↑	30
p47phox	TRAF	JNK ↑	cell apoptosis ↑ oxidant production ↑	16
		ERK1/2; p38 ↑	ND	18
TFAF2/SNX6	ND	TGFβ-R ?	GF traffiking ?	32
Apoptosis-related proteins				
DIAP1/c-IAP-1; c-IAP-2	TRAF	JNK ↓	cell apoptosis ↓	22
Miscellaneous proteins				
TRAF4	TRAF	ND	ND	26
MUL	TRAF	ND	ND	40
USP7/HAUSP	TRAF	ND	ubiquitination ?	40
TFAF1	ND	ND	ND	32
Hic 5	ND	RAFTK/Pyk2	scaffolding ?	16

* ↑ = activation; ↓ = inhibition; ? = putative

Can TRAF4 Transduce Extracellular TNF Signals?

Although in vivo studies show that TRAF4 is involved in important biological functions, how it works at the molecular level remains evasive. Because transient expression of some TRAFs induces nuclear factor kappa B (NFκB) activity, we and others have tested the effects of TRAF4 overexpression on the activation of a NFκB reporter plasmid on transient transfection assays in HEK293T cells. However, no NFκB activity has ever been detected. DTRAF1 does not interact with NFκB signaling either.[23] In order to investigate the signaling pathway(s) involving TRAF4, numerous experiments have been performed to identify its protein partners. Interestingly, upstream TNF-receptors and downstream kinase partners have been found that might engage TRAF4.

TRAF4-Interacting TNF-Rs

Numerous members of the TNF-R family were tested for their interaction with TRAF4. In contrast to other TRAF family members, very few interacted with TRAF4 and only under certain conditions (Table 2).

TRAF4 interacts weakly with the human p75 neurotrophin receptor (p75-NGFR), a member of the TNF-R present in the nervous system, and with the lymphotoxin-beta receptor (LTβ-R).[10,24] The latter receptor mediates an essential signaling system for the development, organization and differentiation of lymphoid tissues.[25] Paradoxically, LTβ-R also induces apoptosis of some epithelial tumors. Moreover, Zapata et al were also able to show an interaction between DTRAF1 and these two receptors.[26]

Recently, it has been shown that TRAF4 increases NFκB activation through the glucocorticoid-induced TNF-R (GITR), a receptor expressed on T cells, B cells and macrophages. This effect is mediated via a TRAF-binding site located in the cytoplasmic domain of GITR, and

is inhibited by the cytoplasmic protein A20, a TNF-inducible zinc finger protein that interacts with TRAF1. This was the first indication that TRAF4 induces GITR signaling, which is presumed to inhibit the suppressive function of regulatory T cells (Treg cells) and to promote the activation of T cells.[27]

TRAF4-Interacting Kinases

TNF-induced signal transduction pathways usually involve kinase cascades (ie : serine/threonine kinases for NFκB and JNK). Knowledge of the TRAF4-interacting kinases is therefore of importance to determine the pathway in which it could be involved.

Misshapen (Msn), a member of the SPS1 protein kinase family, has been shown to probably act as a mitogen-activated protein (MAP)KKKK (MAP4K) in drosophila by activating the JNK pathway. DTRAF1 appears to interact with Msn via its TRAF domain.[28] Moreover, the TRAF domain from DTRAF1 but not DTRAF2 is sufficient to activate JNK. Thus, TRAF4 is a good candidate for an upstream molecule that regulates JNK pathway via interaction and activation of Msn, suggesting that TRAF4 might be involved in regulating Ste20 kinases in mammals. Interestingly, the Trp/Lys/Ile sequence present in the N-TRAF domain, which is responsible for the recruitment of Nck-interacting kinase (NIK), the mammalian homologue of Msn, is conserved in TRAF4. NIK belongs to the germinal center kinase (GCK) subfamily of Ste20 kinases that couples cell surface receptors (ie: Ephrine) to the JNK pathways.[29] Interestingly, it has been shown that interaction of Msn with the Frizzled receptor (Wnt receptor) regulates dorsal closure via JNK pathway.

Another specific association was reported between DTRAF1 and the regulatory N-terminal domain of Pelle, a fly homologue of the mammalian kinase interleukin-1 receptor-associated kinase (IRAK). Individually, Pelle and DTRAF1 are unable to induce NFκB in HEK293T cells, but their coexpression results in significant NFκB activity. Pelle mediates signaling by the cytoplasmic tail of Toll (Interleukin-1 receptor).[26] Whether IRAK can physiologically interact with mammalian TRAF4 has not yet been tested.

Despite intensive research, the function of TRAF4 in signaling pathways triggered by TNF-R-related proteins remains enigmatic. Since the expression pattern of most identified interacting receptors and kinases can be superimposed with that of TRAF4, it is likely that these molecules interact in vivo and lead to functional pathways.

Can TRAF4 Transduce Extracellular Signals via Membrane-Related Partners?

Aside from transmembrane proteins, various membrane-related proteins located more downstream are also implicated in signal transduction. Some have been shown to interact with TRAF4.

p70S6K is a ser/thr kinase localized in the cytosol which, after cytokine stimulation, is also found in the nucleus. The phosphoinositide 3 kinase (PI3K)/p70S6K signaling pathway regulates the translation of key mRNAs of proteins required for cell cycle progression via phosphorylation of the ribosomal S6 protein. Fleckenstein et al identified TRAF4 as a new partner of this kinase,[30] after screening a HeLa cDNA expression library with p70S6K as bait. This interaction was confirmed by several experiments, and complexes were observed in both cytoplasmic and nuclear fractions. These authors also showed that p70S6K/TRAF4 interaction can be induced through activation of LTβ-R in the human TF-1 erythroleukemic cell line. Moreover, in wild-type HEK-293 cells, which do not express endogenous TRAF4, TNFα did not induce p70S6K while cells transfected with TRAF4 showed a strong increase in S6 phosphorylation upon stimulation, suggesting a role for TRAF4 in the activation of this kinase.

Xu et al found TRAF4 in a screen of lung and endothelial libraries for partners of p47phox. p47phox is an adapter subunit of the NAD(P)H oxidase that participates in TNFα signaling, and is associated with the cytoskeleton.[16] p47phox interacts with TRAF4 via a tail-to-tail interaction between the C-terminus of p47phox and the conserved TRAF domain of TRAF4. While these proteins alone have minimal effect, together they constitutively activate JNK and increase oxidant production. The authors postulate that TRAF4 might function to couple p47phox to upstream signaling events.[16] This hypothesis was recently confirmed by Li et al who demonstrated that the

acute response to TNFα involves a rapid PKC-dependent phosphorylation of p47phox, an increase in p47phox-TRAF4 association, translocation of p47phox-TRAF4 to the cell membrane, and activation of the NAD(P)H oxidase, ERK1/2 and p38 MAPK.[18]

Lastly, TRAF4-associated factor 2 (TFAF-2)/sorting nexin 6 (SNX6)[31,32] is a peripheral membrane protein that exhibits a characteristic membrane and cytosolic distribution.[33] It is also localized in endosomal compartments, predominantly in the early endosomes. SNX6 interacts with cargo and is thought to participate in the intracellular trafficking of plasma membrane receptors. SNX6 has been shown to bind to TGFβ-R. Furthermore, the oncogenic serine/threonine kinase Pim1 can phosphorylate TFAF2/SNX6 and induce its translocation from cytoplasm to nucleus.[32]

These membrane-associated TRAF4 partners might determine the subcellular localization of TRAF4, by regulating the proximity of individual signaling complexes to TRAF4, and therefore the activation of specific downstream signals via TRAF4.

Does TRAF4 Regulate Cell Life and Death?

Many TRAF family members negatively regulate apoptotic pathways by increasing the expression of genes which promote cell survival.[2,34] Several groups have hypothesized that TRAF4 might also be involved in apoptosis. However, depending on the study, some authors have proposed a pro-apoptotic function while others an anti-apoptotic function.

TRAF4 as a Pro-Apoptotic Factor

JNK is known to mediate a physiological stress signal that leads to cell death. Two studies[22,23] have reported that DTRAF1 overexpression, notably in S2 cells, can activate the Hep/JNK signaling pathway leading to an increase in JNK phosphorylation, and subsequent apoptosis (30% increase). This activity is independent of the RING finger, as DTRAF1 does not contain a RING finger domain. These authors placed DTRAF1 activity upstream of DTAK1 (drosophila TGFβ-activated kinase). Moreover, DTRAF1 directly interacts with the inhibitor of apoptosis, DIAP1, and its human homologue cIAP-1. c-IAP-1 is normally predominantly localized in the nucleus, but apoptotic stimuli induces its export from the nucleus. Finally, c-IAP-1 associates with mid-bodies in dividing cells.[35] Increased amounts of DIAP1 lower the amount of DTRAF1 in cells. Indeed, DIAP1 (as c-IAP1) contains ubiquitin ligase activity and can stimulate DTRAF1 degradation through ubiquitination. Thus, DIAP1 can prevent DTRAF1-induced activation of JNK as well as cell death.

TRAF4 was also found to be the only TRAF member that is regulated by the tumor suppressor p53, in a microarray analysis of p53-regulated genes.[19] The TRAF4 promoter contains a functional p53 DNA-binding site approximately 1 kb upstream of the initiating methionine residue, and overexpression of TRAF4 induces apoptosis. Since this apoptosis occurs at a slow rate, these authors proposed that TRAF4 is not directly involved but may be a late mediator in a pro-apoptotic signaling pathway. Thus, TRAF4 might play a role in p53-mediated pro-apoptotic signaling in response to cellular stress. Furthermore, TRAF4 suppresses colony formation in 4 cell lines in this study regardless of p53 activity, an activity that is dependent on the TRAF domain.

Finally TRAF4 has been shown to suppress the ability of the common neurotrophin receptor $p75^{NTR}$ dimers to block cell death induced by $p75^{NTR}$ monomers, also suggesting a pro-apoptotic role for TRAF4.[24]

TRAF4 as an Anti-Apoptotic Factor

In Jurkat leukemic T cells expressing IκB-alpha delta N, a super repressor of NFκB activation, treatment by the survival agent and tumor promoter PMA strongly induces apoptosis, indicating that NFκB promotes cell survival. Interestingly, while TRAF4, c-IAP-1 and c-IAP-2 expression is usually induced by PMA, it is not in these cells lacking NFκB activity. This suggests that TRAF4 might be anti-apoptotic like c-IAPs. Among TRAF1-4, TRAF4 is upregulated the most and the fastest to PMA treatment (2h, 3.2X).[17,36]

CD40 has a 62 amino acid long cytoplasmic domain comprising 2 distinct TRAF binding sites.[20] All TRAF proteins except TRAF4 have been reported to associate directly or indirectly with CD40.

However, human multiple myeloma (MM) cells treated with soluble CD40 ligand (gp39) show lower TRAF4 and TRAF6 expression (38% and 32% decrease, respectively) while expression of the other TRAFs remains stable.[37] This was accompanied by inhibition of MM cell growth and apoptosis. These results suggest that TRAF4 is affected downstream of this signaling pathway and not involved in CD40 function. Accordingly, Craxton and colleagues found that TRAF4 mRNA levels are up-regulated following CD40 signaling in B cells.[38] This effect might be cell-specific since it was not found in CD40+ human monocytes or in THP1, a human promonocytic leukemia cell line.[39]

Fleckenstein et al also postulated an anti-apoptotic function for TRAF4 when they found that the anti-Fas antibody, CH-11, induces apoptosis in HEK293 cells, but not when these cells are stably transfected with TRAF4. Thus, TRAF4 confers unresponsiveness to apoptotic stimuli.[30]

Although seemingly paradoxical, these data could all be correct depending on the cells examined. Further experiments are clearly needed to determine the function of TRAF4 in cell life and death.

Miscellaneous TRAF4 Partners

Like all TRAFs, TRAF4 has been shown to homodimerize in fly, fish, mouse and man. Moreover, various TRAF4-interacting proteins have also been reported.

Zapata et al have identified a new family of TRAF-domain containing proteins called TEF, for TRAF domain (TD)-encompassing factors.[40] In vitro, two of these proteins, human MUL/TEF3 and USP7/TEF1 bind to TRAF4 (and five other TRAFs) via their TRAF domains, located at the N-terminal or in the central region, respectively. MUL localizes to cytosolic bodies of unknown nature; USP7/HAUSP is an ubiquitin-specific protease (USP) that localizes in the nucleus in structures positive for promyelocytic leukemia (PML). Such proteins exist in diverse eukaryotic plant and animal species. For example, the TDPOZ subfamily includes more than 30 proteins that associate a TD domain with POZ/BTB domains, and are presumably nuclear scaffold proteins.[41]

Glutathione transferases (GSTs) catalyze the conjugation of glutathione with reactive compounds and is involved in the cellular protection against oxidative stress. They are also proposed to modulate kinases, and GST P1-1 interacts with JNK. GST A1-1 has been shown to interact with TFAF1 (TRAF4-associated factor 1),[42] a protein which has recently been shown to be transcriptionally regulated by nitric oxide.[43]

Xu et al also found a dozen TRAF4-interacting partners among endothelial proteins, which are notably involved in cell proliferation and apoptosis. For example, Hic 5 is a tyrosine kinase scaffold protein that is the paralogue of paxillin, which binds to, and is a substrate for related adhesion focal tyrosine kinase (RAFTK, Pyk2). Such pathways are activated by oxidants.[16]

The in vivo existence and meaning of all these interactions remain to be studied at the functional level.

Is TRAF4 Implicated in Human Diseases?

Very few experiments have been performed to test the involvement of TRAF4 in human diseases.

Malignant Diseases

TRAF4 corresponds to clone MLN62 first identified by differential screening in a human metastatic lymph node from a breast cancer cDNA library.[44] About 17.5% of breast tumors overexpress TRAF4 due to gene amplification, alone or in association with the erbB2/HER oncogene.[45] The positive cells are malignant epithelial cells. This expression suggests that TRAF4 might be involved in the formation and/or progression of primary breast cancers and metastases. In support of this hypothesis, TRAF4 is overexpressed in some human breast tumors, as shown by microarray analysis in one study.[46] However, no extensive study has yet been performed to establish the significance of TRAF4 overexpression in terms of diagnosis or prognosis. On the contrary, a second study has shown that TRAF4 is downregulated in many breast tumors, with less than 10% of the primary tumors expressing TRAF4, while strong TRAF4 expression was observed in normal ducts.[10] These discrepancies might derive from the difference in the differentiation grade of the tumors studied or

from the antibodies used, which might recognize various TRAF4 forms. It has also been reported that Hodgkin disease cell lines L428, KMH2 and HS445 expressed moderately TRAF4.[47]

The implication of TRAF4 in malignant processes has been tested using animal models. It has been shown that TRAF4 is not an oncogene or a tumor suppressor gene. In fact, both gain-of-function (transgenic mice expressing high amounts of TRAF4 under the control of either the ubiquitous promoter CMV or the mammary gland specific promoter MMTV) (our unpublished results) and loss-of-function (TRAF4-deficient mice[11,21]) mouse models for TRAF4 do not result in tumor development.

Neuronal Benign Diseases

Microarray analyses (12000 genes studied) of postmortem temporal cortexes from patients with schizophrenia show that the expression of 38 genes is altered. Notably, decreased expression of myelination related genes (among them erbB3), TRAF4, Neurod1, and histone deacetylase 3 (HDAC3) was observed.[48] The authors hypothesized that TRAF4 decrease might have an important effect on this disease.

To date, no relationship between TRAF4 expression and diagnosis or prognosis has been established in cancers. Moreover, TRAF4 alteration seems to be involved in benign diseases of the CNS. More experiments are needed to determine the clinical significance of TRAF4 alteration in both benign and malignant human diseases.

Conclusion and Perspective

Collectively, data show a strong structural homology among species, suggesting a functional conservation of TRAF4 throughout Metazoan evolution. This conservation strengthens the idea that TRAF4 exerts crucial biological function(s) distinct from those previously assigned to the other TRAF proteins. Various and sometimes opposing functions have been proposed for TRAF4 (ie: pro-apoptotic and anti-apoptotic). Consistent with its wide expression pattern, it might be hypothesized that TRAF4 is pleiotropic and exerts several functions depending on the nature of the cell/organ concerned or even on the cell compartment, each of them driving specific signaling pathways. Accordingly, a great variety of TRAF4 partners have been identified including cytoplasmic adaptors, membrane-related proteins, membrane receptors, apoptosis inhibitors, nuclear proteases. Most of the data suggest that TRAF4 may be preferentially involved in stress-related events. However, to date, TRAF4 has not been placed in a clear signaling cascade(s) and future studies will aim to determine these molecular mechanisms.

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