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FAN, a Novel WD-Repeat Protein, Couples the p55 TNF-Receptor to Neutral Sphingomyelinase

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Summary

The initiation of intracellular signaling events through the 55 kDa tumor necrosis factor–receptor (TNF-R55) appears to depend on protein intermediates that interact with specific cytoplasmic domains of TNF-R55. By combined use of the yeast interaction trap system and a peptide scanning library, the novel WD-repeat protein FAN has been identified, which specifically binds to a cytoplasmic nine amino acid binding motif of TNF-R55. This region has been previously recognized as a distinct functional domain that is both required and sufficient for the activation of neutral sphingomyelinase (N-SMase). Overexpression of full-length FAN enhanced N-SMase activity in TNF–treated cells, while truncated mutants of FAN produced dominant negative effects. The data suggest that FAN regulates ceramide production by N-SMase, which is a crucial step in TNF signaling.

Introduction

Tumor necrosis factor (TNF), a cytokine produced mainly by activated macrophages, mediates pleiotropic inflammatory and immunoregulatory responses, as well as cytotoxicity, antiviral activity, and stimulation of cell growth (for review, see Goeddel et al., 1986; Beutler and Cerami, 1986; Fiers, 1991). These cellular responses to TNF are initiated by its interaction with two distinct cell-surface receptors of 55 kDa (TNF-R55) and 75 kDa (TNF-R75) apparent molecular mass, respectively, which transmit signals to the cytoplasm and nucleus leading to profound alterations in the transcriptional programs (for review, see Krönke et al., 1992; Tartaglia and Goeddel, 1992; Rothe et al., 1992). Although several studies indicate that both TNF receptors are independently active in signaling TNF responses (Tartaglia et al., 1991; Gehr et al., 1992; Barbara et al., 1994; Grell et al., 1994), a large majority of TNF activities can be mediated solely by TNF-R55 (Espevik et al., 1990; Tartaglia et al., 1993a; Wiegmann et al., 1992). As with other membrane receptors, these various activities and their coordinated induction are likely to be mediated by the heterogeneity of functional motifs within the cytoplasmic domain of TNF-R55 and of effector proteins with which these motifs interact. The modular structure of TNF-R55 and the corresponding signaling pathways are just beginning to be elucidated. Recent studies have identified a C-terminal domain of TNF-R55 that sequentially initiates the activation of a phosphatidylcholine-specific phospholipase C and an acidic sphingomyelinase (A-SMase), generating ceramide (Schütze et al., 1992; Wiegmann et al., 1994). Ceramide in turn can trigger further downstream events that ultimately result in proteolytic degradation of nuclear factor κB (NF-κB) inhibitory subunit (IκB) leading to induction of NF-κB (Reddy et al., 1994; Machleidt et al., 1994). The domain of TNF-R55 initiating this A-SMase pathway strikingly corresponds to the so-called death domain responsible for mediating the cytotoxic effects of TNF (Tartaglia et al., 1993b; Brakebusch et al., 1992; Wiegmann et al., 1994). A second, equally important, signaling pathway initiated by TNF-R55 involves a membrane-bound neutral sphingomyelinase (N-SMase; Kim et al., 1991; for review, see Kolesnick and Golde, 1994; Pushkareva et al., 1995). Ceramide production from hydrolysis of plasma membrane sphingomyelin results in activation of proline-directed protein kinases, which might then be responsible for activation of phospholipase A_2 (PLA_2; Lin et al., 1993; Wiegmann et al., 1994). Arachidonic acid production by PLA_2 ultimately leads to the generation of proinflammatory metabolites (reviewed by Heller and Krönke, 1994). Proline-directed protein kinases might include a ceramide-activated protein kinase (Liu et al., 1994), members of the MAP kinase family (Vietor et al., 1993), and the stress-activated protein kinase JNK-1 (Kyriakis et al., 1994). Recently, it has been shown that N-SMase mediates TNF–induced activation of the protein kinase Raf-1 (Belka et al., 1995), possibly involving ceramide-activated protein kinase (Yao et al., 1995) and thereby linking TNF-R55 to the MAP kinase cascade. The domain within the cytoplasmic domain of TNF-R55 responsible for initiating this N-SMase pathway has been functionally mapped to a small region directly adjacent to the death domain and designated NSD (N-SMase activating domain; Adam et al., 1996).

Both TNF receptors belong to the nerve growth factor (NGF)/TNF-R superfamily, which includes among others the Fas antigen, the lymphotoxin-β receptor, CD40, and CD30 (for review, see Smith et al., 1994). Investigation of the intracellular events involved in the signal transduction through members of the TNF receptor superfamily has led to the identification of proteins directly interacting with these receptors (for review, see Bazzoni and Beutler, 1995). So far, four proteins have been identified that bind to TNF-R55. A novel protein, designated TRADD, requires the intact death domain to associate with TNF-R55 (Hsu et al., 1995). In concordance, it has been shown that TRADD recruits FADD and RIP, two other death domain–containing proteins (Chinnaiyan et

al., 1995; Stanger et al., 1995) that trigger apoptosis, and that it also interacts with the RING finger protein TRAF2 (Rothe et al., 1994) leading to NF-κB induction (Hsu et al., 1996a; 1996b). However, TNF-R55–associated proteins involved in initiating the N-SMase signaling pathway remained elusive.

In the present study, we report the isolation and characterization of a novel protein, designated FAN (factor associated with N-SMase activation), which associates with the NSD of human TNF-R55. Sequence analysis classifies FAN as a member of the family of WD-repeat proteins, a growing family of regulatory proteins, many of which are involved in signal transduction. Overexpression of FAN or of N-terminal FAN deletion mutants demonstrates that FAN mediates TNF–induced activation of N-SMase and, therefore, is likely to play an important role in the regulation of major inflammatory cellular responses to TNF.

Results

Identification of a Novel TNF-R55–Associated Protein That Binds to the NSD

To identify proteins that directly interact with the cytoplasmic domain of TNF-R55, we used the yeast interaction trap system (Gyuris et al., 1993). Since we were especially interested in proteins that couple TNF-R55 to the N-SMase pathway, we fused a DNA fragment coding for amino acids 206–345 to the LexA DNA-binding domain. Using deletion mutants of TNF-R55, we have previously demonstrated that this region is sufficient to generate the N-SMase activating signal (Wiegmann et al., 1994). The TNF-R55 bait construct was used to screen a Jurkat cDNA expression library for interacting proteins. A cohort of 18 clones was isolated that showed specific interaction with the cytoplasmic domain of TNF-R55 (data not shown).

To identify a cDNA clone or clones coding for a protein or proteins that mediate the activation of the N-SMase pathway, we took advantage of our previous work, which revealed a small region (amino acids 309–319), directly adjacent to the death domain, which was necessary and sufficient to activate N-SMase (NSD; Figure 1A; Adam et al., 1996). To examine whether this functionally mapped NSD contains binding sites for possible protein interaction, we generated a cellulose-bound peptide scanning library (Kramer et al., 1994; Weihergräber et al., 1996) containing overlapping peptides of the entire cytoplasmic domain of TNF-R55 (Figure 1B). Jurkat cells were metabolically labeled, and extracts were assayed for binding. The NSD (amino acids 309–319) strikingly corresponded to the strong binding site (7-fold above background binding) on peptide 36 containing amino acids 307–321 (Figures 1B and 1C). Since the neighboring peptides, 35 and 37 (amino acids 304–318 and 310–324), were both missing one amino acid at either end, peptide 36 was the only one completely containing the functionally mapped NSD. In addition, at least five more distinct regions were found to bind proteins (Figure 1C). A set of three strong protein binding sites (at least 5-fold above background binding) were found in the C-terminal half of the TNF-R55 cytoplasmic domain on peptides 44–49, 59–60, and 65–67, containing amino acids 331–357, 379–390, and 400–408, respectively, thereby confining an important binding region to amino acids 331–408 (Figures 1B and 1C). This corresponds well to the boundaries of the previously identified death domain, spanning approximately amino acids 326–413 (Tartaglia et al., 1993b). The N-terminal half of the TNF-R55 cytoplasmic domain in general exhibited less binding activity that could be localized to three distinct sites (at least 4-fold above background binding) on peptides 14–17, 26, and the NSD–containing peptide 36, containing amino acids 245–268, 281–295, and 307–321, respectively (Figures 1B and 1C). Notably, the N-terminal halves of human (Schall et al., 1990; Loetscher et al., 1990) and mouse (Lewis et al., 1991) TNF receptor cytoplasmic domains show in general a lower degree of homology than the C-terminal halves. However, the binding sites observed within the N-terminal half fall into the regions of greater conservation (Figure 1D).

Since a peptide scanning library clearly revealed a linear protein binding motif precisely corresponding to the functionally mapped NSD, we used a peptide library staggered by only one amino acid each to identify NSD–interacting proteins within the cohort of cDNA clones obtained by the yeast interaction trap system. Individual cDNAs were in vitro transcribed or translated, and the resulting radiolabeled proteins were individually tested for binding to the staggered peptides. One clone, designated 73.31, showed specific binding to this region significantly greater than background binding (Figure 2). We did not observe specific binding of clone 73.31 to any other peptide outside the NSD throughout the entire cytoplasmic region of TNF-R55 (data not shown). The minimally required amino acid content of the binding motif for this protein could be mapped to amino acids 310–318. Since our novel protein binds to amino acids within the motif previously demonstrated to be associated with N-SMase activation (Adam et al., 1996), it was designated FAN. Sequence analysis of FAN(73.31) revealed a partial cDNA insert of about 1 kb that by comparison with the EMBL nucleotide sequence database appeared to represent the 3′-terminus of a novel gene. A short 3′-terminal stretch of FAN(73.31) was found to be identical with randomly sequenced expressed sequence tags, one of which was assigned to human chromosome 8 (Murakawa et al., 1994).

FAN Is a Ubiquitously Expressed WD-Repeat Protein

Full-length coding sequence for FAN was obtained by screening human cDNA libraries with the partial cDNA fragment of clone FAN(73.31). Sequence analysis of this cDNA clone revealed a 2751 bp open reading frame beginning with a translational initiation consensus sequence (Kozak, 1987) and predicting a polypeptide of 917 amino acids with a M_r of 104,312 (Figure 3A). The 5′-end was confirmed by additionally performed rapid amplification of cDNA ends–polymerase chain reaction on human and mouse mRNA.

A comparison of the predicted amino acid sequence with the National Biomedical Research Foundation-PIR databank revealed significant homologies with a number of WD-repeat proteins, including the human LIS-1 protein, the Drosophila transcription factor IID–associated

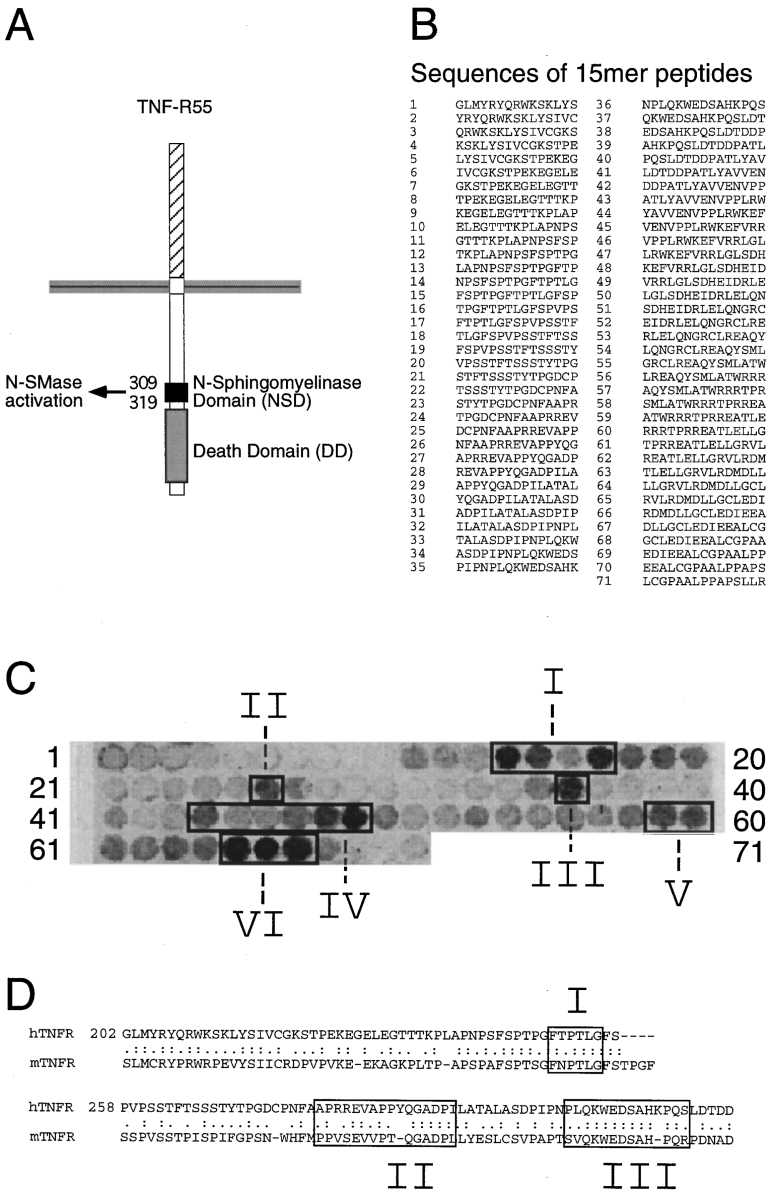

Figure 1. Mapping of Protein Binding Sites on the TNF-R55 Cytoplasmic Domain

(A) Schematic representation of functional domains of TNF-R55.

(B) Overlapping peptides from the TNF-R55 cytoplasmic domain bound to continuous cellulose membrane support.

(C) Protein binding domains of TNF-R55. ^{35}S-methionine–labeled protein extract from Jurkat cells was assayed for binding to the receptor-derived peptide scanning library. Bound radioactivity was visualized by autoradiography and quantified using laser densitometer scanning of the autoradiographs. Regions of increased binding activity (at least 4-fold above background binding) are boxed and marked by Roman numerals.

(D) Homology plot of amino acids 202–326 of the human TNF-R55 (Loetscher et al., 1990) and the mouse TNF-R55 (Lewis et al., 1991). Colons mark identical amino acids; periods mark conservative changes. The binding domains identified in (C) are boxed and marked by corresponding numerals.

protein, and the yeast TUP-1 gene product (reviewed by Neer et al., 1994). Further analysis showed that the C-terminus of FAN contained five WD-repeats (Figure 3B). None of these WD-repeats had more than four mismatches within its core sequence spanning 29–31 amino acids, consistent with the range of 23–41 amino acids in other members of this class of proteins (Neer et al., 1994). Interestingly, the growing family of WD-repeat proteins appears to be composed mostly of regulatory proteins, some of them involved in signal transduction (Neer et al., 1994). In addition, the N-terminus of FAN has significant homology to a human protein homologous to yeast CDC4 (Feuchter et al., 1992), a WD-repeat protein of unknown function.

Northern blot analysis revealed that the mRNA encoding FAN is expressed in a wide variety of human tissues (data not shown). This expression pattern corresponds to the ubiquitous expression of TNF-R55 (Schall et al., 1990; Loetscher et al., 1990) and is consistent with FAN involvement in TNF-R55 signal transduction. The apparent size of the FAN mRNA of 3.8 kb corresponds to the length of the isolated cDNA clones.

FAN Specifically Interacts with the NSD of TNF-R55

Specific interaction of the entire FAN protein (amino acids 3–917) as well as of the truncated FAN protein (FAN[73.31], amino acids 703–917) with the NSD of TNF-R55 was demonstrated in additional yeast interaction trap experiments. Full-length or truncated FAN fused to the transcriptional activation domain was coexpressed with various TNF-R55 deletion mutants or a panel of unrelated proteins fused to the LexA DNA-binding domain (Table 1). Both full-length and truncated FAN(703–917) strongly interacted with TNF-R55 constructs containing the NSD. As expected, the cytoplasmic domain of the deletion mutant TNF-R55Δ308–340 lacking the

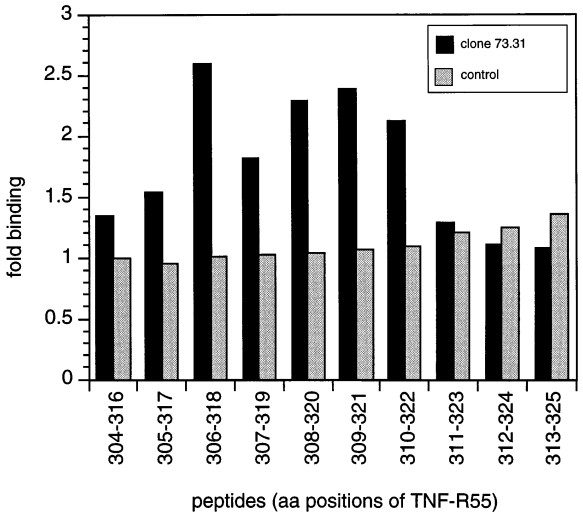

Figure 2. Clone 73.31 Binds Specifically to Peptides Containing the N-SMase Activation Domain

The cDNA of clone 73.31 was in vitro transcribed or translated, and the radiolabeled protein was incubated with a receptor-derived cellulose-bound peptide scanning library. The binding activity resulting from laser densitometer scanning of autoradiographs is shown for peptides containing the indicated amino acids in comparison with a nonspecific protein (clone 39.1) used as a control.

NSD (Adam et al., 1996) failed completely to interact with full-length or truncated FAN. Control baits consisting of either the unrelated protein tyrosine kinase SYK or related membrane receptors such as TNF-R75, Fas, and the Interleukin-1 receptor did not associate with the FAN proteins. FAN(703–917) fused to the LexA DNA-binding domain showed a minor constitutive transactivating effect in yeast by itself, even without an activation-domain partner. This effect, however, was significantly enhanced in the presence of the TNF-R55Δ345 cytoplasmic domain fused to the activation domain, confirming specific interaction of FAN and TNF-R55. Taken together, the interaction trap assay strongly suggested a highly specific interaction between FAN and TNF-R55, strictly requiring the presence of the NSD. The NSD–binding site of FAN is apparently located within the C-terminal WD-repeat region (703–917).

To demonstrate physical interaction between FAN and TNF-R55 in intact cells, we left COS-1 cells untransfected or transfected with the FLAG-tagged full-length FAN fusion construct or the vector pFLAG.CMV2. Identical amounts of cellular lysates were immunoprecipitated using the α–TNF-R55 monoclonal antibody htr-9 or an isotype-matched monoclonal control antibody against human CD28. The reactivity of htr-9 with simian TNF-R55 was previously confirmed by FACS analysis (data not shown). Western blotting with the α-FLAG antibody MAb5 detected a protein of approximately 100 kDa, corresponding to the predicted size of the FLAG–FAN fusion protein, only in FLAG-FAN–transfected cells immunoprecipitated with the α–TNF-R55 antibody htr-9 (Figure 4). FLAG–FAN was detected neither in immunoprecipitations with the control antibody, nor in lysates from cells transfected with the vector pFLAG.CMV2 or from untransfected cells (Figure 4).

A

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1    MAFIRKKQQEQQLQLYSKERFSLLLLNLEEYYFEQHRANHILHKGSHHERKIRGSLKICS
61   KSVIFEPDSISQPIIKIPLRDCIKIGKHGENGANRHFTKAKSGGISLIFSQVYFIKEHNV
121  VAPYKIERGKMEYVFELDVPGKVEDVVETLLQLHRASCLDKLGDQTAMITAILQSRLART
181  SFDKNRFQNISEKLHMECKAEMVTPLVTNPGHVCITDTNLYFQPLNGYPKPVVQITLQDV
241  RRIYKRRHGLMPLGLEVFCTEDDLCSDIYLKFYEPQDRDDLYFYIATYLEHHVAEHTAES
301  YMLQWQRGHLSNYQYLLHLNNLADRSCNDLSQYPVFPWIIHDYSSSELDLSNPGTFRDLS
361  KPVGALNKERLERLLTRYQEMPEPKFMYGSHYSSPGYVLFYLVRIAPEYMLCLQNGRFDN
421  ADRMFNSIAETWKNCLDGATDFKELIPEFYGDDVSFLVNSLKLDLGKRQGGQMVDDVELP
481  PWASSPEDFLQKSKDALESNYVSEHLHEWIDLIFGYKQKGSDAVGAHNVFHPLTYEGGVD
541  LNSIQDPDEKVAMLTQILEFGQTPKQLFVTPHPRRITPKFKSLSQTSSYNASMADSPGEE
601  SFEDLTEESKTLAWNNITKLQLHEHYKIHKEAVTGITVSRNGSSVFTTSQDSTLKMFSKE
661  SKMLQRSISFSNMALSSCLLLPGDATVITSSWDNNVYFYSIAFGRRQDTLMGHDDAVSKI
721  CWHDNRLYSASWDSTVKVWSGVPAEMPGTKRHHFDLLAELEHDVSVDTISLNAASTLLVS
781  GTKEGTVNIWDLTTATLMHQIPCHSGIVCDTAFSPDSRHVLSTGTDGCLNVIDVQTGMLI
841  SSMTSDEPQTCFVWDGNSVLSGSQSGELLVWDLLGAKISERIQGHTGAVTCIWMNEQCSS
901  IITGGEDRQIIFWKLQY
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B

WD-repeats: $\{X_{6\text{-}94} — (GH - X_{23\text{-}41} - WD)\}$

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FAN repeat1   IH KEAVTGI T V SR N   G  S  SVF TTSQ D STLK M FS
FAN repeat2   GH DDAVSKI C W  H D   N     RLY SASW D STVK V WS
FAN repeat3   EH DVSVDTI S L    N   A AST LLV SGTK E GTVN I WD
FAN repeat4   SD EPQTCFV  W    D G N     SVL SGSQ S GELL V WD
FAN repeat5   GH TGAVTCI  W  M N   E QCS SII TGGE D RQII F WK
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Figure 3. Predicted Amino Acid Sequence of FAN Showing Five WD-Repeats

(A) The amino acid sequence deduced from the sequence of a cDNA clone isolated by screening a human muscle cDNA library with the 1 kb cDNA fragment of clone 73.31. The WD-repeats are underlined, and the 5′-ends of the deletion mutants (FAN[448–917] and FAN[703–917]) are indicated.

(B) Alignment of the five WD-repeats located in the C-terminus of FAN. The WD-consensus sequence according to Neer et al. (1995) is shown above. Mismatches in the FAN repeats are indicated in bold.

Taken together, these results confirm the specific interaction of FAN and TNF-R55 in intact cells.

FAN Mediates Activation of N-SMase

To address the role of FAN for the activation of N-SMase, we investigated the ability of the NSD–derived peptide containing amino acids 307–321 (D2A) to interfere with TNF–induced sphingomyelin hydrolysis. Increasing amounts of the peptide D2A or a control peptide containing death domain–derived amino acids (E2A) were incubated with membrane fractions of Jurkat cells prior to TNF treatment. As shown in Figure 5, N-SMase activation was effectively inhibited by peptide D2A but not by the control peptide. Thus, the NSD–derived peptide specifically blocked the activation of N-SMase, probably owing to competitive binding of FAN, which provides the first means of selective and specific interference with N-SMase activation.

To demonstrate directly the involvement of FAN in the

Table 1. Specific Interactions between FAN and TNF-R55

DNA-binding–domain hybrid[a]	Activation-domain hybrid	Growth on Leu⁻ medium[b]	Colony color	Relative β-Galactosidase activity[c]
TNF-R55 (aa 206–345)	–	−	White	<1
–	TNF-R55 (aa 206–345)	−	White	<1
TNF-R55 (aa 206–345)	FAN (aa 703–917)	+	Blue	27.3
TNF-R55 (aa 206–345)	FAN (aa 3–917)	+	Blue	n.d.
TNF-R55 (aa 206–426)	FAN (aa 703–917)	+	Blue	24.7
TNF-R55 (aa 206–426)	FAN (aa 3–917)	+	Blue	n.d.
TNF-R55 (Δ 308–340)	FAN (aa 703–917)	−	White	<1
TNF-R55 (Δ 308–340)	FAN (aa 3–917)	−	White	n.d.
SYK	FAN (aa 3–917)	−	White	n.d.
FAS (aa 191–335)	FAN (aa 3–917)	−	White	n.d.
TNF-R75 (aa 288–461)	FAN (aa 3–917)	−	White	n.d.
IL1-R (aa 342–557)	FAN (aa 3–917)	−	White	n.d.
FAN (aa 703–917)	–	(+)/−	Light blue	4.0
FAN (aa 703–917)	TNF-R55 (aa 206–345)	+	Blue	36.1

[a] Yeast cells were sequentially transformed with expression vectors encoding various lexA DNA-binding domain and activation domain fusion proteins.
[b] Each double transformant was plated on Ura⁻His⁻Trp⁻Leu⁻ galactose plates and on Ura⁻His⁻Trp⁻ galactose plates containing X-Gal.
[c] At least 100 colonies each were combined, grown in liquid culture, and β-galactosidase activity was measured in triplicate as described in Experimental Procedures.

activation pathway of N-SMase, we transiently cotransfected COS-1 cells with pEF.CD4, containing only extracellular and transmembrane portions of CD4 to avoid any functional interference, together with expression constructs containing cDNAs either encoding the complete FAN protein, an N-terminal deletion mutant FAN (703–917), or a C-terminal deletion mutant FAN(1–547). Transfected cells were enriched by a magnetic column after staining with α-CD4 magnetic beads. Cells expressing the C-terminal 215 amino acids of FAN (amino acids 703–917), containing WD-repeats 2–5 (see Figure 3B) exhibited a dominant negative effect on N-SMase activation by TNF (Figure 6A). In contrast, cells expressing the complete FAN protein showed enhanced N-SMase activation. The C-terminal deletion mutant FAN(1–547) did not influence the TNF–induced N-SMase activation. The basal level of N-SMase activity without TNF treatment remained unchanged in all transfected cells, and the expression of the different FAN proteins did not lead to a different kinetics of TNF–induced N-SMase activation (Figure 6A). Ceramide levels in TNF–stimulated cells transfected with FAN(703–917) or complete FAN corresponded to the changes of N-SMase activities (Figure 6B). Measurements of ceramide steady-state levels, however, may not precisely reflect N-SMase activities, because ceramide can be rapidly metabolized to ceramide-1-phosphate, sphingosine, or gangliosides. In addition, ceramide can be produced by other SMases like A-SMase or by ceramide synthase. The effects of TNF on enzymes involved in ceramide metabolism are not known. As a minimal approach, cells have been pretreated with D609, which has been shown to prevent ceramide production by a TNF–responsive A-SMase activity (Schütze et al., 1992).

To investigate the specificity of FAN action, enzymatic activities of A-SMase and N-SMase were measured in

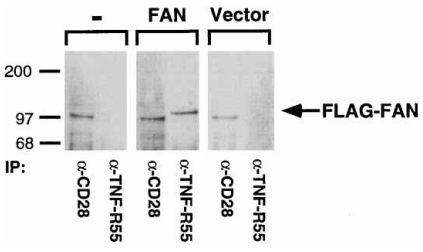

Figure 4. Association of TNF-R55 and FAN in Intact Cells

COS-1 cells (1×10^7) were left untransfected or were transiently transfected with 20 μg of pFLAG.CMV2 or pFLAG.FAN(3–917). After 48 hr, cells were lysed and cellular lysates were immunoprecipitated with the α–TNF-R55 antibody htr-9 or an isotype-matched control antibody (α-CD28). Western blots were performed with the α–FLAG-antibody MAb5. The coprecipitating FLAG–FAN fusion protein is indicated by an arrow. The positions of molecular mass markers (in kDa) are indicated on the left.

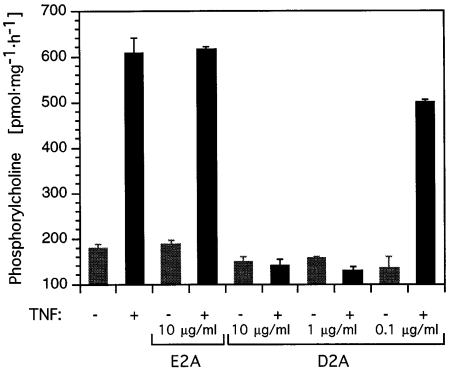

Figure 5. The Peptide Containing the FAN Binding Motif Specifically Suppresses N-SMase Activation

Membrane fractions of Jurkat cells were obtained by mild lysis (see Experimental Procedures) and left untreated or incubated for 30 min, with the indicated amounts of peptide D2A containing the FAN binding motif or with a death domain–derived control peptide (E2A). After stimulation for 0 or 1.5 min with TNF, N-SMase activity was measured at pH 7.4. TNF–induced N-SMase activities are expressed in production of phosphorylcholine [pmol × mg^{-1} × h^{-1}]. Shown are values of one representative experiment (n = 3); the bars indicate the respective standard deviations from triplicate determinations.

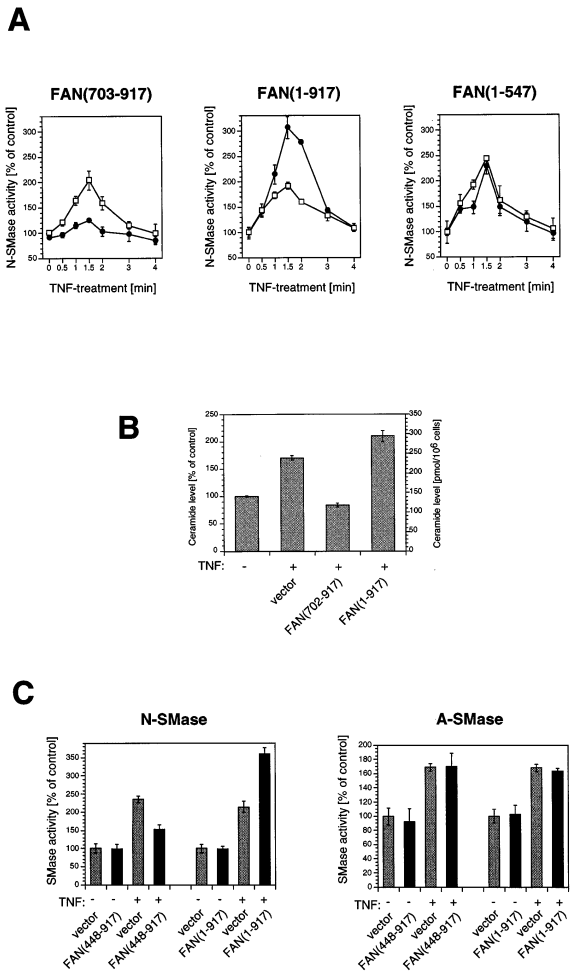

Figure 6. FAN Mediates N-SMase Activation

(A) COS-1 cells (1×10^7) were transiently cotransfected with 20 μg of pEF.CD4 and with either 20 μg of the control vector (pEF.ATG, open square) or 20 μg of the indicated FAN expression constructs (closed circle). After 24 hr, transfected cells were enriched by α-CD4 staining followed by magnetic purification. After an additional 24 hr, cells were treated in triplicate with 100 ng/ml of TNF for the indicated times, and N-SMase activity was determined. TNF–induced N-SMase activities are expressed as percent of untreated vector-transfected cells. Basal levels of phosphorylcholine production were 234–242 pmol $\times$ $mg^{-1} \times h^{-1}$. The results are representative for three independent experiments.

(B) COS-1 cells (2×10^7) were transiently cotransfected with 20 μg of pEF.CD4 together with 20 μg of pEF.ATG or with 20 μg of the indicated FAN expression constructs. After 24 hr, transfected cells were enriched by α-CD4 staining followed by magnetic purification. After an additional 24 hr, cells were treated in triplicate with 100 ng/ml of TNF for 1.5 min, and ceramide steady-state levels were determined. The results are representative for three independent experiments.

(C) COS-1 cells (1×10^7) were transiently transfected with 20 μg of pEF.ATG or with the indicated FAN expression constructs. After 48 hr, cells were treated in triplicate with 100 ng/ml of TNF for 1.5 min for N-SMase assays or 3 min for A-SMase assays, and N-SMase activity at pH 7.4 or A-SMase activity at pH 5.0 was determined. TNF–induced SMase activities are expressed as percent of untreated vector-transfected cells. Basal levels of phosphorylcholine production were [$mg^{-1} \times h^{-1}$]: 133–194 pmol for N-SMase and 582–670 pmol for A-SMase. The results are representative for three independent experiments, respectively.

parallel in COS-1 cells transiently transfected with FAN or a truncated version of FAN. Neither an N-terminal deletion mutant FAN(448–917) nor the entire FAN protein had any effect on A-SMase activation after TNF treatment (Figure 6C). Similar to the results shown in Figure 6A, expression of FAN(448–917) exhibited a dominant negative effect on TNF–induced N-SMase activation, while complete FAN enhanced TNF–induced N-SMase activation (Figure 6C). FAN-induced changes of N-SMase activities were less pronounced when compared with Figure 6A, because transfectants were not enriched by CD4.

Taken together, our results clearly show that the interaction of FAN with the NSD is responsible for mediating TNF–induced N-SMase activation, while the A-SMase pathway remains unaffected.

Discussion

During the last few years, the intracellular events by which TNF-R55 transmits signals through the cell membrane into cytoplasm and nucleus after ligand binding have begun to be elucidated. There are two major pathways that have been shown to involve phospholipases: the A-SMase pathway and the N-SMase pathway, both leading to different cellular responses (Wiegmann et al., 1994). The structural motifs within the cytoplasmic domain of TNF-R55 initiating these signals have been identified: a C-terminal domain signals the A-SMase pathway (Wiegmann et al., 1994), which seems to be identical to the death domain–signaling apoptosis (Brakebusch et al., 1992; Tartaglia et al., 1993b). Another functional domain, the N-terminal NSD, is responsible for the N-SMase pathway (Adam et al., 1996). However, direct links between TNF-R55 and N-SMase, which could provide hints on the activation mechanisms, have not been described yet.

Here we describe a novel protein, FAN, that is shown to interact with human TNF-R55. By use of the yeast interaction trap system, we demonstrate that binding of FAN to TNF-R55 is highly specific and does require the presence of the NSD. The specific binding of FAN to NSD–containing peptides suggests that, in contrast to proteins binding to the death domain, the tertiary structure of TNF-R55 does not seem to play an important role for interaction with FAN. The binding motif for the association between FAN and TNF-R55 was mapped to amino acids 310–318, which corresponds perfectly to the functionally mapped NSD (amino acids 309–319; Adam et al., 1996). The interaction of FAN and TNF-R55 was confirmed in intact cells by coimmunoprecipitation of transfected FLAG-tagged full-length FAN with the endogenous TNF-R55 of COS-1 cells. A synthetic NSD–derived peptide competes for binding of a signal-transducing protein or proteins and thereby completely inhibits its N-SMase activation. This underscores the essential role of the NSD and the NSD–binding protein FAN for the initiation of the N-SMase pathway. Moreover, overexpression of full-length FAN enhances N-SMase activation in a TNF–dependent manner, while N-terminal truncations of FAN that retain only the TNF-R55 binding WD-repeats are dominant-negative inhibitors of

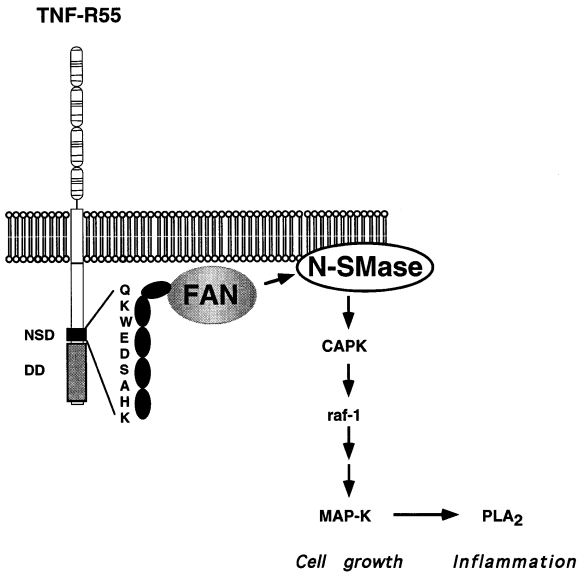

Figure 7. TNF-R55 Initiates the N-SMase Pathway via Interaction between the NSD and FAN

NSD, N-SMase activation domain. DD, death domain. The amino acid sequence of the FAN binding motif is indicated.

N-SMase activation. Interestingly, a C-terminal deletion mutant of FAN lacking the WD-repeats required for interaction with TNF-R55 was unable to influence the TNF–induced N-SMase activation when overexpressed in COS-1 cells. This suggests that the function of FAN may require binding to TNF-R55, which is mediated by the C-terminal WD-repeats of FAN. A possible explanation for the TNF–dependence of FAN function could be that binding of FAN to TNF-R55 requires oligomerization of the receptor after ligand binding. The interaction of FAN with the aggregated TNF-R55 might then induce a conformational change in FAN that is not achieved solely by overexpressing FAN or by binding of FAN to the competing peptide. FAN action is apparently strictly specific for N-SMase, since none of the FAN constructs showed any effect on the activation of A-SMase. Together, our data indicate that FAN represents an important functional intermediate between the TNF-R55 and the activation of N-SMase (Figure 7).

It should be emphasized that FAN is clearly distinct from any other TNF-R55–associated protein so far described. Characterizations of TRADD (Hsu et al., 1995) and, in turn, TRADD–associated proteins (Hsu et al., 1996a; 1996b), have provided a possible link of the death domain with the activation of PC-PLC, A-SMase, induction of NF-κB, and apoptosis. Despite the identification of proteins binding to TNF-R55 outside the death domain, none of these proteins can provide a possible link between the NSD and activation of N-SMase. TRAP1 and TRAP2, proteins that possess sequence homology to the 90 kDa family of heat-shock proteins, display binding properties not consistent with binding to the functionally defined NSD (Song et al., 1995). The binding site of TNF-R55 for a protein with homology to the yeast equivalent of the p112 subunit of the 26S proteasome (55.11) has been mapped to amino acids 243–308, upstream of the NSD (Boldin et al., 1995).

Structure of FAN

Sequence analysis of the predicted open reading frame encoding FAN revealed its structural homology to the family of WD-repeat proteins (for review, see Neer et al., 1994). These proteins have in common the presence of at least four WD-repeats. All so far described WD-repeat proteins appear to serve regulatory functions in various cellular processes. Notably, none of them contains an intrinsic enzymatic function. Many WD-repeat proteins are involved in signal transduction, such as the β-chains of heterotrimeric G proteins (Watson et al., 1994), RACK1 (Ron et al., 1994), the PLA_2-activator protein (PLAP; Clark et al., 1991), the regulatory subunit of phosphatase 2A (Mayer et al., 1991), or the recently described protein associated with the type II TGF-β–receptor (Chen et al., 1995). Some of them consist only of WD-repeats (for example, RACK1; Ron et al., 1994), others contain N- or C-terminal extensions of various lengths (for example, PLAP; Clark et al., 1991). The WD-repeat structure seems to be a functional motif that may facilitate defined protein–protein interactions, sometimes leading to multiprotein complexes, as shown for the β-subunit of heterotrimeric G proteins (Neer et al., 1994). The additional amino acid sequences may contribute to the regulatory function. This has been shown for the PLA_2-activator protein, PLAP, in which a short region in the C-terminal extension activates PLA_2 (Clark et al., 1991). This modular structure might also apply to FAN. The region of FAN required for association with TNF-R55 has been mapped to the C-terminal 214 amino acids, since this portion of FAN is sufficient to interact with TNF-R55 in the yeast interaction trap system. Within this region, four out of five WD-repeats are located, suggesting that the interaction with TNF-R55 is mediated by the WD-repeats. The expression of a truncated FAN molecule, containing only the WD-repeats (FAN[448–917]), acts in a dominant-negative manner on TNF–induced N-SMase activation, suggesting that it lacks an effector domain in the N-terminus. However, TNF-R55 binding seems to be required for the function of FAN, since expression of the C-terminal FAN deletion mutant containing amino acids 1–547 by itself had no effect on either the basal level of the N-SMase activity or on the TNF–induced N-SMase activation. The sequence of the putative FAN effector domain spanning the 627 amino acids N-terminal of the WD-repeats does not provide any evidence for potential enzymatic activities of FAN. The N-terminal extension of FAN shares a sequence homology of amino acids 304–575 (53.8% sequence identity) with another human WD-repeat protein of unknown function (Feuchter et al., 1992), which suggests the existence of a family of FAN-related proteins.

Biological Implications of FAN-Associated Nonapoptotic Signals

While a more detailed picture is beginning to emerge for the signals leading to the A-SMase/apoptotic pathway by identifying the death domain and its major interaction partners (Tartaglia et al., 1993b; Hsu et al., 1995, 1996a, 1996b), the N-SMase pathway has been investigated less intensively. We have recently suggested a

sequential activation of N-SMase, proline-directed protein kinases, and PLA_2 as an independent signaling cascade (Wiegmann et al., 1994). This N-SMase pathway involves the activation of various important signaling systems. Activation of membrane-bound N-SMase itself is a very early step in TNF-R55 signaling, leading to production of the second messenger molecule ceramide (Dressler et al., 1992; Wiegmann et al., 1994). Ceramide production by N-SMase has been shown to lead to a sequential activation of a ceramide-activated kinase, ceramide-activated protein kinase (Liu et al., 1994), raf-1 kinase (Belka et al., 1995; Yao et al., 1995), and MAP kinases (Vietor et al., 1993). The MAP kinase cascade represents an essential pathway of mitogenic signaling in many cell types. The activation of MAP kinases by raf-1 could thus explain the growth-stimulatory function of TNF observed in select cell types (Vietor et al., 1993). One important proinflammatory enzyme downstream of MAP kinase is PLA_2. MAP kinase activates PLA_2 by phosphorylation (Lin et al., 1993), which leads to production of arachidonic acid and finally to the secretion of prostaglandine E_2 and leukotrienes, important mediators of the inflammatory response (for review, see Heller and Krönke, 1994). Similar observations have been recently reported for the signaling through the Fas antigen, another member of the TNF receptor superfamily. Analogous to the functional dichotomy of TNF-R55, Fas initiates an A-SMase pathway, which was linked to the cytotoxic signal of Fas. Like TNF-R55, Fas independently signals for an N-SMase pathway, leading to activation of the MAP kinase ERK2 and of PLA_2 (Cifone et al., 1995). Although the Fas antigen does not contain the FAN-binding motif and does not interact with FAN (Table 1), it harbors a region immediately upstream of its death domain (S. A.-K. et al., unpublished data), which displays minimal homology to the NSD of TNF-R55 and which might serve as binding motif for a FAN-related protein.

We have previously shown that the signaling cascades initiated by the death domain do not overlap with the N-SMase pathway (Wiegmann et al., 1994). Indeed, FAN does not seem to interfere with death-domain signaling. None of the FAN expression constructs had any effect on TNF–induced CAT gene transcription directed from four HIV–related κB sites (data not shown). Neither expression of FAN nor expression of FAN deletion mutants changed the cytotoxic action of TNF (data not shown).

Taken together, our results indicate that FAN is a novel WD-repeat protein that is distinct from death domain–associated proteins and any other TNF-R55–binding protein so far described. Instead, FAN appears to be crucially involved in mediating TNF–induced activation of the N-SMase pathway, which in turn regulates important mitogenic and proinflammatory responses.

Experimental Procedures

Cell Culture and Biological Reagents

The human leukemic T cell line Jurkat and COS-1 cells were originally obtained from the American Type Culture Collection. All cell lines were grown in Click's RPMI culture medium (Biochrom) supplemented with 10% fetal calf serum, 10 mM glutamine, 0.1 mM β-mercaptoethanol, and 50 μg/ml each of streptomycin and penicillin. Highly purified recombinant human TNF (3×10^7 U/mg) was provided by G. Adolf (Boehringer Research Institute). The α–TNF-R55 monoclonal antibody htr-9 (Brockhaus et al., 1990) was a gift from W. Lesslauer and H. Loetscher, and the α-CD28 monoclonal antibody was purchased from Dianova. The monoclonal α–FLAG-antibody (MAb5) was purchased from Kodak International Biotechnologies. Peptides were synthesized using an automated synthesizer (Abimed) according to the standard Fmoc machine protocols. After cleavage of the protection groups, the products were purified by High Pressure Liquid Chromatography to greater than 95%.

Plasmid Construction

The LexA DNA-binding domain fusions were constructed in the vector plex202 (a gift from R. Brent). The cytoplasmic domains of the human TNF-R55 and the D345 and D308–340 deletion mutants were cloned by polymerase chain reaction into plex202. The cDNAs of human TNF-R75, human Fas antigen, and mouse Interleukin-1 receptor were provided by A. Himmler (Boehringer Research Institute), M. Peter, and W. Falk, respectively. The corresponding cytoplasmic domains were cloned by polymerase chain reaction into plex202. The plex.SYK construct was obtained by cloning the entire coding sequence for the protein tyrosine kinase SYK into plex202 restricted by MluI/NotI. The generation of the Jurkat cDNA library fused to a synthetic activation domain has been described elsewhere (Stanger et al., 1995). The plex.FAN constructs were generated by cloning FAN cDNAs containing amino acids 3–917 and 703–917, respectively, into plex202. For in vitro transcription or translation, the cDNA inserts isolated from clones derived from the two-hybrid screen were subcloned into pBluescript.KS^+ (Stratagene). For the in vivo interaction assay, the FAN cDNA containing amino acids 3–917 was subcloned into pFLAG.CMV2 (Kodak International Biotechnologies). For eukaryotic expression studies, the vector pEF.ATG was constructed by inserting an oligonucleotide containing a start codon in-frame with an EcoRI site followed by a NotI site into pEF.BOS (Mizushima and Nagata, 1990), cut with EcoRI and blunt-ended with Klenow polymerase. Complete or truncated FAN cDNAs were subcloned from phagemid clones isolated by hybridization screening of cDNA libraries into pEF.ATG. The pEF.CD4 expression construct was generated by amplifying the extracellular and transmembrane domains of the human T cell surface molecule CD4 by polymerase chain reaction and cloning into pEF.BOS.

Yeast Interaction Trap System

Transformation of the yeast strain EG48/JK103 (Gyuris et al., 1993) with bait constructs and, subsequently, with the library DNA or selected activation domain fusion constructs, was performed as described (Stanger et al., 1995). Transformants were grown on Ura^- His^-Trp^- glucose plates, before selection for leucine prototrophy on $Ura^-His^-Trp^-Leu^-$ galactose plates was used to test for positive interaction. Testing for β-galactosidase expression was performed either on $Ura^-His^-Trp^-$ galactose X-Gal plates or, for quantitation, in a liquid assay. Yeast cells were grown overnight at 30°C in Ura^- His^-Trp^- galactose medium to an OD_{600} of about 1.8, diluted 1:5 in the same medium and grown at 30°C to an OD_{600} of 1.5 to 1.8. Cells were washed twice in phosphate-buffered saline (PBS), lysed in PBS by freezing/thawing, and diluted 1:8 with PBS containing 0.36% β-mercaptoethanol. The reaction was started by adding 160 μl of PBS containing 2.8 mg/ml of o-nitrophenyl-β-D-galactoside and stopped after incubation for 90 min at 37°C with 400 μl of 1M NA_2CO_3. After precipitating the cells, the absorbance at 429 nm of the supernatants was determined. β-galactosidase units were calculated by the equation: units = $OD_{420} \times 1000/$(time [min.] × vol. [min.] × O D_{600}).

Protein Binding to a TNF-R55–Derived Cellulose-Bound Peptide Scanning Library

The TNF-R55–derived peptide scanning libraries were automatically prepared by spot synthesis (Frank, 1992) using a spot synthesizer (Abimed) and the software DIGEN (Jerini Bio Tools), as described in detail elsewhere (Kramer et al., 1994; Reineke et al., 1996). We prepared two different scans covering the entire cytoplasmic domain of TNF-R55: one library containing 15 mer (12 amino acids

overlapping) and a second library consisting of 13 mer (11 amino acids overlapping). To generate radiolabeled whole-cell extracts from Jurkat cells, 3×10^7 cells were incubated with 2.5 mCi in vitro cell labeling mix (Amersham; more than 1000 Ci/mmol of L-[^{35}S]-methionine and L-[^{35}S]-cysteine) in methionine/cysteine-free medium for 4 hr, washed twice in PBS, and lysed in HDP (30 mM HEPES [pH 7.9], 10% glycerol, 7 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1% NP40, 10 μg/ml of aprotinin and leupeptin) by freezing/thawing. The cell lysate was incubated on peptide filters, prewashed twice in methanol and twice in PBS in 1 × blocking reagent (Cambridge Research Biochemicals) and in HDP overnight at 4°C with continuous shaking. After five washes at room temperature in NET (150 mM NaCl, 50 mM Tris [pH 7.5], 5 mM EDTA, 0.05% NP40), filters were air-dried and autoradiographed. Radiolabeled proteins encoded by the clones derived from the interaction trap screening were generated using the TNT T7-coupled Reticulocyte Lysate System (Promega) and ^{35}S-methionine (Amersham). Screening of the peptide libraries with the in vitro translated proteins was performed as described above.

cDNA Cloning

The 1 kb cDNA insert of clone FAN(73.31), originally isolated by the two-hybrid screen, was used as a probe to screen a human testis and a human skeletal muscle cDNA library (both from Stratagene) by standard methods (Sambrook et al., 1989). The cDNA inserts were in vivo excised according to the instructions of the manufacturer and sequenced using an automated sequencer. All clones isolated shared the same 3′-end with a putative polyadenylation site. cDNA clones isolated from the testis and the muscle library were shown to terminate at their 5′-ends at almost the identical nucleotide.

In Vivo Interaction Assay

For the in vivo interaction assay, 1×10^7 COS-1 cells were transiently transfected with 20 μg of pFLAG.CMV2 or pFLAG.FAN by electroporation at 280 V, 960 μF, in 0.4 mm cuvettes. After 48 hr, cells were detached using 2 mM EDTA, lysed in TNB (20 mM Tris [pH 8.0], 140 mM NaCl, 0.5% Brij58), and 1.5 mg of cellular lysates were precleared with γ–bind-sepharose (Pharmacia). Immunoprecipitation with identical amounts of different antibodies was performed overnight on ice, followed by collection of the immunecomplexes by a 1 hr incubation with γ–bind-sepharose. After five washes in TNB, immunoprecipitated proteins were separated on 8% polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes. Western blots were performed using 10 μg/ml of α–FLAG-antibody MAb5, a peroxidase-coupled rabbit–α-mouse antiserum (Dianova), and the ECL immunodetection system (Amersham).

Assays for Neutral and Acidic SMase

COS-1 cells were transfected with various FAN expression constructs or with identical amounts of the expression vector, either alone or in combination with the pEF.CD4 expression construct. To enrich for CD4-expressing cells, 24 hr after transfection cells were detached with 2 mM EDTA, stained with α-CD4 Magnetic Microbeads (Miltenyi Biotech), and purified using MiniMACS separation columns (Miltenyi Biotech), according to the protocol supplied by the manufacturer. After an additional 24 hr, cells were harvested for SMase assays. Cells without α-CD4 enrichment were also harvested 48 hr after transfection. Activation of neutral and acidic SMase after TNF treatment was measured as recently described (Wiegmann et al., 1994), with a minor modification of the N-SMase lysis buffer containing 0.5% CHAPS instead of Triton X-100.

For N-SMase assays in membrane fractions after preincubation with peptides, serum-starved Jurkat cells were lysed in N-SMase lysis buffer for 10 min at 4°C, homogenized by repeatedly squeezing through an 18 gauge needle, and centrifuged at 2000 rpm for 10 min to obtain nuclei-free supernatants containing the cytosolic and membrane fractions. Protein concentration was determined, and triplicates of 25 μg of protein per reaction were incubated with or without peptide for 30 min at 4°C. The reactions were shifted to 37°C, followed by TNF stimulation for 1.5 min. Thereafter, cellular lysates were assayed for N-SMase activity as described above.

Ceramide Quantitation

COS-1 cells were cotransfected with pEF.CD4 and various FAN expression constructs and enriched for CD4-expressing cells after 24 hr. After a further 24 hr, cells were treated with 50 μg/ml of D609 for 30 min to prevent the activation of A-SMase (Schütze et al., 1992). Thereafter, cells were stimulated with TNF. Ceramide was quantitated by the diacylglycerol kinase assay as described by Dressler and Kolesnick (1990). Cells were extracted with chloroform : methanol : 1N HCl (100 : 100 : 1, v/v/v), and lipids were dried under N_2. The extracts were incubated with 0.1 N methanolic KOH for 1 hr at 37°C to remove glycerophospholipids. After reextraction, the organic phase was dried under N_2. Ceramide was measured using the sn-1,2-diacylglycerol (DAG) assay reagents system (Amersham), following the protocol provided by the manufacturer. Ceramide 1-phosphate levels were determined by two-dimensional laser densitometry of autoradiographs. Ceramide levels were quantified by comparison with a standard curve of ceramide 1-phosphate generated by subjecting 30–500 pmol of ceramide (ceramide type III, Sigma) to the DAG kinase reaction.

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References

Adam, D., Wiegmann, K., Adam-Klages, S., Ruff, A., and Krönke, M. (1996). A novel cytoplasmic domain of the p55 TNF receptor initiates the neutral sphingomyelinase pathway. J. Biol. Chem. *271*, 14617–14622.

Barbara, J.A.J., Smith, W.B., Gamble, J.R., Vanostade, X., Vandenabeele, P.; Tavernier, J., Fiers, W., Vadas, M.A., and Lopez, A.F. (1994). Dissociation of TNF cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor–selective TNF mutants. EMBO J. *13*, 843–850.

Bazzoni, F., and Beutler, B. (1995). How do tumor necrosis factor receptors work? J. Inflammation *45*, 221–238.

Belka, K., Wiegmann, K., Adam, D., Holland, R., Neuloh, M., Herrmann, F., Krönke, M., and Brach, M.A. (1995). Tumor necrosis factor (TNF) induces c-raf-1 Kinase via the p55 TNF receptor engaging neutral sphingomyelinase. EMBO J. *14*, 1156–1165.

Beutler, B., and Cerami, A. (1986). Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature *320*, 584–588.

Boldin, M.P., Mett, I.L., and Wallach, D. (1995). A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its "death domain." FEBS Lett. *367*, 39–44.

Brakebusch, C., Nophar, Y., Kemper, O., Engelmann, H., and Wallach, D. (1992). Cytoplasmic truncation of the p55 tumour necrosis factor (TNF) receptor abolishes signaling but not induced shedding of the receptor. EMBO J. *11*, 943–950.

Brockhaus, M., Schoenfeld, H.J., Schlaeger, E.J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990.) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc. Natl. Acad. Sci. USA *87*, 3127–3131.

Chen, R.-H., Miettinen, P.J., Maruoka, E.M., Choy, L., and Derynck, R. (1995). A WD-domain protein that is associated with and phosphorylated by the type II TGF-β receptor. Nature *377*, 548–552.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain–containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Cifone, M.G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G., and Testi, R. (1995). Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J. *14*, 5859–5868.

Clark, M.A., Özgür, L.E., Conway, T.M., Dispoto, J., Crooke, S.T., and Bomalaski, J.S. (1991). Cloning of a phospholipase A2–activating protein. Proc. Natl. Acad. Sci. USA *88*, 5418–5422.

Dressler, K.A., and Kolesnick, R.N. (1990). Ceramide 1-phosphate, a novel phospholipid in human leukemia (HL-60) cells. J. Biol. Chem. *265*, 14917–14921.

Dressler, K.A., Mathias, S., and Kolesnick, R.N. (1992). Tumor necrosis factor–α activates the sphingomyelin signal transduction pathway in a cell-free system. Science *255*, 1715–1718.

Espevik, T., Brockhaus, M., Loetscher, H., Nonstad, U., and Shalaby, R. (1990). Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. J. Exp. Med. *171*, 415–426.

Feuchter, A.E., Freeman, J.D., and Mager, D.L. (1992). Strategy for detecting cellular transcripts promoted by human endogenous long terminal repeats: identification of a novel gene (CDC4L) with homology to yeast CDC4. Genomics *13*, 1237–46.

Fiers, W. (1991). Tumor necrosis factor: characterization at the molecular, cellular and in vivo level. FEBS Lett. *285*, 199–212.

Frank, R. (1992). Spot synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron *48*, 9217–9232.

Gehr, F., Gentz, R., Brockhaus, M., Loetscher, H., and Lesslauer, W. (1992). Both tumor necrosis factor receptor types mediate proliferative signals in human mononuclear cell activation. J. Immunol. *149*, 911–917.

Goeddel, D.V., Aggarwal, B.B., Gray, P.W., Leung, D.W., Nedwin, G.E., Palladino, M.A., Patton, J.S., Pennica, D., Shepard, H.M., Sugarman, B.J., and Wong, G.H.W. (1986). Tumor necrosis factors: gene structure and biological activities. Cold Spring Harbor Symp. Quant. Biol. *51* Pt. 1, 597–609.

Grell, M., Zimmermann, G., Hulser, D., Pfizenmaier, K., and Scheurich, P. (1994). TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. J. Immunol. *153*, 1963–1972.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell *75*, 791–803.

Heller, R.A., and Krönke, M. (1994). Tumor necrosis factor receptor–mediated signaling pathways. J. Cell Biol. *126*, 5–9.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor-1–associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495–504.

Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D.V. (1996a). TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor-1 signal transduction pathways. Cell *84*, 299–308.

Hsu, H., Huang, J., Shu, H.-B., Baichwal, V., and Goeddel, D.V. (1996b). TNF–dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity *4*, 387–396.

Kim, M.Y., Linardic, C., Obeid, L., and Hannun, Y. (1991). Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α- and γ-interferon: specific role in cell differentiation. J. Biol. Chem. *266*, 484–489.

Kolesnick, R., and Golde, D.W. (1994). The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell *77*, 325–328.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucl. Acids Res. *15*, 8125.

Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R., Landgraf, C., and Schneider-Mergener, J. (1994). Combinatorial cellulose-bound peptide libraries: screening tool for the identification of peptides that bind ligands with predefined specificity. Methods (Comp. Meth. Enzymol.) *6*, 388–395.

Krönke, M., Schütze, S., Scheurich, P., and Pfizenmaier, K. (1992). TNF signal transduction and TNF–responsive genes. Immunol. Ser. *56*, 189–216.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T.A., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-jun kinases. Nature *369*, 156–160.

Lewis, M., Tartaglia, L.A., Lee, A., Bennett, G.L., Rice, G.C., Wong, G.H., Chen, E.Y., and Goeddel, D.V. (1991). Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species-specific. Proc. Natl. Acad. Sci. USA *88*, 2830–2834.

Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., and Davis, R.J. (1993). $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell *72*, 269–278.

Liu, J., Mathias, S., Yang, Z.H., and Kolesnick, R.N. (1994). Renaturation and tumor necrosis factor–α stimulation of a 97 kDa ceramide-activated protein kinase. J. Biol. Chem. *269*, 3047–3052.

Loetscher, H., Pan, Y.C., Lahm, H.W., Gentz, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. (1990). Molecular cloning and expression of the human 55 kDa tumor necrosis factor receptor. Cell *61*, 351–359.

Machleidt, T., Wiegmann, K., Henkel, T., Schütze, S., Baeuerle, P., and Krönke, M. (1994). Sphingomyelinase activates proteolytic I-κ B-α degradation in a cell-free system. J. Biol. Chem. *269*, 13760–13765.

Mayer, R.E., Hendrix, P., Cron, P., Matthies, R., Stone, S.R., Goris, J., Merlevede, W., Hofsteenge, J., and Hemmings, B.A. (1991). Structure of the 55 kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal-specific isoform. Biochemistry *30*, 3589–3597.

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. Nucl. Acids Res. *18*, 5322.

Murakawa, K., Matsubara, K., Fukushima, A., Yoshii, J., and Okubo, K. (1994). Chromosomal assignments of 3′-directed partial cDNA sequences representing novel genes expressed in granulocytoid cells. Genomics *123*, 379–389.

Neer, E.J., Schmidt, C.J., Nambudripad, R., and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature *371*, 297–300.

Pushkareva, M., Obeid, L.M., and Hannun, Y.A. (1995). Ceramide: an endogenous regulator of apoptosis and growth suppression. Immunol. Today *16*, 294–297.

Reddy, S.A., Chaturvedi, M.M., Darnay, B.G., Chan, H., Higuchi, M., and Aggarwal, B.B. (1994). Reconstitution of nuclear factor κB requires membrane-associated components: comparison with pathway activated by ceramide. J. Biol. Chem. *269*, 25369–25372.

Reineke, U., Sabat, R., Kramer, A., Stigler, R.-D., Seifert, M., Michel, T., Volk, D., Schneider-Mergener, J. (1996). Mapping protein–protein interactions using hybritope and peptide scanning libraries. Mol. Diversity, in press.

Ron, D., Chen, C.-H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994). Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. Proc. Natl. Acad. Sci. USA *91*, 839–843.

Rothe, J., Gehr, G., Loetscher, H., and Lesslauer, W. (1992). Tumor necrosis factor receptors: structure and function. Immunol. Res. *11*, 81–90.

Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell *78*, 681–692.

Sambrook, J., Frisch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schall, T.J., Lewis, M., Koller, K.J., Lee, A., Rice, G.C., Wong, G.H., Gatanaga, T., Granger, G.A., Lentz, R., Raab, H., Kohr, W.J., and Goeddel, D.V. (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell *61*, 361–370.

Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992). TNF activates NF-κB by phosphatidylcholine-specific phospholipase-C–induced acidic sphingomyelin breakdown. Cell *71*, 765–776.

Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959–962.

Song, H.Y., Dunbar, J.D., Zhang, Y.X., Guo, D., and Donner, D.B. (1995). Identification of a protein with homology to hsp90 that binds the type-1 tumor necrosis factor receptor. J. Biol. Chem. *270*, 3574–3581.

Stanger, B.Z., Leder, P., Lee, T.H., Kim, E., and Seed, B. (1995). RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell *81*, 513–523.

Tartaglia, L.A., and Goeddel, D.V. (1992). Two TNF receptors. Immunol. Today *13*, 151–153.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A., Jr., and Goeddel, D.V. (1991). The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc. Natl. Acad. Sci. USA *88*, 9292–9296.

Tartaglia, L.A., Rothe, M., Hu, Y.F., and Goeddel, D.V. (1993a). Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. Cell *73*, 213–216.

Tartaglia, L.A., Ayres, T.M., Wong, G.H.W., and Goeddel, D.V. (1993b). A novel domain within the 55 kDa TNF receptor signals cell death. Cell *74*, 845–853.

Vietor, I., Schwenger, P., Li, W., Schlessinger, J., and Vilcek, J. (1993). Tumor necrosis factor–induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. J. Biol. Chem. *268*, 18994–18999.

Watson, A.J., Katz, A., and Simon, M.I. (1994). A fifth member of the mammalian G protein β-subunit family. J. Biol. Chem. *269*, 22150–22156.

Weihergräber, O., Schneider-Mergener, J., Grötzinger, J., Wollmer, A., Küster, A., Exner, M., and Heinrich, P.C. (1996). Use of immobilized synthetic peptides for the identification of contact sites between human interleukin-6 and its receptor. FEBS Lett. *379*, 122–126.

Wiegmann, K., Schütze, S., Kampen, E., Himmler, A., Machleidt, T., and Krönke, M. (1992). Human 55 kDa receptor for tumor necrosis factor coupled to signal transduction cascades. J. Biol. Chem. *267*, 17997–18001.

Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994). Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell *78*, 1005–1015.

Yao, B., Zhang, Y.H., Delikat, S., Mathias, S., Basu, S., and Kolesnick, R. (1995). Phosphorylation of Raf by ceramide-activated protein kinase. Nature *378*, 307–310.

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