

Activation of p42/p44 mitogen-activated protein kinases (MAPK) and p38 MAPK by tumor necrosis factor (TNF) is mediated through the death domain of the 55-kDa TNF receptor

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Abstract In the mouse fibrosarcoma cell line L929sA, tumor necrosis factor (TNF) stimulates activation of the stress-responsive p38 mitogen-activated protein kinase (MAPK), as well as the classical p42 and p44 MAPK. TNF signaling can be mediated by p55 or p75 TNF receptors. Here, we demonstrate that TNF-R55 is sufficient to activate p42/p44 MAPK and p38 MAPK. Moreover, by expressing different membrane-bound or purely cytoplasmic truncations of TNF-R55, we show that the intracellular death domain of TNF-R55 is the crucial domain involved. The cytoplasmic membrane-proximal region of TNF-R55, known to induce neutral sphingomyelinase activation, is not required for activation of p38 MAPK or p42/p44 MAPK.

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Key words: Death domain; Mitogen-activated protein kinase; Receptor; Signaling; Tumor necrosis factor

1. Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine mediating activities as diverse as cell proliferation, cell differentiation and cell death. It is also an important mediator of inflammation and cellular immune responses, and is involved in the pathology of many diseases [1]. TNF effects are initiated by binding of the cytokine to specific cell surface receptors, namely p55 (TNF-R55) and p75 (TNF-R75), which are present on most cell types [2]. Although the receptors are deprived of any intrinsic kinase activity, TNF clearly activates several kinases, such as the classical protein kinases (PK)C α [3] and PKA [4], but also ceramide-activated PK (CAPK) [5], PKC ζ [6] and β -casein kinase [7]. Another family of TNF-stimulated kinases is the MAPK family. The three MAPK subfamilies, namely c-jun N-terminal kinase (JNK), p38 MAPK and p42/p44 MAPK, become activated by phosphorylation on threonine and tyrosine residues by upstream dual-specificity kinases. The stress-responsive JNK and p38 MAPK are potently activated in response to cellular stress and inflammatory cytokines, such as TNF and interleukin (IL)-1, in almost every cell type [8–11]. p42/p44 MAPK are mainly activated by growth factors via a Ras-Raf-1-dependent pathway. However, this subdivision of MAPK-activating stimuli is certainly not absolute, since several reports clearly demonstrated the activation of p42/p44 MAPK by TNF in endothelial cells,

macrophages, primary fibroblasts, etc. [12–16]. Furthermore, in the murine fibrosarcoma cell line L929sA, we have previously shown that TNF also activates classical MAPK [17] and p38 MAPK [10]. Moreover, making use of specific MAPK inhibitors, we demonstrated that both classes of MAPK are involved in TNF-induced IL-6 and GM-CSF gene induction [10,18].

A major breakthrough in elucidating TNF signaling came from the identification of several cytosolic proteins that, directly or indirectly, associate with the intracellular domains of the TNF receptors [19]. Two distinct domains have been characterized for TNF-R55. The N-terminal, membrane-proximal region (MPR) plays an important role in signaling to the neutral (N) sphingomyelinase pathway [20], as well as in inducing nitric oxide synthase [21] and in the TNF-induced translocation of mitochondria [22]. N-Sphingomyelinase activation can be linked to MAPK activation, via ceramide-activated protein kinase (CAPK), which initiates the Ras-Raf-1-signaling cascade [23,24]. However, in the case of TNF-induced N-sphingomyelinase, subsequent activation of Raf-1 kinase activity is still controversial and probably cell type-specific [15,20,25]. No clear link between TNF-induced activation of Raf-1 and p42/p44 MAPK has been demonstrated so far.

The C-terminal, 80 amino acids long death domain (DD) of TNF-R55 mediates induction of cell death, activation of the transcription factor NF- κ B and IL-6 gene transcription [21,26]. Following receptor oligomerization, TNF-R55-DD interacts with a TNF-R55-associating DD protein (TRADD). In turn, TRADD recruits an apoptosis-inducing signaling complex FADD/FLICE and/or RIP, on the one hand, and an NF- κ B-activating complex TRAF2/NIK and/or RIP on the other hand [19]. Some of these conclusions were drawn from overexpression studies with dominant-negative TRAF2 mutants, capable of inhibiting NF- κ B-driven reporter gene constructs. However, experiments with TRAF2 knockout mice recently revealed that TNF can still induce NF- κ B translocation to the nucleus in the absence of TRAF2 [27,28]; this tempers the unique role of TRAF2 in TNF-induced NF- κ B release and subsequent transcriptional activity. Overexpression assays have also demonstrated that TRAF2 is necessary for activation of JNK induced by TNF-R55 [29] and TNF-R75 [11]. This phenomenon was indeed confirmed in cells derived from TRAF2 knockout mice. In addition, TNF-induced activation of p38 MAPK can be blocked by dominant-negative TRAF2, which also points to a possible role for TRAF2 in p38 MAPK activation [29,30]. However, for the activation of both p42/p44 MAPK and JNK by TNF another DD-containing protein was recently described,

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namely MAPK-activating DD (MADD) [31]. Clearly, the exact role of TRAF2 and/or MADD in activating these kinases is still not fully understood.

We here now demonstrate unambiguously, by stable expression of physiological amounts of human (h) TNF-R55, that hTNF-R55 alone is sufficient to activate both p42/p44 MAPK and p38 MAPK in L929sA cells. Various mutants revealed that these activations require a structurally active DD, and do not involve *N*-sphingomyelinase activations. This clearly demonstrates that TRAF2 and/or MADD, and not the *N*-sphingomyelinase-Ras-Raf-1 pathway, are concerned.

2. Materials and methods

2.1. Cell lines and cell culture

TNF-sensitive murine fibrosarcoma L929sA cells [32] and all stable cell clones derived thereof were cultured as previously described [26].

2.2. Cytokines and antisera

Purified *Escherichia coli*-derived murine TNF was produced in our laboratory and had a specific biological activity of 1.3×10^8 IU/mg as determined in a TNF cytotoxicity assay; it contained < 4 ng endotoxin/mg protein. Recombinant human interferon (IFN)- α (A/D), which is also active on mouse cells, was a generous gift from Dr. C. Weissmann (University of Zürich, Switzerland) and had a specific activity of 7.9×10^7 U/mg as determined on murine cells in an L929/vesicular stomatitis virus assay. htr1 is an agonistic mouse monoclonal IgM antibody directed against the extracellular domain of hTNF-R55 and was generously provided by Dr. M. Brockhaus (F. Hoffmann-La Roche, Basel, Switzerland [33]). R32WS86T is a hTNF-R55-specific mAb [34]. PD98059, a specific inhibitor of p42/p44 MAPK, was supplied as powder (Alexis Corporation, Nottingham, UK) and was dissolved in DMSO.

The phospho-specific p38 (Thr-180/Tyr-182) and p42/p44 (Thr-202/Tyr-204) MAPK polyclonal rabbit antibodies detect only the dual phosphorylated form of MAPK. Polyclonal rabbit IgG antibodies which detect total MAPK levels, independently of the phosphorylation state of the protein, were used as controls; they were purchased from New England Biolabs (Beverly, MA, USA) as part of a kit, which also includes anti-rabbit IgG coupled to horseradish peroxidase, used as second antibody for Western blotting.

2.3. Plasmids and generation of stable cell lines

Generation of stable cell lines from the parental L929sA cell line by transfection of different expression plasmids was previously described [22,26]. The Leu-351 $\rightarrow$ Ala mutation in hTNF-R55-DD was created with a Transformer Site-directed Mutagenesis kit (Clontech Laboratories, Palo Alto, CA, USA). The mutator oligonucleotide 5'-GGTCGCTCGCCCCCTAGGCGCCGAC-3' was used to introduce the L351A mutation and to remove the *Esp*I restriction site. Mutated sequences were screened by restriction digest analysis with *Esp*I. After sequencing, the cDNA of one correct clone was used to construct pMxCAT-R55i-L351A, an IFN- α -inducible eukaryotic expression vector for mutated CAT-R55i at position 351. The stable cell line L929sA-CAT-R55i-L351A was tested for CAT-R55i-L351A expression in a radioactive CAT assay [26] and for cytotoxicity after production of CAT-R55i-L351A protein.

2.4. p38 MAPK activation assay

All stable cell clones were seeded at 3.5×10^5 cells/well in 6-well plates. After 24 h, cells were triggered with 100 ng/ml htr1, 1000 IU/ml IFN- α or 2000 IU/ml TNF. At the end of the incubation period, cells were washed in PBS. Cell extracts were essentially prepared as described in the protocol of a PhosphoPlus p38 MAPK antibody kit (New England Biolabs). One fifth of the total cell lysate (20 μ l) was separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. Western blot analysis was performed to detect either phosphorylated or, as a control for equal loading, non-phosphorylated p38 MAPK.

2.5. p42/p44 MAPK activation assay

Stable cell clones were seeded at 5×10^5 cells/well in 6-well plates.

After 24 h, the growth medium was replaced with DMEM, supplemented with 0.1% newborn calf serum for 48 h to starve the cells. Subsequently, inductions were carried out in the starved medium. Western analysis was essentially carried out with a PhosphoPlus p42/p44 MAPK antibody kit (New England Biolabs), according to the manufacturer's instructions.

3. Results

3.1. TNF-R55 constructs and their characterization in L929sA cells

Fig. 1A gives an overview of the various receptor constructs used in the present study. hR55wt contains the full-length wild-type hTNF-R55 cDNA, driven by an SV40 early promoter. In the construct hR55 Δ MPR, the region corresponding to the membrane-proximal amino acids 203–304 has been removed, leaving mainly the C-terminal-coding part of hTNF-R55, including the DD, as the intracellular receptor cDNA. Several cell clones, stably transfected with these constructs, could be selectively killed, upon treatment with the hTNF-R55-specific monoclonal agonistic antibody htr1 (data not shown). From these, two independent clones, called L929sA-hR55wt and L929sA-hR55 Δ MPR, expressing full-length hTNF-R55 and the MPR-deleted mutant, respectively, were retained for further characterization of TNF-R55 and/or DD-mediated signaling events. Both cell lines were reported to express physiological relevant levels of hTNF receptors and to induce cell death upon triggering with htr1 [22].

We also constructed a fusion protein between chloramphenicol acetyltransferase (CAT) and the intracellular domain of the hTNF-R55 (CAT-R55i) [26]. CAT is a bacterial protein, which spontaneously trimerizes to give the biologically active form of the enzyme. By linking the cytoplasmic part of hTNF-R55 to the C-terminal end of CAT, placed under the control of the IFN- α -inducible Mx promoter [26], we can generate a cytoplasmic, structural equivalent of the activated trimerized intracellular part of hTNF-R55. Induced expression of this CAT-R55i fusion protein in L929sA cells was shown to elicit an efficient TNF response, i.e. cytotoxicity, NF- κ B activation and IL-6 induction [26]. The CAT-R55i-L351A construct, containing the Leu-351 $\rightarrow$ Ala mutation, i.e. the TNF-R55 equivalent of the *lpr* mutation in the Fas antigen, shown to abolish TNF-induced signaling to cytotoxicity, NF- κ B activation and gene expression [21], was also stably transfected to L929sA cells. Although expression of the protein could clearly be demonstrated upon triggering of the transfected cells with IFN- α (Fig. 1B), no cytotoxicity could be generated (data not shown). In the construct CAT-linkDD, only the coding portion of TNF-R55-DD was fused, over a flexible linker, to CAT cDNA. This construct is again fully active in signaling to cell killing, NF- κ B activation and IL-6 expression [26].

3.2. p38 MAPK activation is mediated by the TNF-R55 death domain

Activation of p38 MAPK by TNF, shown to be a very rapid and transient event, is achieved by dual phosphorylation of the protein on threonine and tyrosine. This phosphorylation can be demonstrated by the use of specific antibodies recognizing only the phosphorylated, activated form of the protein. All the constructions described above were used to analyse the involvement of TNF-R55 and/or its DD in activation of the p38 MAPK. hR55WT- or hR55 Δ MPR-express-

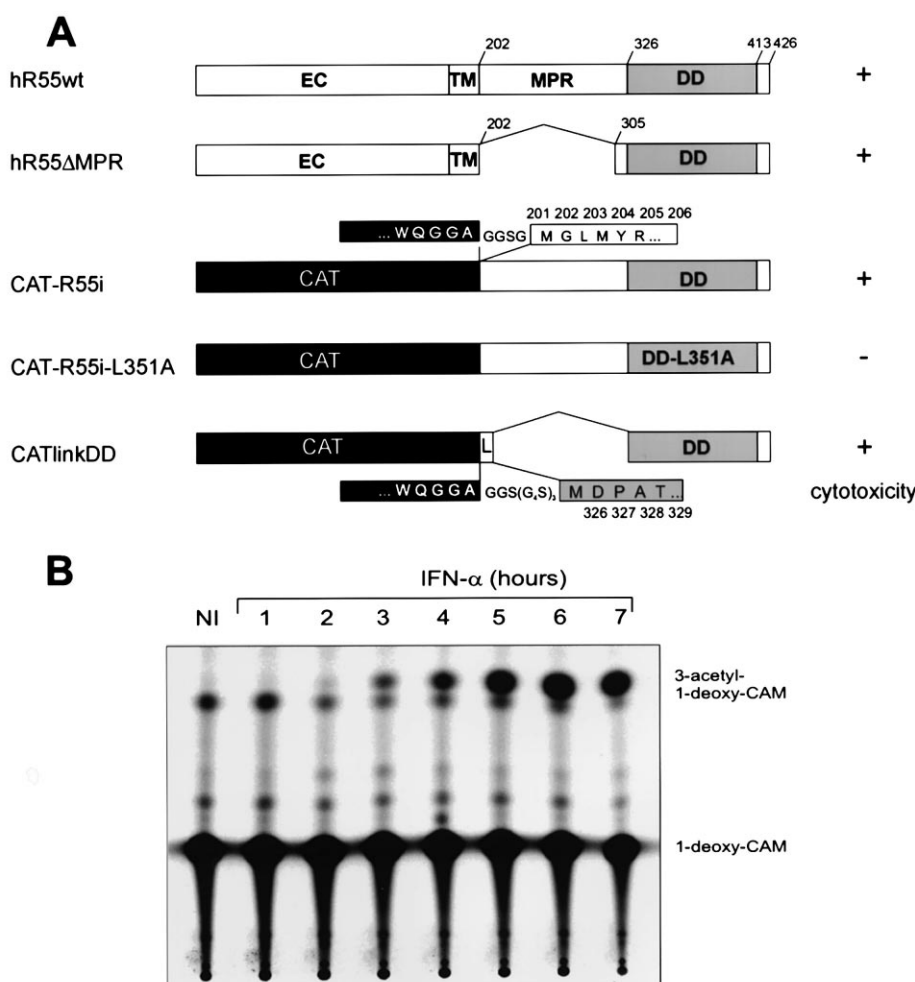


Fig. 1. A: Schematic representation of the different hTNF-R55 proteins. hR55wt: wild-type hTNF-R55; hR55ΔMPR: hTNF-R55 lacking the membrane proximal region; CAT-R55i: fusion protein between CAT and the intracellular domain of TNF-R55; CAT-R55i-L351A: CAT-R55i where Leu-351→Ala; CATlinkDD: fusion protein between CAT and TNF-R55-DD with a 15-amino acid linker (Gly₄Ser)₃ between the two polypeptides. EC: Extracellular domain; TM: transmembrane domain; MPR: membrane-proximal region; DD: death domain; CAT: chloramphenicol acetyltransferase. B: Expression of the CAT-R55i-L351A fusion protein in L929sA cells by inductions with IFN-α. L929sA-CAT-R55i-L351A cells were either not treated (NI) or treated with IFN-α (1000 IU/ml) for different time periods. Extracts were made and used in an enzymatic CAT assay [26].

ing cells were treated with TNF or htr1 for different time periods. Western blot analysis with phospho-specific p38 MAPK antibody demonstrates that phosphorylation of p38 MAPK can be observed already 5 min after treatment with TNF (Fig. 2A). Exclusive triggering of full-length hTNF-R55 with htr1 also resulted in the activation of p38 MAPK after 15 min of treatment (Fig. 2A). Clearly, triggering of the hTNF-R55 alone is sufficient to activate p38 MAPK. When the cells expressing hR55ΔMPR were incubated with htr1, activation of p38 MAPK could still be achieved after 15 min of triggering (Fig. 2A). Treatment of parental L929sA cells with htr1 did not result in activation of p38 MAPK (data not shown). From these results we can conclude that in L929sA cells hTNF-R55 activates p38 MAPK through its DD. Recently, it was shown that a novel region of 11 amino acids at positions 309–319 in hTNF-R55, called NLD (for *N*-sphingomyelinase-linked domain), is necessary and sufficient for activating *N*-sphingomyelinase [35]. As the deletion variant hR55ΔMPR still contains this region, it might be possible that this domain plays a role in the activation of p38 MAPK.

In CAT-R55i-expressing cells, p38 MAPK is activated after

3–3.5 h of IFN-α treatment, the time needed for the production of the fusion protein (Fig. 2B). However, with the CAT-R55i-L351A construct, which does contain an active NLD, but has a deficient DD, it is no longer possible to activate p38 MAPK (Fig. 2B). This proves that activation of p38 MAPK is solely mediated by a functional DD, without a contribution of the NLD. As a control, TNF treatment of the same cells is included, showing that the p38 MAPK pathway in these cells is intact. Together, our data clearly demonstrate that hTNF-R55 is sufficient to trigger p38 MAPK activation and that this activation is the result of clustering of an active form of the DD.

3.3. p42/p44 MAPK activation is mediated by the TNF-R55 death domain

Parallel studies were performed to assess the involvement of TNF-R55 in the activation of p42/p44 MAPK. Previously, it was demonstrated that TNF activates p42/p44 MAPK in L929sA cells [17]. However, the receptor type involved and the exact signaling pathways are unknown so far. Fig. 3 demonstrates that TNF triggering of hR55wt-expressing cells in-

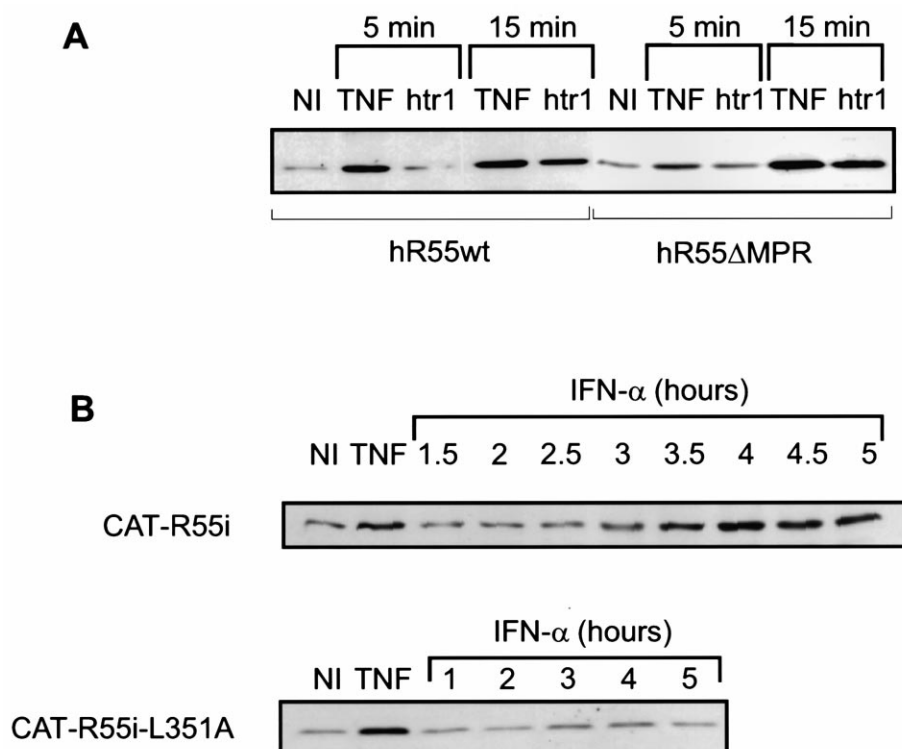


Fig. 2. p38 MAPK activation assay. Activation of p38 MAPK was assayed with a phospho-specific p38 (Thr-180/Tyr-182) MAPK antibody. Equal loading was confirmed by anti-p38 MAPK antibody (data not shown). A: L929sA-hR55wt and L929sA-hR55ΔMPR cells were either not treated (NI), or treated with TNF (2000 IU/ml) or htr1 (100 ng/ml) for 5 or 15 min. B: L929sA-CAT-R55i and L929sA-CAT-R55i-L351A cells were not treated (NI), treated with TNF (2000 IU/ml) as a control or treated with IFN-α (1000 IU/ml) for various times to induce expression of the CAT fusion products.

deed activates p42/p44 MAPK, as demonstrated via phospho-specific p42/p44 MAPK antibodies. This activation can be reproduced by treatment of the same cells with the hTNF-R55-specific TNF-mutien R32WS86T, showing the involvement of the TNF-R55 in MAPK activation. We could not longer make use of the agonistic antibody htr1 to trigger the human receptors, due to activation of p42/p44 MAPK by the htr1 solution in the parental mouse L929sA cells in the absence of human receptors (data not shown). Probably remaining serum proteins in the htr1 preparation are responsible for this activation. Treatment of hR55ΔMPR-expressing cells with TNF or R32WS86T also results in p42/p44 MAPK activation. In order to explore a possible involvement of the *N*-spinghomyelinase pathway, expression of CAT-R55i, CAT-R55i-L351A or CATlinkDD was induced by treatment of the relevant cells with IFN-α. In both CAT-R55i- and CAT-linkDD-expressing cell lines this treatment resulted in a clear activation of p42/p44 MAPK, which could be abolished by the specific inhibitor PD98059 (Fig. 4). However, the mutant coding for an inactive DD lacked any ability to activate MAPK. This demonstrates that, besides activation of the p38 MAPK activation, TNF-R55-DD can also stimulate this second class of MAPK, without the need for *N*-spinghomyelinase activation.

4. Discussion

MAPK are, among the recently discovered kinases, almost the most complex ones with multiple and diversified signaling functions. External cellular stresses, such as ultraviolet irradi-

ation, chemical heat, osmotic shock, inhibitors of protein synthesis and bacterial lipopolysaccharide, are all able to activate the stress p38 MAPK and JNK. In addition, the pro-inflam-

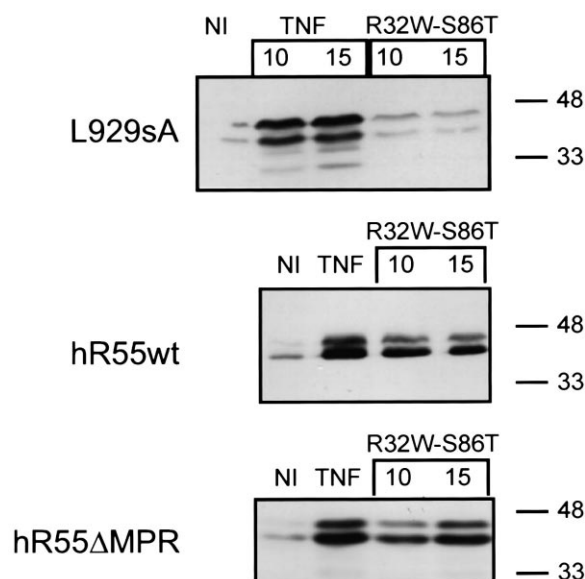


Fig. 3. p42/p44 MAPK activation assay. Parental L929sA cells, L929sA-hR55wt and L929sA-hR55ΔMPR cells were serum-starved as described, followed by treatment of the cells with TNF (2000 IU/ml) or R32WS86T (10 ng/ml) for 10 or 15 min. Cell lysates were made and activated p42/p44 MAPK was detected using a phospho-specific p42/p44 (Thr-202/Tyr-204) MAPK antibody. Equal loading was confirmed by anti-p38 MAPK antibody (data not shown).

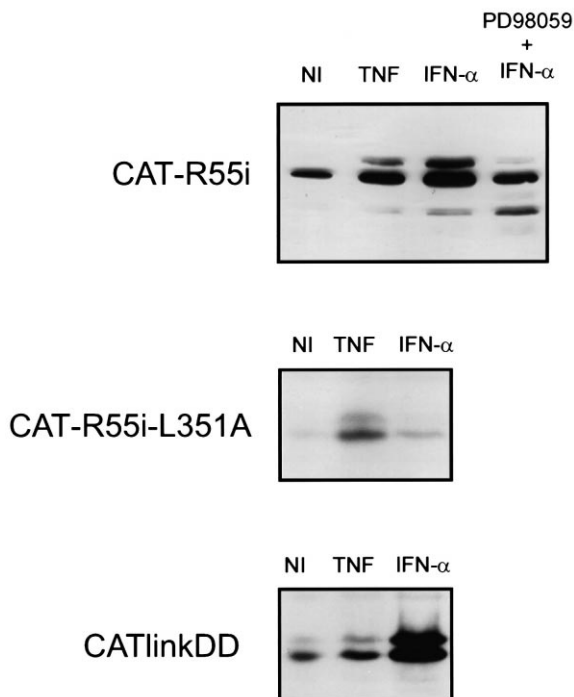


Fig. 4. p42/p44 MAPK activation assay in cells with the CAT fusion constructs. L929sA-CAT-R55i, L929sA-CAT-R55i-L351A and L929sA-CATlinkDD cells were serum-starved, followed by treatment of the cells with TNF (2000 IU/ml) for 10 min, IFN- α (1000 IU/ml) for 3.5 h or the combination of IFN- α and PD98059 (10 μ M, added 2 h before IFN- α). Cell lysates were made and activated, and phosphorylated p42/p44 MAPK were detected as well as total p42/p44 MAPK levels (data not shown).

matory cytokines IL-1 and TNF were shown to be strong inducers of these kinases. Furthermore, the so-called mitogen p42/p44 MAPK do also play a prominent role in the pleiotropic action of TNF and IL-1.

In this paper we show that TNF strongly activates p38 MAPK as well as p42/p44 MAPK through hTNF-R55. Moreover, we clearly demonstrate that their activation is triggered by clustering of a functional TNF-R55-DD.

Both the p55 and p75 TNF receptor have been shown to activate JNK via aggregation of the adaptor molecule TRAF2 [11,29]. Also the activation of p38 MAPK after TNF stimulation was reported to be mediated via TRAF2 [30]. However, in all of these experiments the involvement of TRAF2 in TNF signaling was demonstrated by overexpression of the dominant negative variant of TRAF2 which was able to block TNF-R55 and TNF-R75-mediated JNK and p38 MAPK activation. As mentioned in the introduction, only JNK activation, and not NF- κ B translocation, by TNF was indeed abrogated in cells lacking endogenous TRAF2 [27,28]. Therefore, we investigated the role of TNF-R55 and, more particularly, of its DD, in MAPK activation via a more physiologically relevant system, i.e. by stably expressing the hTNF-R55 or various recombinant mutants in the mouse fibrosarcoma cell line L929sA. In these cells TNF-R55 seems to be the sole mediator of p38 MAPK activation by TNF, as specific triggering of hTNF-R55 activates p38 MAPK as strongly as TNF itself. Moreover, specific triggering of human TNF-R75 in TNF-R75-transfected L929sA cells could not induce phosphorylation of p38 MAPK (E. Boone, unpub-

lished data). Apparently, the reported TNF-R75-mediated p38 MAPK activation is highly cell-specific [11]. As reported here, in hTNF-R55 the DD is clearly the crucial domain for p38 MAPK activation in L929sA cells. However, whether it activates p38 MAPK solely via TRAF2 in these cells needs further investigation. As a matter of fact, the lack of TNF-R75-mediated p38 MAPK activation in L929sA cells argues that TRAF2 might not be the most important mediator of TNF-induced p38 MAPK activation. One possible candidate for an alternative p38 MAPK signaling pathway is the TNF-R55-associated protein MADD [31], which has been shown to activate not only JNK and p42/p44 MAPK but also p38 MAPK (personal communication of Drs. A. Goldfeld and B. Brinkman, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA).

Growth factor-mediated activation of p42/p44 MAPK occurs by activation of the Ras-Raf-1 pathway. TNF signaling via Raf-1 is, however, still controversial in that in some systems TNF effects are Raf-1 dependent [36] and in others clearly Raf-1 independent [13,15,37]. Activation of Raf-1 by TNF is assumed to be generated through activation of *N*-sphingomyelinase and recruitment of Raf-1 by Ras to the membrane [20,36]. However, Müller and coworkers recently published that the observed TNF-induced Ras-Raf-1 binding does not lead to the subsequent activation of Raf-1 kinase [25]. Correspondingly, TNF-induced p42/p44 MAPK activity was shown to be Raf-1 independent in macrophages [15]. Moreover, the involvement of Raf-1 in some cell lines was only suggested by mimicking the TNF signaling pathway with exogenously added ceramide or sphingomyelinase-C [38]. In this respect we now communicate that the membrane-proximal region required for activation of Raf-1 is not involved in TNF-R55-mediated activation of p42/p44 MAPK and that the DD is necessary and sufficient. These data counteract the generally assumed idea that activation of p42/p44 MAPK by TNF would be mediated by Raf-1. Again, the recently cloned MADD protein could be the true mediator of TNF signaling to p42/p44 MAPK. Future experiments with MADD knockout mice will establish the exact involvement of MADD in p42/p44 and p38 MAPK activation.

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