Tumor necrosis factor α -induced activation of c-jun N-terminal kinase is mediated by TRAF2

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Tumor necrosis factor α (TNF α) a pro-inflammatory cytokine is an endogenous mediator of septic shock, inflammation, anti-viral responses and apoptotic cell death. TNF α elicits its complex biological responses through the individual or cooperative action of two TNF receptors of mol. wt 55 kDa (TNF-RI) and mol. wt 75 kDa (TNF-RII). To determine signaling events specific for TNF-RII we fused the extracellular domain of the mouse CD4 antigen to the intracellular domain of TNF-RII. Crosslinking of the chimeric receptor using anti-CD4 antibodies initiates exclusively TNF-RII-mediated signals. Our findings show that: (i) TNF-RII is able to activate two members of the MAP kinase family: extracellular regulated kinase (ERK) and c-jun N-terminal kinase (JNK); (ii) TRAF2, a molecule that binds TNF-RII and associates indirectly with TNF-RI, is sufficient to activate JNK upon over-expression; (iii) dominant-negative TRAF2 blocks TNF α -mediated JNK activation and (iv) TRAF2 signals the activation of JNK and NF- κ B through different pathways. Our findings suggest that TNF α -mediated JNK activation in fibroblasts is independent of the cell death pathway and that TRAF2 occupies a key role in TNF receptor signaling to JNK.

Keywords: JNK/NF- κ B/signal transduction/TNF receptor/TRAF2

Introduction

Tumor necrosis factor α (TNF α) induces a heterogeneous array of biological effects that are the result of complex signaling events initiated by two different transmembrane receptors of mol. wt 55 kDa (TNF-RI or p55) and mol. wt 75 kDa (TNF-RII or p75) respectively, which are expressed in most cell types (Bazzoni and Beutler, 1996). The trimeric ligand TNF α is able to bind to TNF-RI or TNF-RII thereby engaging three receptor chains per ligand. Although TNF α exhibits higher affinity binding to TNF-RII (K_d : 100 pM) than to TNF-RI (K_d : 500 pM) most of the biological responses of TNF α are thought to be mediated through TNF-RI. TNF-RII, which shows an inducible expression in most cell types, can modulate the sensitivity to TNF by its ability to capture and pass ligand to the lower affinity TNF-RI and also contributes to the cytotoxic or proliferative response to TNF (Banner *et al.*, 1993; Tartaglia *et al.*, 1993c; Vandenabeele, 1995).

Past research efforts have focused mainly on TNF-RI signaling which predominantly leads to cytotoxic and inflammatory responses (Tartaglia *et al.*, 1993a). Its intracellular domain shares a region of homology with other TNF receptor family members like CD95 (Fas/Apo-1), and is also competent to signal cell death (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993a). This conserved region, the so-called 'death domain', consists of an 80 amino acid (aa) motif found in other TNF receptor family members or signal transducers of the apoptotic pathway (Nagata and Suda, 1995; Wallach *et al.*, 1995). The recent cloning of TNF-RI associated molecules is starting to shed light on how the receptor contributes to the complex biological responses triggered by TNF α . The intracellular death domain of TNF-RI associates in response to TNF α crosslinking with the TNF Receptor Associated Death Domain protein (TRADD) (Hsu *et al.*, 1995). TRADD in turn serves as an adapter molecule for the Fas Associated Death Domain protein (FADD) and Receptor Interacting Protein (RIP) (Hsu *et al.*, 1996a,b), two molecules that also associate with the death domain of Fas (Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995). Both of these death domain containing proteins induce apoptosis when ectopically expressed and FADD may be a direct link to the apoptotic ICE protease cascade through its association with an ICE-like protease termed FLICE/MACH (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Signaling events downstream of FADD have also been linked to the generation of ceramide, a possible consequence of ICE-protease activation (Hannun, 1994; Chinnaiyan *et al.*, 1996; Pronk *et al.*, 1996). In addition, the N-terminus of TRADD associates with TRAF2, a molecule shown to be involved in NF- κ B activation (Rothe *et al.*, 1995b; Hsu *et al.*, 1996b). TNF-RI is also connected to the sphingomyelin pathway leading to the activation of neutral and acidic sphingomyelinase, which both convert sphingomyelin to ceramide (Hannun, 1994; Kolesnick, 1994; Schutze *et al.*, 1995). Neutral sphingomyelinase associates directly with a membrane proximal region of TNF-RI and most likely can activate the ERK signaling pathway through the ceramide activated kinase (CAP) (Yao *et al.*, 1995; Adam *et al.*, 1996). Acidic sphingomyelinase seems to be activated by a phosphatidylcholine dependent phospholipase C (PC-PLC) and linked to the activation of NF- κ B (Schutze *et al.*, 1995).

Much less is known about signaling events associated with TNF-RII. TNF-RII contains a unique intracellular domain and in addition to its cooperating function in TNF-mediated cytotoxicity it alone has been shown to trigger thymocyte proliferation or apoptosis in response to TNF α stimulation (Tartaglia *et al.*, 1993b,c). The TNF-RII cytoplasmic domain associates with the TRAF family members TRAF1 and TRAF2. TRAF2 binds directly to the TNF-RII cytoplasmic domain whereas TRAF1 associates indirectly through binding to TRAF2 (Rothe *et al.*, 1994).

In addition, TNF-RII associates indirectly with c-IAPs, the mammalian homologs of the apoptotic inhibitor protein encoded by baculovirus, which can bind to the TRAF1/TRAF2 heterodimer (Birnbaum *et al.*, 1994; Clem and Miller, 1994; Rothe *et al.*, 1995a). The main characteristic of the TRAF family is a common C-terminal TRAF domain that interacts with the intracellular 'non-death domain' regions of distinct members of the TNF receptor family (TNF-RII, CD40 and CD30) and other TRAF molecules (Cheng *et al.*, 1995; Rothe *et al.*, 1995b; Lee *et al.*, 1996). TRAF2 is characterized by the presence of a unique Zn binding motif (C3HC4) called a 'RING finger' that has been shown to mediate protein-DNA or protein-protein interactions (Cheng *et al.*, 1995; Saurin *et al.*, 1996). In the case of TRAF2 the N-terminal RING finger is essential for TRAF2-induced NF- κ B activation (Rothe *et al.*, 1995b). In contrast, no function could be assigned to TRAF1, which lacks a RING finger at the N-terminus.

To investigate TNF-RII-mediated signaling in more detail we constructed a chimeric receptor by replacing the extracellular domain (ECD) of human TNF-RII with the ECD of murine CD4. This approach enables an anti-CD4-induced crosslinking of TNF-RII without engaging TNF-RI. Our specific interest focused on the activation of protein kinase cascades that could be involved in proliferative as well as cytotoxic responses, both functions that can be triggered by TNF-RII. TNF α is a potent inducer of Jun N-terminal kinase (JNK/SAPK) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sluss *et al.*, 1994) and also stimulates the activation of the extracellular regulated kinase (ERK) (Davis, 1994; Belka *et al.*, 1995; Winston *et al.*, 1995). The JNK/SAPK signaling cascade is initiated by a variety of stress factors such as UV (Derijard *et al.*, 1994), heat shock (Adler *et al.*, 1995), cycloheximide (Cano *et al.*, 1994), ionizing radiation (Kharbanda *et al.*, 1995b; Chen *et al.*, 1996), osmotic stress and pro-inflammatory cytokines (Ringeaud *et al.*, 1995; Kyriakis and Avruch, 1996) and also through mitogenic stimuli like oncogenic ras and T cell activation (Su *et al.*, 1994). The upstream signaling events leading to JNK/SAPK activation are rather complex. They seem largely mediated through the GTP bound form of the small GTPases Rac and Cdc42 (Coso *et al.*, 1995; Minden *et al.*, 1995) which transmit the signal through the p21 activated protein kinase PAK (Bagrodia *et al.*, 1995; Brown *et al.*, 1996) and subsequently through the MEK kinases MEKK1, 2 and 3 (Minden *et al.*, 1994; Blank *et al.*, 1996) to MKK4/SEK/JNKK (Yan *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995) which acts as the direct activator of JNK/SAPK. In addition, other tyrosine kinases such as c-Abl and PYK2 or ser/thr kinases such as GC kinase (Kharbanda *et al.*, 1995a; Pombo *et al.*, 1995; Tokiwa *et al.*, 1996), activate the JNK/SAPK pathway upon overexpression. To date, the only connection between TNF receptor associated molecules and the JNK/SAPK cascade is the observation that generation of ceramide or exogenous ceramide is sufficient to activate the JNK/SAPK cascade in selected cell types (Westwick *et al.*, 1995; Verheij *et al.*, 1996).

By using a chimeric TNF-RII carrying a CD4 extracellular domain we demonstrate the ability of TNF-RII to activate both ERK and JNK and subsequently identified and characterized TRAF2 as an important mediator of TNF α -induced activation of the JNK/SAPK cascade.

Fig. 1. Chimeric CD4-TNF-RII: (A) Schematic representation of the CD4-TNF-RII. The mouse CD4 extracellular and transmembrane domain (aa 1-419) was fused to the intracellular domain of human TNF-RII (aa 288-462). The control construct CD4-TM consists only of the mCD4 extracellular and transmembrane domain (aa 1-419). (B) Western blot analysis of CD4-TNF-RII and CD4-TM expression. Anti-CD4 immunoprecipitates of stably transfected CHO cell lysates were separated by SDS-PAGE and immunoblotted using the same anti-mCD4 antibody. Expression of CD4-TM (lane 1) and CD4-TNF-RII (lane 2) were detected at 45 kDa and 85 kDa respectively. Cross-reactivity with the IgG heavy chain from the immune complex is indicated by an arrow. (C) FACS analysis of stable CHO cell lines. Staining of the CD4 extracellular domain with a phycoerythrin coupled anti-mCD4 confirmed the surface expression of CD4-TM (CHO-CD4-TM) and CD4-TNF-RII (CHO CD4-TNF-RII). No staining could be detected on CHO wild type cells (CHO).

Results

Stable expression of a CD4-TNF-RII chimera

Treatment of cells with TNF α results in a variety of different cellular responses mediated through TNF-RI and/or TNF-RII. Signaling through TNF-RII is of special interest as the p75 TNF receptor is capable of inducing cell death or proliferation dependent on the cell type (Vandenabeele, 1995). To identify signaling events associated exclusively with the p75/TNF-RII we constructed a chimeric receptor by fusing the ECD and transmembrane domain (TM) of the mouse CD4 receptor to the intracellular domain (ICD) of human TNF-RII (Figure 1A). Signaling through this chimera can be triggered by receptor crosslinking using anti-mCD4 antibodies as demonstrated for a chimeric CD4-Fas receptor (Chu *et al.*, 1995). The CD4-TNF-RII chimera and a CD4-TM control construct (Figure 1A) were stably transfected into Chinese hamster ovary cells (CHO) and single cell clones were selected. Expression of the receptors

Fig. 2. Activation of JNK and ERK following CD4-TNF-RII crosslinking. CHO cells stably expressing either the chimeric CD4-TNF-RII (A) or CD4-TM (B) were serum deprived for 24 h and left untreated (no treatment), treated with cycloheximide (20 μ g/ml) for 20 min (CHX), crosslinked with primary anti-CD4 antibody for 30 min (anti-CD4), crosslinked with secondary anti-IgG for 30 min (anti-IgG) or double crosslinked with anti-CD4 for 30 min then anti-IgG for 15 min (anti-CD4/IgG15') or 30 min (anti-CD4/IgG30'). The activity of endogenous JNK was determined by an *in vitro* kinase assay (insert) using GST-jun as substrate and quantitated on a molecular imager (Bio-Rad). (C) Serum deprived CHO cells stably expressing the CD4-TNF-RII were left untreated (no treatment), treated with 10% FCS for 10 min (FCS), crosslinked with primary anti-CD4 antibody for 30 min (anti-CD4), crosslinked with secondary anti-IgG for 30 min (anti-IgG) or double crosslinked with anti-CD4, 30 min then anti-IgG for 5 min (anti-CD4/IgG5'), 15 min (anti-CD4/IgG15') or 30 min (anti-CD4/IgG30'). The activity of endogenous ERK was determined by an *in vitro* kinase assay (insert) using MBP as substrate and molecular imager quantitated results shown graphically as fold activation of ERK. (D) Same treatment as described above on CHO cells stably expressing the CD4-TM control construct.

was verified by immunoprecipitation using a polyclonal antiserum raised against the ECD of mCD4 and visualization after immunoblotting using the same antibody (Figure 1B). Surface expression levels of CD4-TNF-RII and CD4-TM were found to be comparable as determined by FACS analysis using a phycoerythrin labeled anti-mCD4 monoclonal (Figure 1C). Attempts to select stable CD4-TNF-RII cell lines were unsuccessful since the chimera exhibited constitutive cytotoxic activity reminiscent of a previously described epo-TNF-RII chimera (Bazzoni *et al.*, 1995).

CD4-TNF-RII-mediated signaling to JNK1 and ERK2

The p75 TNF-RII has been shown to be a potent activator of the transcription factor NF- κ B, however, little is known about its ability to signal activation of specific protein kinases (Rothe *et al.*, 1995b). TNF α treatment of cells can initiate signaling through two important protein kinase

cascades leading to activation of the JNK/SAPK and ERK, which join together to induce AP-1 activity (Karin, 1995). To determine whether CD4-TNF-RII is capable of mimicking the activation of JNK1 and ERK2 we induced receptor dimerization through a primary rat anti-mouse CD4 antibody followed by the induction of larger aggregates using a secondary goat anti-rat IgG. A prominent induction of JNK1 (Figure 2A) and ERK2 (Figure 2C) activity can be observed following double crosslinking (anti-CD4/IgG). Maximum JNK activation was 4.5-fold at 15 min after addition of the secondary antibody consistent with the kinetics of TNF α -induced JNK activation (Rangaud *et al.*, 1995). Maximal ERK2 activation (9-fold) was reached after 5 min. However, little or no activation of JNK1 (Figure 2A) and ERK2 (Figure 2C) was observed following secondary antibody alone (anti-IgG) or dimerization with anti-mouse CD4 (anti-CD4) indicating that receptor dimerization alone is not sufficient to induce receptor signaling. The activation

Fig. 3. Overexpression of TRAF2 induces JNK activity. (A) CHO cells (lanes 1–3) or COS-7 cells (lanes 4–9) were transiently transfected with empty vector (lanes 1 and 4), HA-tagged TRAF2 (lane 2), HA- Δ TRAF2 (lanes 3 and 6), HA-TRAF1 (lane 7), HA-FADD (lane 8) and HA-RIP (lane 9). The expression levels of HA-tagged proteins were determined by anti-HA immunoblot (anti-HA). The activity of cotransfected Glu-JNK1 was measured following an *in vitro* kinase assay (GST c-jun). The amount of JNK1 in each immune complex was controlled by anti-JNK immunoblot (anti-JNK). The amount of 32 P-labeled GST-jun was quantitated on a molecular imager and is represented graphically as the mean and standard deviation of at least three independent experiments. (B) TRAF2 does not trigger an autocrine activation loop. COS-7 cells were transfected with Glu-JNK1 followed by 24 h expression and subsequently serum-starved for 24 h. Cells were either left untreated (lane 1) or incubated for 15 min with the conditioned medium of COS-7 cells expressing Glu-JNK1 (lane 3), Δ TRAF2 (lane 4) or wild type TRAF2 (lane 5). Subsequently the activity of Glu-JNK1 determined in an *in vitro* kinase assay. As a comparison, the extent of TRAF2-mediated JNK1 activation (lane 2) is shown.

of JNK1 and ERK2 can be considered to be specific for the TNF-RII intracellular domain as no activation of either JNK1 (Figure 2B) or ERK2 (Figure 2D) was observed following crosslinking of the CD4-TM cell line lacking the TNF-RII intracellular domain. Double crosslinking for 24 h in the presence or absence of cycloheximide did not result in the induction of apoptosis (data not shown). These data demonstrate that the CD4-TNF-RII chimera is able to initiate signaling events in response to antibody crosslinking and that the intracellular domain of TNF-RII can activate the JNK signaling pathway. Furthermore, activation of the ERK signaling pathway, which is believed to be mainly mediated by TNF-RI can also be triggered by TNF-RII in CHO cells (Belka *et al.*, 1995; Winston *et al.*, 1995).

Overexpression of TRAF2 induces JNK1 activation

The stress activated protein kinase subfamily of MAP kinases, JNK/SAPK and p38 (Raingeaud *et al.*, 1995), have attracted recent interest as their activation might be a promoting factor for apoptotic cell death but can also be involved in proliferative responses (Su *et al.*, 1994; Verheij *et al.*, 1996). In order to understand which of the TNF-RII associated molecules are responsible for the JNK activation in response to CD4-TNF-RII crosslinking we tried to identify molecules shared between TNF-RII and other mem-

bers of the TNF receptor family known to activate JNK. TNF-RII associates with the TRAF family members TRAF1 and TRAF2 (Rothe *et al.*, 1994). The CD40 receptor associates with TRAF2 and TRAF3 (Hu *et al.*, 1994; Cheng *et al.*, 1995; Rothe *et al.*, 1995b; Sato *et al.*, 1995) and receptor crosslinking by its ligand CD40L induces activation of JNK (Sakata *et al.*, 1995; Berberich *et al.*, 1996) and NF- κ B (Rothe *et al.*, 1995b). The involvement of TRAF2 in both of these pathways identifies it as a possible activator of the JNK signal cascade. To test whether TRAF2 is the molecule responsible for TNF receptor-mediated activation of JNK we cotransfected CHO or COS-7 cells with a plasmid expressing a Glu-epitope tagged JNK1 and either wild type TRAF2 or Δ TRAF2 which lacks the N-terminal RING finger motif and is unable to activate NF- κ B (Rothe *et al.*, 1995b). The extent of JNK1 activation was determined by immunoprecipitation of JNK1 followed by an *in vitro* kinase assay using GST c-jun(1–79) as substrate. Transfection of TRAF2 alone was sufficient to induce an 8.3-fold increase in JNK activity in CHO and an 8-fold activation in COS-7 cells (Figure 3A). However, Δ TRAF2 was unable to elevate JNK activity in either cell type (Figure 3A). In addition, low level expression of TRAF2 resulted in a 4.3-fold induction of JNK activity that could be further increased after a 15 min stimulation with TNF α to a final 8-fold induction of

JNK activity (Figure 4A). This TRAF2 dependent potentiation of TNF α -induced JNK activation further supports a central role for TRAF2 as a signaling molecule that links TNF receptor crosslinking to the activation of the JNK/SAPK cascade. In contrast, overexpression of either TRAF2 or Δ TRAF2 did not result in any activation of ERK2 (Figure 4C). Transfection of the second TNF-RII associated molecule, TRAF1, which forms heterodimers with TRAF2 (Rothe *et al.*, 1994), resulted in no significant increase in the activity of cotransfected JNK1 (Figure 3A, lane 7). Overexpression of FADD, a cell death inducing protein which binds to the death domain of TRADD, failed to increase the activity of cotransfected JNK (Figure 3A, lane 8). Consistent with this result we also find that dominant-negative FADD, a potent inhibitor of TNF and Fas-mediated cell death (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996b) does not interfere with TNF-mediated JNK activation (data not shown). Cotransfection of JNK1 and RIP, a protein that binds to TRAF2 and the TRADD death domain, showed only little stimulation of JNK activity (Figure 3A, lane 9), although it has been shown to induce NF- κ B activity upon overexpression (Hsu *et al.*, 1996a). Our data show that TRAF2 is the first TNF receptor associated molecule described to independently activate the JNK/SAPK pathway and that the early TNF α -induced activation of JNK appears to be distinct from signals leading to cell death.

TRAF2 has been shown to induce the activation of the transcription factor NF- κ B (Rothe *et al.*, 1995b) which could lead to the induction of an autocrine loop through the secretion of NF- κ B-induced factors that in turn could activate JNK (McCarthy *et al.*, 1995; Vercammen *et al.*, 1995). To address this possibility we used the conditioned medium of cells expressing JNK, Δ TRAF2 or TRAF2 to induce JNK activation in serum deprived COS-7 cells. None of the conditioned media (CM) was able to induce JNK activity above background (Figure 3B) making it unlikely that the JNK activation observed following overexpression of TRAF2 is the result of autocrine stimulation.

Δ TRAF2 blocks TNF α -induced activation of JNK

Overexpression of Δ TRAF2 has been shown to exert a dominant-negative effect on TNF α -induced activation of NF- κ B (Hsu *et al.*, 1996b). In order to test whether Δ TRAF2 is also able to interfere with TNF α -induced JNK activation we overexpressed Δ TRAF2 in COS-7 cells and observed a >50% reduction in TNF α -induced JNK1 activity, indicating that Δ TRAF2 exerts a partial dominant-negative effect on TNF α -mediated JNK activity in COS cells (Figure 4A). To further substantiate this finding we performed a similar experiment in HeLa cells, which are highly responsive to TNF α . JNK1 activation was assessed in the presence or absence of TNF α stimulation after cotransfection with either empty vector or increasing concentrations of Δ TRAF2 (Figure 4B). TNF α -treatment of vector transfected cells for 15 min resulted in a 22-fold induction of JNK1 activity compared with untreated cells (Figure 4B; lanes 1 and 2). However, transfection of either 0.5 μ g or 2 μ g Δ TRAF2 led to a marked decrease of TNF stimulated JNK activity. Low amounts of Δ TRAF2 expression already reduced the maximal TNF α -induced JNK activation by 37% to 13.9-fold compared with 22-fold in vector transfected cells. Furthermore, higher amounts of Δ TRAF2

resulted in a nearly complete suppression of TNF α -stimulated JNK1 activity.

The requirement for large amounts of Δ TRAF2 to elicit its dominant-negative effect has also been observed for the inhibition of TNF α -mediated activation of NF- κ B (Hsu *et al.*, 1996b). This might indicate that TRAF2 is bound in a stable complex with other cytoplasmic proteins and that high concentrations or long expression times of Δ TRAF2 are needed to compete with wild type TRAF2 (Hsu *et al.*, 1996b; Rothe *et al.*, 1996). Transfection of Δ TRAF2 without TNF α treatment caused only a negligible increase in JNK1 activity. These findings demonstrate clearly that TRAF2 is essential for TNF α -mediated JNK activation in HeLa cells.

Overexpression of TRAF2 does not lead to activation of ERK

TNF α has been shown to trigger a weak stimulation of ERK2 in B cells and macrophages (Belka *et al.*, 1995; Winston *et al.*, 1995). ERK2 activation is also observed following crosslinking of the CD4-TNF-RII chimera (Figure 2C). To determine whether TRAF2 or Δ TRAF2 can also modulate the TNF α -induced activation of ERK, we cotransfected COS-7 cells with HA-tagged ERK2 and empty vector, wild type TRAF2 or Δ TRAF2 (both Glu-tagged) and subsequently monitored the activation of ERK2 in the presence or absence of TNF α by an *in vitro* kinase assay using myelin basic protein (MBP) as substrate (Figure 4C). Overexpression of TRAF2 or Δ TRAF2 did not lead to an activation of cotransfected ERK2, indicating that the ERK activation observed following CD4-TNF-RII crosslinking is most likely mediated by a molecule different from TRAF2. Similar results have been obtained in CHO cells (data not shown). These results imply that TRAF2 is both necessary and sufficient for TNF α -induced JNK activation but is not involved in the activation of ERK2.

TRAF2 activates NF- κ B and JNK through different pathways

The results presented above suggest that the RING finger of TRAF2 is essential for the efficient activation of JNK/SAPK. Interestingly, the TRAF2-mediated activation of NF- κ B is also dependent on the RING finger (Rothe *et al.*, 1995b) raising the possibility that both pathways are activated by a common mechanism or are functionally interconnected. Furthermore, overexpression of MEKK, an upstream activator of JNK, has recently been shown to elevate NF- κ B dependent promoter activity of cotransfected reporter plasmids (Meyer *et al.*, 1996). To address the question whether TRAF2-induced JNK activity is necessary for TRAF2-mediated NF- κ B activation we overexpressed a catalytically inactive mutant of the JNK activating kinase MKK4 (MKK4-KR) to disrupt the activation of JNK by cotransfected TRAF2. High level expression of TRAF2 together with a JNK1 reporter plasmid resulted in a 17-fold induction of JNK activity as measured by an *in vitro* kinase assay (Figure 5A). Cotransfection of wild type MKK4 and TRAF2 results in a dramatic increase in JNK activity (43-fold) compared with expression of TRAF2 alone, indicating that endogenous MKK4 could be limiting in TRAF2-induced JNK activation. MKK4 alone causes only a minor increase in JNK activity (data not shown). In contrast, cotransfection of TRAF2 with a catalytically inactive MKK4 (MKK4-KR) leads to a nearly complete suppression

Fig. 4. TRAF2 modulates TNF α -mediated JNK activation. **(A)** COS-7 cells were co-transfected with a Glu-JNK1 reporter plasmid and empty vector (lanes 1 and 2), HA-TRAF2 (lanes 3 and 4) or HA- Δ TRAF2 (lanes 5 and 6). Serum deprived cells were left untreated (lanes 1, 3 and 5) or stimulated with TNF α (10 ng/ml) for 15 min (lanes 2, 4 and 6) and JNK1 activity determined by an *in vitro* kinase assay (GST c-jun). 32 P-labeled GST-jun was quantified on a molecular imager, corrected for the amount of JNK in the immune complex (α -JNK) and represented as fold JNK activation. Expression levels of HA-tagged proteins were determined by anti-HA immunoblot (anti-HA). **(B)** Δ TRAF2 blocks TNF α -induced JNK activation. HeLa cells were transfected with empty vector (lanes 1 and 2) or Glu- Δ TRAF2 at 2 μ g (lanes 3 and 5) or 0.5 μ g (lane 4). Serum deprived cells were left untreated (lanes 1 and 3) or stimulated with TNF α (10 ng/ml) for 15 min (lanes 2, 4 and 5) and the activity of cotransfected HA-JNK1 determined in an *in vitro* assay. The amount of JNK1 in the assay was determined by α -JNK immunoblot (α -JNK). The quantitated GST-jun phosphorylation is the mean of at least two experiments and is represented as fold JNK activation. **(C)** TRAF2 does not modulate TNF α -stimulated ERK activation. COS-7 cells were transfected with empty vector (lanes 1 and 2), Glu-TRAF2 (lanes 3 and 4) and Glu- Δ TRAF2 (lanes 5 and 6). The activity of cotransfected HA-ERK was determined in untreated cells (lanes 1, 3 and 5) or after a 15 min TNF α treatment (10 ng/ml) (lanes 2, 4 and 6) by an *in vitro* kinase assay using MBP as substrate. Expression of Glu-tagged proteins was verified by α -Glu immunoblot (α -Glu). The amount of ERK in the immune complex was determined by anti-ERK immunoblot (anti-ERK). MBP phosphorylation was quantitated on a Bio-Rad phosphoimager and is represented as relative ERK activation.

Fig. 5. Activation of JNK and NF- κ B are not interconnected. (A) MKK4-KR inhibits TRAF2 mediated JNK activation. COS-7 cells were transfected with empty vector (lane 1), 1 μ g (lane 2) or 2 μ g (lane 3) of HA-TRAF2, 2 μ g HA-TRAF2 plus Flag-MKK4 (lane 4) and 2 μ g HA-TRAF2 plus Flag-MKK4-KR (lane 5) and the activity of co-transfected Glu-JNK1 determined by an *in vitro* kinase assay (GST c-jun). JNK activity in the immune complex was corrected for the amount of JNK shown by the anti-JNK immunoblot (anti-JNK), and is represented graphically. (B) TRAF2 mediated NF- κ B activation is not affected by MKK4-KR. COS-7 cells were transfected with NF- κ B dependent luciferase reporter plasmid and an internal control plasmid carrying TK-Renilla luciferase together with empty vector (lane 1), HA-TRAF2 (lane 2), HA-TRAF2 plus Flag-MKK4 (lane 3), HA-TRAF2 plus Flag-MKK4-KR (lane 4), Flag-MKK4 alone (lane 5) and HA- Δ TRAF2 (lane 6). Expression levels were determined by immunoblot (anti-HA and anti-Flag). The internal Renilla luciferase standard was used to correct for variations in transfection efficiency. NF- κ B dependent luciferase activity is represented as fold induction of vector transfected cells (lane 1).

of TRAF2-mediated JNK activation (Figure 5A). However, coexpression of TRAF2 and MKK4 together with an NF- κ B dependent luciferase reporter plasmid (see Materials and methods) only resulted in a minor increase in NF- κ B activity compared with the 14-fold induction observed by expression of TRAF2 alone (Figure 5B). Furthermore coexpression of TRAF2 with the dominant-negative MKK4-KR mutant led only to a minor decrease (14-fold to 12-fold) in TRAF2-mediated NF- κ B induction. NF- κ B reporter activity was unaffected by expression of either wild type MKK4 or Δ TRAF2.

These results indicate that TRAF2 signals the activation of two independent pathways: one leading to JNK activation through MKK4, and a different, JNK independent, pathway leading to NF- κ B activation.

The TRAF2-mediated activation of NF- κ B and JNK is independent of TNF receptor association

The N-terminal RING finger domain of TRAF2 has so far been characterized as the major signaling module within TRAF2 (Cheng *et al.*, 1995; Rothe *et al.*, 1995b). However, little is known about the significance of the C-terminal TRAF domain in TRAF2-mediated signaling. The TRAF domain can be divided into a C-terminal TRAF C domain (aa 355–501) and a TRAF N domain (aa 264–355). TRAF C is responsible for the association of TRAF2 with the ICD of TNF-RII (Rothe *et al.*, 1995a) and TRADD (Hsu *et al.*, 1996b) whereas TRAF N, an α -helical structure, is mainly involved in the binding of c-IAPs and might also serve as

an interaction site for other TRAF family members (Cheng *et al.*, 1995; Rothe *et al.*, 1995a). To determine the contribution of these domains to the activation of JNK or NF- κ B, we constructed deletion mutants lacking either TRAF C (T358) or TRAF N and TRAF C (T264) (Figure 6A) and compared their ability to activate both JNK and NF- κ B pathways (Figure 6B and C respectively), with that of wild type and Δ TRAF2 in COS-7 cells. Surprisingly, deletion of most of the TRAF C domain (T358) resulted in a pronounced increase in JNK activation compared with wild type TRAF2. An average from six independent transfections established that T358 induces a 12-fold increase in JNK activity compared with an 8-fold induction for wild type TRAF2 (Figure 6B). This difference is even more pronounced considering the lower expression levels of T358. These results have been confirmed in CHO cells (data not shown). Removal of the TRAF C domain had a comparable effect on the extent of NF- κ B activation that nearly doubled compared with the TRAF2 wild type (Figure 6C). In contrast, removal of the TRAF C and TRAF N domain (T264) reduced the ability of TRAF2 to activate JNK almost to basal levels (Figure 6B). Removal of the TRAF N domain resulted in a >2 -fold reduction of NF- κ B activity compared with the TRAF2 wild type, but did not reach the basal level of Δ TRAF2 (Figure 6C) as observed for JNK activation (Figure 6B). These results show that TRAF2 signaling to JNK and NF- κ B does not require association with TNF-RII or TRADD and further indicates a strong requirement for the TRAF N domain.

Fig. 6. TRAF C and TRAF N modulate TRAF2 signaling. **(A)** Schematic representation of wild type and mutant TRAF2 constructs. Amino acid positions in the murine TRAF2 is indicated numerically above the construct. The functional domains of TRAF2 are marked. The RING finger is represented by a black rectangle, the zinc fingers are numbered 1–5 in a white rectangle, the α -helical TRAF N domain is dotted and the C-terminal TRAF C domain is shown hatched. **(B)** Activation of JNK by TRAF2 mutants. COS-7 cells were transfected with empty vector (lane 1), HA-TRAF2 (lane 2), HA- Δ TRAF2 (lane 3), HA-T264 (lane 4) and HA-T358 (lane 5), and the extent of cotransfected Glu-JNK1 activity determined in an immune complex assay (GST c-jun). Protein expression was verified by immunoblot (anti-HA). JNK activity is represented relative to vector transfected cells and is the mean and standard deviation of at least four independent transfections. **(C)** Activation of NF- κ B by TRAF2 mutants. An NF- κ B luciferase reporter and a TK-Renilla luciferase control plasmid were cotransfected with empty vector (lanes 1 and 5), HA-TRAF2 (lane 2), HA- Δ TRAF2 (lane 3) or HA-T264 (lane 4). A lower amount of TRAF2 (lane 6) was transfected separately to compensate for the low level expression of T358 (lane 7). NF- κ B activity was quantitated as described in Figure 5B.

Discussion

The results presented here show the ability of a chimeric CD4–TNF–RII to induce the activation of the MAP kinase family members JNK and ERK and the identification of TRAF2 as the TNF receptor I and II-associated molecule

that connects receptor crosslinking to the activation of the stress activated protein kinase cascade.

CD4–TNF–RII activates JNK and ERK

TNF α has a trimeric structure and induces receptor oligomerization rather than dimerization upon receptor binding

(Banner *et al.*, 1993; Bazzoni and Beutler, 1996). We find that simple dimerization using an anti-CD4 monoclonal antibody is not sufficient to trigger receptor signaling to either JNK or ERK with kinetics comparable to the natural ligand. Receptor signaling can only be efficiently initiated by multimerization of the receptors through additional crosslinking using an anti-IgG antibody. Although we observe the activation of JNK, we do not detect apoptotic cell death after TNF-RII crosslinking in the presence or absence of cycloheximide indicating that activation of TNF-RII-specific signaling pathways (i.e. JNK) is not sufficient to trigger apoptosis in fibroblasts. Activation of JNK has been reported to be the result of the generation of ceramide, a lipid second messenger and potent inducer of cell death in selected cell types (Jarvis *et al.*, 1994; Ji *et al.*, 1995; Verheij *et al.*, 1996). In addition, TNF α -induced apoptosis in U937 and BAE cells is dependent on JNK activity and heat shock or cis-platinum-induced apoptosis is reduced following inactivation of the JNK pathway (Verheij *et al.*, 1996; Zanke *et al.*, 1996). However, the consequences of JNK activation vary considerably among cell types. Crosslinking of the CD4–TNF-RII in CHO cells induces, in addition to the activation of JNK, the activation of ERK. ERK activation has been shown to counteract Fas-induced apoptosis in Jurkat cells (Wilson *et al.*, 1996) and could be responsible for the suppression of an apoptotic phenotype in the CD4–TNF-RII CHO cells. In addition, TNF-RII-mediated ERK activation could contribute to the TNF-RII proliferative response observed in CT6 cells (Tartaglia *et al.*, 1993b). The TNF α -induced activation of ERK has largely been attributed to the activation of TNF-RI associated neutral sphingomyelinase (nSM). The ceramide generated by nSM can activate CAP which in turn activates Raf leading to ERK activation. (Belka *et al.*, 1995; Winston and Riches, 1995; Yao *et al.*, 1995; Adam *et al.*, 1996). Our results show that ERK can also be activated by TNF-RII in CHO cells indicating a connection of TNF-RII to the neutral sphingomyelinase in CHO cells or the existence of an alternative pathway to activate MAP kinase.

TRAF2 is necessary and sufficient for TNF α -mediated JNK activation

Our results identify TRAF2 as the mediator between TNF receptor crosslinking and the activation of JNK in all cell types tested. Overexpression of TRAF2 was sufficient to induce JNK activation independent of TNF receptor crosslinking (Figure 3A) and could synergize with TNF α -induced activation of JNK if expressed at low levels (Figure 4A). In contrast, overexpression of TRAF1, a molecule that binds to TNF-RII in a heterodimer with TRAF2 showed no significant induction of JNK (Figure 3A). Some supporting evidence for a role of TRAF2 in JNK activation emerged from recent findings which show that the CD40 receptor, which also associates with TRAF2, is also a potent activator of JNK (Sakata *et al.*, 1995; Berberich *et al.*, 1996). Furthermore, the TNF α -induced Zn finger protein A20 has been shown to associate with TRAF2 thereby blocking its signaling capabilities (Krikos *et al.*, 1992; Song *et al.*, 1996). A20 is known to suppress TNF-mediated activation of the transcription factors AP-1 and NF- κ B (Jaattelae *et al.*, 1996) as well as TNF α -mediated cytotoxicity (Opipari

et al., 1992). Therefore A20 might be part of a feedback mechanism to downregulate TRAF2 signaling to JNK and NF- κ B. TRAF2 is characterized by an N-terminal RING finger that has been shown to be essential for TRAF2-mediated activation of NF- κ B (Rothe *et al.*, 1995b) and JNK (Figure 3A) and a C-terminal TRAF domain involved in homo and heterodimerization as well as TNF-RII and TRADD binding (Cheng *et al.*, 1995; Rothe *et al.*, 1995a; Hsu *et al.*, 1996b). The binding of TRAF2 to TNF-RII and its association with TNF-RI through TRADD suggests that both TNF receptors are able to activate JNK. Indeed, we have shown JNK activation by TNF-RII (Figure 2A) and Sanchez *et al.* have shown a strong TNF-RI-mediated activation of JNK in response to TNF α in human embryo kidney fibroblasts 293 which are devoid of endogenous TNF-RII (Pennica *et al.*, 1992; Sanchez *et al.*, 1994; Rothe *et al.*, 1995a). Furthermore, a signaling-deficient TRAF2 mutant lacking the N-terminal RING finger is able to suppress TNF α -mediated JNK activation in HeLa cells (Figure 4B). These findings imply that both TNF-RII and TNF-RI are able to signal activation of JNK and that TRAF2 is necessary and sufficient for TNF receptor I and II-mediated JNK activation in fibroblasts.

TNF α -mediated activation of JNK is independent of the cell death pathway

TNF α -induced cell death in fibroblasts requires the presence of inhibitors of translation or transcription and cells treated with TNF α in the absence of these inhibitors do not enter apoptosis. TNF α -mediated activation of JNK in fibroblasts occurs rapidly after receptor crosslinking and is independent of the above inhibitors, suggesting that the activation of JNK is not a consequence of the activation of the cell death pathway in fibroblasts (Ringeaud *et al.*, 1995; our unpublished observations). In contrast, Fas-mediated JNK activation occurs with much slower kinetics and parallels the onset of apoptosis indicating that TNF α and Fas most likely activate JNK through a different mechanism (Wilson *et al.*, 1996). In addition, the ability of a dominant-negative TRAF2 to inhibit TNF α -induced activation of JNK indicates that other TNF receptor associated molecules should have little or no potential to activate JNK. Consistently, overexpression of the cell death-associated molecules FADD and RIP had no effect on the activation of cotransfected JNK1 (Figure 3A) and a dominant-negative mutant of FADD, which is a potent inhibitor of TNF and Fas-induced cell death (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996b), is unable to block TNF-mediated activation of JNK (C.Reinhard, B.Shamoon and L.T.Williams, unpublished observation) and NF- κ B (Hsu *et al.*, 1996b). Taken together it appears that the TNF α -induced early activation of JNK occurs independently of the cell death pathway rather than through the cell death pathway as observed during Fas-induced apoptosis.

Interestingly, overexpression of TRAF2 did not induce apoptosis in HeLa cells emphasizing that JNK activation is not sufficient to force these cells into the apoptotic pathway. Consistently, Δ TRAF2 expression did not interfere with TNF α -induced apoptosis in HeLa cells (Hsu *et al.*, 1996b). However, TRAF2 has recently been shown to bind CD30 and is most likely to be inducing cell death during CD30 and T cell receptor engagement (Lee *et al.*, 1996). Together, these findings emphasize the importance

of the signaling context in determining the consequences of TRAF2 signaling.

TRAF2 activates NF- κ B and JNK through different pathways

Recently a connection between JNK and NF- κ B activation has been suggested based on the observation that overexpression of MEKK1, a potent inducer of JNK activity, increases NF- κ B transcriptional activity of cotransfected reporter plasmids (Meyer *et al.*, 1996). We used a dominant-negative approach to test whether TRAF2-mediated JNK and NF- κ B activation are interconnected. Overexpression of dominant-negative MKK4-KR, a kinase dead mutant of the upstream activating kinase for JNK, completely disrupts TRAF2-mediated signaling to JNK (Figure 5A). In contrast, TRAF2-mediated NF- κ B activation is not affected by MKK4-KR (Figure 5B), indicating that TRAF2-mediated activation of NF- κ B is not a result of the concomitant induction of JNK activity. The discrepancy between our results and those described above, might be due to the ability of MEKK1 to activate multiple pathways or due to autocrine activation of NF- κ B by MEKK1-induced and secreted factors. However, JNK has been shown to interact with the NF- κ B subunit c-Rel in a two-hybrid system and *in vivo* but the functional implications of that interaction remain to be determined (Meyer *et al.*, 1996). Both JNK and NF- κ B can be activated by ceramide indicating that they might share some of their upstream activators, however, the pathway most likely bifurcates at a point upstream of MEKK and leads to the independent activation of JNK and NF- κ B (Schutze *et al.*, 1992; Lozano *et al.*, 1994; Westwick *et al.*, 1995; Verheij *et al.*, 1996).

The TRAF C and TRAF N domains modulate TRAF2 signaling

The signaling of TRAF2 to either JNK or NF- κ B seems to be strictly dependent on the presence of the N-terminal RING finger; however, little is known about the contribution of the C-terminal TRAF domain. Removal of the TRAF C domain (aa 359–501, T358) generates a TRAF2 molecule that is no longer able to associate directly with TNF-RII or to associate to TNF-RI through the adapter molecule TRADD. Surprisingly, this mutant not only maintains its signaling activity but shows a pronounced increase in its ability to activate JNK (Figure 6B) or NF- κ B (Figure 6C) compared with wild type TRAF2. This increase has also been observed by Takeuchi *et al.* in a mutational analysis of TRAF2-mediated NF- κ B activation (Takeuchi *et al.*, 1996). This result shows that the TRAF2-mediated activation of JNK and NF- κ B are independent of the TRAF2 association with TNF-RII or TRADD. Furthermore, the increased signaling activity observed compared with wild type TRAF2 could indicate a favorable conformational change of the mutant molecule or the loss of a binding site for an inhibitory molecule. Interestingly, TRAF-I, a recently identified molecule binding to the TRAF C domain of TRAF2 has been shown to be an inhibitor of TRAF2-mediated NF- κ B activation (Rothe *et al.*, 1996). Consequently, removal of the TRAF C domain would result in a loss of the TRAF-I binding site, leading to a stronger NF- κ B and JNK activation than observed with the wild type TRAF2. The same protein,

independently described as TANK, acts as an activator of TRAF2-mediated NF- κ B activation (Cheng and Baltimore, 1996), however, our data are more consistent with the notion that TRAF-I/TANK is an inhibitor. Further removal of the TRAF N (aa 264–357, T264) completely abolished the ability of TRAF2 to activate JNK (Figure 6B) and severely reduced its ability to activate NF- κ B (Figure 6C). These findings indicate that TRAF N has an essential function during the formation of signaling-competent TRAF2 complex. TRAF N is involved in homo- and heterodimerization of TRAF family members and also in binding to c-IAPs (Rothe *et al.*, 1995a; Takeuchi *et al.*, 1996). Indeed, deletion of TRAF N results in a loss of association with TRAF2 as determined by co-immunoprecipitation (data not shown). The replacement of the TRAF N region by an artificial dimerization or oligomerization domain may shed more light on the requirement for dimer or multimer formation in TRAF2-mediated signaling to JNK and NF- κ B.

The data presented here suggest a central role for TRAF2 as a mediator of JNK and NF- κ B activation by various members of the TNF receptor family like TNF-RI and II, CD40 and possibly CD30. A future challenge will be to determine the immediate downstream targets of TRAF2 and to define the bifurcation point for the signaling pathways leading to the activation of JNK or NF- κ B.

Materials and methods

Cell lines

Chinese Ovary Hamster cells (CHO-K1) and COS-7 cells were obtained from the American Type Culture Collection (ATCC). CHO cells were maintained in HAM F12 medium containing 10% bovine calf serum (CS), 50 μ g/ml penicillin and 50 μ g/ml streptomycin. COS-7 and HeLa cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine fetal calf serum (FCS), 8 mM glutamine, 50 μ g/ml penicillin and 50 μ g/ml streptomycin.

Plasmid constructs

TRAF2 cDNA (Rothe *et al.*, 1994) was obtained by the polymerase chain reaction (PCR) from a mouse B cell cDNA library (Clontech) using primers flanking the entire coding region and then cloned into the cytomegalovirus (CMV) promoter-driven mammalian expression vector pCG (Klippel *et al.*, 1996) using *Xba*I and *Bam*HI restriction sites. Δ TRAF2 has been constructed by PCR, deleting aa 1–87 as described (Rothe *et al.*, 1995b) (Figure 6A). The C-terminal deletion mutants T264 and T358 were generated by PCR spanning aa 1–264 and aa 1–358 respectively. TRAF1 cDNA (Rothe *et al.*, 1994) was obtained by RT-PCR from mouse spleen total RNA (Clontech) using an RT-PCR kit (Perkin Elmer) according to the manufacturer's instructions. The coding region was inserted into pCG as described above. FADD (Chinnaiyan *et al.*, 1995) and RIP (Stanger *et al.*, 1995) cDNAs were kindly provided by A. Roulston and X. Niu (Chiron Corporation, Emeryville, CA). All constructs were modified by addition of an N-terminal influenza virus hemagglutinin (HA)-tag. In addition TRAF2 and Δ TRAF2 versions carrying an N-terminal Glu-tag (EEEEYMPME) were constructed by PCR. Flag tagged MKK4 cDNA (Derijard *et al.*, 1995) and pcDNA3-JNK as well as GST c-jun(1–79) were a generous gift of Roger Davis (Howard Hughes Medical Institute, Worcester, MA). The Flag tag of JNK was replaced by an N-terminal Glu-tag by PCR and cloned into pCG. The HA-tagged JNK has been described (Klippel *et al.*, 1996). A kinase deficient, dominant-negative MKK4 was constructed by replacing lysine 131 in the ATP binding site with arginine using wt MKK4 as template for a PCR mutagenesis (Ho *et al.*, 1989). A HA-tagged-ERK2 expression plasmid (Klippel *et al.*, 1996) was kindly provided by Anke Klippel (Chiron Corporation, Emeryville, CA). To generate the chimeric CD4-TNF-RII the extracellular and transmembrane domains of murine CD4 (nucleotides 170–1426) (Littman and Gettner, 1987) were isolated by PCR using pCD.L3T4.25, carrying full-length mCD4 cDNA (kindly provided by D. Littman, UCSF), as template and cloned into the eukaryotic

expression vector pcDNA3 (Invitrogen). The cytoplasmic domain of human TNF-RII (Smith *et al.*, 1990) (nucleotides 951–1475) was isolated by PCR from a HepG2 cDNA library and cloned in-frame with the above described CD4-TM sequences. In the pcDNA3-CD4-TNF-RI construct, the cytoplasmic domain of human TNF-RI (Loetscher *et al.*, 1990) (nucleotides 883–1551) was isolated by PCR from the same HepG2 cDNA library and cloned downstream of the CD4-TM fragment. All constructs were verified by sequence analysis.

Stable cell lines

CHO cells were transfected with pcDNA3-CD4-TM or pcDNA3-CD4-TNF-RII at 30% confluency using 10 µl lipofectamin (Gibco-BRL) per µg DNA in serum-free medium (OptiMEM, Gibco-BRL) for 5 h. After 24 h expression in regular medium selection was started by adding 800 µg/ml G418 (Gibco-BRL). Single colonies were isolated at day 10 and subjected to single cell cloning. Expressing cell lines were maintained in HAM F12 containing 10% CS, 50 µg/ml penicillin, 50 µg/ml streptomycin and 400 µg/ml G418 (Gibco-BRL).

Antibodies

Rabbit polyclonal anti-ERK2 and anti-JNK1 were obtained from Santa Cruz Biotechnology. Mouse monoclonal 12CA5 (Wilson *et al.*, 1984) anti-HA antibody was obtained from Boehringer; mouse monoclonal anti-Flag antibody was from Kodak. The mouse monoclonal anti-Glu-Glu antibody was provided by L.Conroy (Chiron Corporation, Emeryville, CA). The rabbit polyclonal anti-mCD4 was kindly provided by A.Truhnay (Smith Kline Beecham).

Crosslinking of chimeric receptors

CHO cells expressing CD4 chimeric receptors were grown in 10 cm dishes to 70% confluency in regular HAM F12 and starved 16 h prior to crosslinking in HAM F12 containing 0.1% bovine serum albumin (BSA, Sigma). The rat monoclonal anti-mCD4 (L3/T4, CALTAG) was applied at 0.5 µg/ml in 5 ml starving medium. After 30 min the supernatant was removed and replaced with 5 ml starving medium containing 2.5 µg/ml of secondary anti-rat IgG/IgM (Jackson Immuno-Reagents) for the desired time of stimulation. Control treatment for JNK or ERK activation was cycloheximide (20 µg/ml, Sigma) for 20 min and 10% FCS for 10 min respectively. Cells were washed twice in cold PBS prior to extraction. Cytoplasmic extracts were prepared by scraping cells in Triton lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β-glycerophosphate, 50 mM NaF, 10 mM NaPPi, 15% (v/v) glycerol and 1% (v/v) Triton X-100. Lysates were cleared by centrifugation at 13 000 g and quick frozen in liquid nitrogen.

Transient transfection

COS-7 and CHO cells were grown in 10 cm dishes to 60% confluency and transfected using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Various amounts of constructs were cotransfected with 1 µg of pCG-JNK1, pCG-ERK2 or pcDNA3-NF-κB-luciferase reporter plasmids at 5 µg total DNA. Incubation lasted 3 h (COS-7) to 6 h (CHO) in serum-free medium (OptiMEM, Gibco-BRL). Alternatively, COS-7 cells were transfected with 5 µg DNA using DEAE-dextran (Pharmacia mol. wt: 500 000) at 0.4 mg/ml and chloroquine 0.1mM final concentration in 2.5% FCS DMEM for 2 h at 37°C. HeLa cells were transfected using the lipid formulation TransIT-LT1 (Mirus Corp) using 2 µl/µg DNA according to the manufacturer's instructions. After removal of the DNA mixture, cells were incubated for 24 h at 37°C in either DMEM/10% FCS (COS-7, HeLa) or HAM F12/10% FCS (CHO). Following 24 h expression cells were starved for 20 h in DMEM/0.1% BSA (COS-7) or HAM F12/0.1% BSA (CHO). During some experiments cells were subjected to a 15 min TNFα treatment (10 ng/ml). Extraction condition for JNK and ERK reporters were as described above. Cells containing luciferase reporter plasmids were extracted using a hypotonic lysis buffer supplied with the dual luciferase reporter assay system (Promega). All cell extracts were controlled for equal expression of transfected constructs and reporter plasmids by immunoblotting prior to further analysis.

In vitro protein kinase assays

Equal protein amounts of cell extracts containing HA-JNK1 or Glu-JNK1 were incubated with 1 µg 12CA5 (Boehringer) or 1 µg anti-Glu respectively and incubated for 4 h on ice. Immune complexes were subsequently precipitated for 30 min at 4°C on a shaker using protein A-Sepharose beads (Sigma) for 12CA5 or protein G-Sepharose beads

(Sigma) for the anti-Glu monoclonal antibodies. The beads were washed twice with Triton lysis buffer and twice with JNK reaction buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid (HEPES, pH 7.4), 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol and 0.1 mM sodium vanadate. Determination of JNK activity was carried out as described (Derijard *et al.*, 1994) using 3 µg GST c-jun(1–79) as substrate, and 50 µM [γ-³²P]ATP (10 Ci/mmol) in 30 µl JNK reaction buffer and the reaction was allowed to proceed for 25 min at 30°C. *In vitro* kinase assays for HA-ERK2 were carried out as described (Ming *et al.*, 1994) using 2 µg MBP (Upstate Biotechnologies) as substrate. The reactions were stopped by 10 µl Laemmli sample buffer, boiled for 5 min and phosphoproteins resolved by SDS-PAGE (Laemmli, 1970) and transferred on nitrocellulose (Schleicher & Schuell). Incorporated radioactivity was quantitated using a Bio-Rad phosphorimager and the amount of JNK1 or ERK2 in the immune complex determined by immunoblot. Endogenous JNK and ERK activity was determined using rabbit polyclonal antisera (Santa Cruz biotechnology) following the above protocol. All findings have been confirmed by three independent experiments.

Determination of NF-κB-luciferase activity

COS-7 cells were cotransfected with an NF-κB-luciferase reporter plasmid containing three copies of the Igκ NF-κB binding site [(G)GGG-ACTTTCC(G)] (Fujita *et al.*, 1992) and an internal control plasmid under control of a TK promoter leading to low constitutive expression of Renilla luciferase (Promega). The reporter and control plasmid were cotransfected with various constructs using Lipofectamin (Gibco) as described above and expression allowed for 24 h. Prior to extraction the cells were starved in DMEM/0.5% FCS for 24 h and cytosolic extracts prepared and analysed according to the instructions of the dual-luciferase reporter assay system (Promega). Luciferase units reflecting NF-κB activity were corrected for the activity of the internal standard, Renilla luciferase light units, and protein concentration. The fold induction was calculated with respect to the light units generated by the vector control. All experiments have been confirmed by three independent experiments.

Immunoblot analysis

The protein concentration of the cell extracts was determined by Coomassie protein assay (Pierce) and 30 µg of total protein resolved by SDS-PAGE and transferred to nitrocellulose filters (Schleicher & Schuell). Membranes were blocked by a 30 min incubation in TBST [10 mM Tris-Cl pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20] containing 5% (w/v) skim milk. Antibodies were applied in TBST at the appropriate dilutions for at least 4 h. Complexed IgGs were detected by either anti-mouse or anti-rabbit conjugated to alkaline phosphatase (Promega) and developed using nitroblue tetrazolium and 5-bromo-4-3-indolylphosphate (Promega). Immunoblots of immune complex assays were quantitated using a Bio-Rad imaging system.

Acknowledgements

We are grateful to Drs Roger Davis, Dan Littman, Anne Roulston and A.Truhnay for generously providing reagents. We are also grateful to Hamid Khoja for his support and preparation of the GST-c-jun(1–79). We thank Lauri Gorda for synthesis and purification of oligonucleotides and Amanda Goodsell for help with the FACS analysis. We thank Drs Bert Pronk, Anne Roulston, Steven Harrison and Anke Klippel for many helpful comments on the manuscript.

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Received on October 7, 1996; revised on November 8, 1996