Tumor Necrosis Factor Receptors (Tnfr) in Mouse Fibroblasts
Deficient in Tnfr1 or Tnfr2 Are Signaling Competent and
Activate the Mitogen-activated Protein Kinase Pathway with
Differential Kinetics*

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To dissect tumor necrosis factor receptor (Tnfr)-1 (CD120a) and Tnfr2 (CD120b)-dependent signal transduction pathways, primary fibroblasts isolated from inguinal adipose tissue of wild type (wt), tnf−/−, tnf+/−, and tnf−/−/tnfr2+/− mice were studied. The mitogen-activated protein kinases Erk1 and Erk2 were found to be tyrosine-phosphorylated and activated by Tnf treatment in all wt, tnf−/−, and tnf−/−/tnfr2+/− fibroblasts; the activation was down-regulated 60 min after the start of steady state Tnf treatment. Distinct kinetics of Erk1 and Erk2 activation were detected; the Tnfr1-mediated activation of Erk1 and Erk2 started more slowly and persisted for more prolonged times as compared with Tnfr2 activation. Raf-1, Raf-B, Mek-1, Mek kinase, and p90rsk kinases were also shown to be activated independently in a distinct time-dependent pattern through the two Tnfr receptors. In addition, both Tnfr1 and Tnfr2 mediated independently the activation of the transcription factor Ap-1 albeit with parallel activation kinetics. In contrast, Tnfr1 exclusively mediated activation of NF-κB and fibroblast proliferation; however, Tnfr2 enhanced proliferation triggered through Tnfr1. These findings indicate distinct but also overlapping roles of Tnfr1 and Tnfr2 in primary mouse fibroblasts and suggest different regulation mechanisms of signal transduction pathways under the control of both Tnfr receptors.

Tumor necrosis factor α (Tnfa) is one of the most pleiotropic cytokines, which exerts cytotoxicity as well as differentiation and growth modulatory activities on many different target cells (for review see Refs. 1–3). Tnfa activities are elicited by binding to at least two distinct surface receptors of 55 kDa (Tnfr1, CD120a) and 75 kDa (Tnfr2, CD120b), which are ubiquitously coexpressed on almost all cell types in various proportions (reviewed in Refs. 1, 4, 5). Both Tnf receptors have similar extracellular domains, but their intracellular parts are entirely unrelated suggesting distinct functions by addressing different signal pathways (5–8). Tnfr1 and Tnfr2 bind Tnfa and Tnfb with similar high equilibrium affinity, but they differ significantly in their Tnfa binding kinetics; Tnfa bound to Tnfr2 is exchanged at a significantly faster rate than when complexed with Tnfr1 (9–12).

Most of the cellular responses to Tnfa such as cytotoxicity, cell growth, activation of NF-κB, and up-regulation of adhesion molecules are triggered by Tnfr1 engagement (for review see Refs. 13–16), but a small subset of Tnf activities, mainly proliferation of lymphoid cells, is mediated by direct Tnfr2 signaling; in some cell lines Tnfr2-mediated activation of NF-κB and cytotoxicity has also been reported (17–23). The predominant role of Tnfr2 has been proposed to be an accessory function in enhancing or synergizing Tnfr1 signaling, e.g. by ligand passing (5, 10, 24). Studies of Tnfr1- and Tnfr2-deficient mice revealed a decisive role in vivo of Tnfr1 in the host defense against intracellular pathogens, whereas Tnfr2 played a role in Tn-induced necrosis (16, 25, 26).

More recently, new insight in Tnfa postreceptor mechanisms has been gained with the identification of a number of presumable signal transducing molecules that bind to the intracellular domains of Tnfr1 and Tnfr2 (27–35). The role in the activation of the transcription factor NF-κB of one of these molecules, Traf2, which associates with the Tnfr2 intracellular domain has been demonstrated in transfection studies and using a κB element-driven reporter construct (29, 35).

In fibroblasts, Tnfa has been reported to activate several kinases such as c-Jun kinase, a member of the Mapk family, the phosphorylation of cytosolic proteins, and the transcription factors Ap-1 and NF-κB (36–44). Furthermore, it induces c-jun and c-fos gene expression (45). Mitogen-activated protein kinases (Mapk) play a central role in the early signal transduction events after receptor engagement of a variety of growth factors, cytokines and hormones; Tnfa has been found to activate c-Raf1 and the Map kinases Erk1 and Erk2 (46–49).

In the present study, primary fibroblasts isolated from mice deficient in Tnfr1 (tnfr1−/−), in Tnfr2 (tnfr2−/−), and in both Tnfr1 and Tnfr2 (tnfr1−/−/tnfr2−/−) were used to further investigate signal pathways under Tnfr1 and Tnfr2 control (16, 25, 26). Tnfr1 and Tnfr2 were found to activate distinct but also overlapping signal pathways.

MATERIALS AND METHODS

Mice — The generation of homozygous Tnfr1-deficient (tnfr1−/−) or Tnfr2-deficient (tnfr2−/−) mice by gene targeting has been described else-

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‡ The abbreviations used are: Tnf, tumor necrosis factor; Tnfr, tumor necrosis factor receptor; Traf, Tnfr receptor-associated factor; Mapk, mitogen-activated protein; Mapk, mitogen-activated protein kinase; Erk, extracellular regulated kinase; Mek, Mapk kinase; Mek, Mek kinase (Mapk kinase kinase); n, mouse; p90rsk, p90 ribosomal-S6 kinase; HRP, horseradish peroxidase; PMFSF, phenylmethylsulfonyl fluoride; DME, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; h, human; IL-1, interleukin-1; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; wt, wild type; PVDF, polyvinylidene fluoride; Mbp, myelin basic protein; Trap, Tnfr receptor-associated protein; Tradd, Tnfr1-associated death domain protein.
Rabbits—Rabbit polyclonal anti-Map kinase R2 (Erk1-CT; recognizing Erk1 and Erk2 independent of phosphorylation state), rabbit polyclonal anti-Map kinase R3 (Erk1-NT; recognizing Erk1), and mouse monoclonal anti-Map kinase antibodies (Erk2; recognizing Erk2) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-Mek-1, anti-Mek, anti-Raf-1, anti-Raf-B, and anti-p90(RSK) antibodies were purchased from Santa Cruz (CA). Rabbit polyclonal antibodies to tyrosine-phosphorylated Erk1 and Erk2 (phospho-Map) were purchased from New England BioLabs (Beverly, MA). Horseradish peroxidase (HRP)-labeled secondary anti-rabbit antibodies, [γ-32P]ATP (>3000 Ci/mmol), [γ-32P]ATP (2000–3000 Ci/mmol), and (methyll-32H)thymidine (5 Ci/mmol) were purchased from Amersham Corp. (Buckinghamshire, UK).

Protocols—All samples were purchased from Pharmacia (Uppsala, Sweden). Peptatin A, leupeptin-hemisulfate, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, and dithiothreitol (DTT) were from Fluka (Milano, Italy). Pepstatin A, leupeptin-hemisulfate, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, and dithiothreitol (DTT) were from Fluka (Milano, Italy). The preparation of nuclear extracts was determined using a BCA protein assay (Pierce, Rockford, IL). Incorporation of radioactivity was measured in a Betaplate harvester. The homogenate was filtered through a 100-mesh nylon membrane, Millipore, Bedford, MA). The preparation of cell lysates was determined using a bovine serum albumin binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) in a total volume of 15 μl for 20 min at room temperature. As NF-κB probe, a double-stranded oligonucleotide containing two tandemly arranged κB elements of the human immunodeficiency virus long terminal repeat (5′-ATCAGG-GACTTTCGGCCTGGOGGGACTTTCG-3′) was used. A double-stranded oligonucleotide containing a single nucleotide deletion (5′-AGGATGGAGAATGTTGATATCCTTGACAGCC-3′) was included as controls in the NF-κB and Ap-1 assays, respectively. The membranes were then washed and subjected to enhanced chemiluminescence detection (Amersham Corp.) under standard conditions. Autoradiograms were analyzed with a laser densitometer ( Molecular Dynamics, Sunnyvale, CA).
the beads with bound immunocomplexes in 20 \mu l of Mbp substrate solution (4 mg/ml) and 20 \mu l of reaction buffer (10 mM ATP, 40 mM MgCl2, 40 mM HEPES) supplemented with 5 \mu Ci of [32P]ATP and subsequent incubation under agitation (30 min, 30°C). The kinase reaction was stopped with 40 \mu l of 2 \times Laemmli’s sample buffer containing DTT. The samples were heated to 95°C for 4 min and then subjected to SDS-PAGE. The labeled Mbp substrate was quantitated by PhosphorImager analysis of the dried gels.

RESULTS
Predominant Role of Tnfr1 in Tnf-stimulated Mouse Fibroblast Proliferation—To dissect the activity of the two Tnf receptors in proliferation, primary mouse fibroblasts from wild type (wt) and homozygous tnfr1-o or tnfr2-o mice were stimulated for 48 h with various amounts of mTnfα, and thymidine incorporation was measured. Confirming previous studies (24, 52), proliferation of wt fibroblasts was stimulated by mTnfα, reaching a maximum at about 5–10 ng/ml mTnfα (Fig. 1). tnfr2-o fibroblasts similarly showed enhanced proliferation with mTnfα stimulation, but the maximal proliferative response achieved was reproducibly lower than with wt fibroblasts. In contrast, mTnfα had no effect on tnfr1-o fibroblast proliferation (Fig. 1). These findings confirm that Tnfr1 is essential in mediating proliferative signals, whereas Tnfr2 engagement merely enhances proliferation triggered through Tnfr1 (24).

Tnfr1 Exclusively Activates Transcription Factor NF-κB in Mouse Fibroblasts—Several previous studies had attributed NF-κB activation by Tnf to Tnfr1 signaling, but in some cell lines NF-κB activation was also reported to be under Tnfr2 control (16, 20, 21, 28, 53–55). To investigate the role of Tnfr1 and Tnfr2 in NF-κB activation in the present cell systems, primary wt and Tnfr-deficient fibroblasts were stimulated for 15 and 30 min with predetermined optimal doses of mTnfα, and equal amounts of nuclear protein extracts were tested for activated and translocated NF-κB in electrophoretic mobility shift assays (EMSA). NF-κB was found strongly activated by mTnfα treatment in wt fibroblasts (Fig. 2A). In contrast, mTnfα completely failed to activate NF-κB in tnfr1-o fibroblasts (Fig. 2B), confirming similar results with other cells from tnfr1-o mice (16). However, NF-κB could be activated in tnfr1-o fibroblasts by hIL-1α treatment, demonstrating that NF-κB activation in general was not affected. Tnfα stimulation of tnfr2-o fibroblasts resulted in NF-κB activation (Fig. 2C), for a more quantitative evaluation, the integrated relative peak intensities of the κB-specific bands were determined by densitometry of the autoradiograms of three independent series of experiments, each using all four cell types in parallel. Despite non-negligible variation among experiments, the data support the conclusion that NF-κB activation in wt fibroblasts increases from the 15- to the 30-min time point, whereas it has decreased after 30 min, when compared with 15 min, of Tnfα stimulation of tnfr2-o fibroblasts, suggesting that Tnfr2 signals are required to achieve a more sustained activation of NF-κB. In tnfr1-o/tnfr2-o fibroblasts, no κB binding activity was detected in the nuclear extracts stimulated with mTnfα, whereas hIL-1α treatment still elicited NF-κB activation (Fig. 2D).

Both Tnfr Receptors Signal in Ap-1 Activation—Tnf has previously been reported to be a potent activator of transcription factor Ap-1 through prolonged activation of the c-Jun kinase (44, 45). The role of the two Tnf receptors in Ap-1 activation in wt, tnfr1-o, tnfr2-o, and tnfr1-o/tnfr2-o fibroblasts was therefore investigated by EMSA. Interestingly, Ap-1 was found to be activated by mTnfα in all wt, tnfr1-o, and tnfr2-o fibroblasts, showing that Ap-1 was independently activated through both Tnfr receptors (Fig. 3, A–C). Increased Ap-1 probe binding activity in nuclear extracts from wt, tnfr1-o, and tnfr2-o fibroblasts was already detected after 30 min and was still apparent 3 h after mTnfα stimulation (Fig. 3, A–C). The intensity of the shifted bands appeared to be comparable in the nuclear extracts of all these cell types, since the integrated relative intensities of the Ap-1 bands determined by densitometry of autoradiograms of three independent series of parallel experiments with all four cell types were not significantly different. As expected, treatment of tnfr1-o/tnfr2-o fibroblasts with hIL-1α, but not with mTnfα, resulted in Ap-1 activation (Fig. 3D).

Increased Phosphorylation of Erk1 and Erk2 after Tnf Treatment—To investigate the control of the Map kinases Erk1 and Erk2 through Tnfr1 and Tnfr2 in the primary mouse fibroblasts, Western blot analyses were performed. Equal amounts of total cell lysate protein of wt, tnfr1-o, tnfr2-o, and tnfr1-o/tnfr2-o fibroblasts treated for various times with mTnfα and hIL-1α were separated by SDS-PAGE, transferred to PVDF membranes, and stained with erk1-CT antibody. Two distinct bands in the range of 40–45 kDa, assigned to Erk1 and Erk2, were visualized with unstimulated, mTnfα, and hIL-1α-stimulated cells of all four mouse types (Fig. 4A). The amount of Erk1 and of Erk2 protein staining was approximately constant in all fibroblast types and did not significantly change with the
Cell types is presented. was apparent 5 min after mTnf creased tyrosine phosphorylation of Erk2 and, to a lesser ex-
blasts, whereas tnfr1o hIL-1, and tnfr2o stimulation;only a min-
treatment in wt, tnf1r1/tnfr2o fibroblasts. Erk1 and Erk2 tyrosine phosphorylation was tran-
activation kinetics in the Tnf treatment. In contrast, in wt fibroblasts, both Erk1 and Erk2 showed
increased tyrosine phosphorylation of Erk2 and, to a lesser ex-
treatment in wt, tnf1r1/tnfr2o fibroblasts, whereas tnfr1r1/tnfr2o fibroblasts did not respond. In
contrast, phosphorylation of Erk1 and Erk2 was induced by hIL-1a in all wt, tnf1r1, tnf2r2, and
contrast, phosphorylation of Erk1 and Erk2 was more short-lived in tnf1r1 fibroblasts when compared with wt fibroblasts, since phosphorylated forms of Erk1 or Erk2 were evident 5 min, but
no longer detected 15 min after mTnf treatment (Fig. 4B). In tnf2r2 fibroblasts, a relatively weak Erk2 phosphorylation was apparent 5 min after mTnf stimulation, but it became stronger, and Erk1 phosphorylation became evident 15 min after mTnf stimulation. Similar to wt fibroblasts, a weak Erk2, but no Erk1, phosphorylation persisted 60 min after mTnf stimulation in tnf2r2 fibroblasts. hIL-1a, but not Mtno, elicited phosphorylation of Erk1 and Erk2 in tnf1r1/tnfr2o fibroblasts (Fig. 4B).

\[ \text{Tnf1r1 and Tnf2r2 Control Erk1 and Erk2 Kinase Activity—To corroborate the above findings, the Tnf-stimulated kinase activity of Erk1 and Erk2 in lysates of wt, tnf1r1, tnf2r2, and tnf1r1/tnfr2o fibroblasts was analyzed in Mbp-SDS-PAGE kinase assays (Fig. 5), and by in-solution kinase assays using specific Erk1 and Erk2 immunoprecipitates (Fig. 6). First, Mbp-SDS-PAGE kinase assays of lysates of mTnf or hIL-10-treated fibroblasts revealed strongly inducible kinase activities of two proteins in the typical locations of Erk1 and Erk2 of about 40–45 kDa (Fig. 5). The Mtno concentration chosen in these studies was 10 ng/ml; preliminary studies had shown that the Mapk responses saturated at Tnf concentrations of 0.5 ng/ml. Furthermore, no significant decrease in the active Tnf concentration in the culture medium could be measured at the end of the experiment. The two kinases, tentatively assigned to Erk1 and Erk2, in wt fibroblasts showed similar activity 5 and 15 min after Tnf stimulation and some residual activity after 60 min. Two further kinases of about 90 and 140 kDa were also found strongly activated by mTnf treatment, following a similar activation time dependence. Both presumptive Erk1 and Erk2 were triggered through Tnfr1 and Tnfr2 as demonstrated with the tnf1r1 and tnf2r2 fibroblasts (Fig. 5). In tnf1r1 fibro-
blasts, the activity of these kinases was strongly stimulated after 5 min and had decayed to a large extent after 15 min of Tnf treatment. In contrast, in tnf2r2 fibroblasts the time dependence of kinase activation was reversed, the signal intensity increasing from a relatively low level at 5 min to a maximum at 15 min. The significance of the differential Tnfr1- and Tnfr2-mediated time dependence of kinase activity was further supported by the 90- and 140-kDa kinases following similar activation kinetics in the tnf1r1 and tnf2r2 fibroblasts. As expected, tnf1r1/tnfr2o fibroblasts showed kinase activation after hIL-1a but not after mTnf stimulation.

In the second approach, Erk1/Erk2 in-solution kinase assays were performed with erk1-CT polyclonal rabbit antibodies binding Erk1 and Erk2 (1:2500 diluted) (A) and with polyclonal rabbit antibodies exclusively binding tyrosine-phosphorylated Erk1 and Erk2 (1:1000 diluted) (B). Bands were visualized by autoradiography and enhanced chemiluminescence detection under standard conditions. Erk1- and Erk2-specific bands are indicated by arrows.

**Fig. 4.** Phosphorylation of Erk1 and Erk2 in mTnf- and hIL-10-treated primary mouse fibroblasts. Parallel primary mouse fibroblast cultures of wt, tnf1r1, tnf2r2, and tnf1r1/tnfr2o mice were stimulated with 10 ng/ml mTnf for various times, with 10 ng/ml hIL-1α (15 min), or left untreated. Cell lysates (calibrated to 25 μg of total cellular protein) were separated by SDS-PAGE, and protein bands were trans-
ferred to PVDF membranes. Western blot analyses were performed with erk1-CT polyclonal rabbit antibodies binding Erk1 and Erk2 (1:2500 diluted) (A) and with polyclonal rabbit antibodies exclusively binding tyrosine-phosphorylated Erk1 and Erk2 (1:1000 diluted) (B).
**Tnfr1 and Tnfr2 Signaling**

**Fig. 5. Time dependence of Erk1 and Erk2 activation in mTnft- and hIL-1α-stimulated primary mouse fibroblasts.** Erk1 and Erk2 activation in mTnft- and hIL-1α-stimulated wt, tnfr1<sup>a</sup>, tnfr2<sup>a</sup>, and tnfr1<sup>a</sup>/tnfr2<sup>a</sup> fibroblasts in parallel cultures were analyzed by Mbp-SDS-PAGE kinase assays. Cell lysates (calibrated to 20 μg of total protein) were added to Mbp (10 ng/ml) or hIL-1α (10 ng/ml, 15 min) stimulated primary fibroblasts were separated by SDS-PAGE in a 10.5% gel containing 0.1 ng/ml Mbp. After renaturation, the gels were assayed for kinase activity using [γ-<sup>32</sup>]<sub>P</sub>-Mbp (see “Materials and Methods”), dried, and analyzed by a PhosphorImager under standard conditions. The two bands at 40-45 kDa (indicated by arrows) were tentatively assigned Erk1 and Erk2.

**Fig. 6. Time-dependent activation of Erk1 and Erk2 in mTnft- and hIL-1α-treated primary mouse fibroblasts.** Wt, tnfr1<sup>a</sup>, tnfr2<sup>a</sup>, and tnfr1<sup>a</sup>/tnfr2<sup>a</sup> fibroblasts in parallel cultures were stimulated with 10 ng/ml mTnfta (5-60 min), 10 ng/ml hIL-1α (15 min), or left untreated. Cell lysates (calibrated to 30 μg of total protein) were immunoprecipitated with 1 μg of erk1-CT antibody recognizing Erk1 and Erk2. In-solution kinase assays were performed with immunoprecipitates, using Mbp and [γ-<sup>32</sup>]<sub>P</sub>-ATP. Phosphorylated Mbp substrate was visualized by SDS-PAGE and PhosphorImager analyses under standard conditions. Dried gels were exposed to PhosphorImager screens, and <sup>32</sup>P incorporation was quantified by volume integration (Imagequant software package, Molecular Dynamics).

The ubiquitous and simultaneous expression of two distinct Tnf receptors on almost all cells is intriguing (6, 67-70). While their extracellular domains have sequence similarity, their intracellular domains are unrelated, suggesting distinct modes of activation of signal transduction pathways (6, 71). Studies using Tnfr1- and Tnfr2-selective agonists, such as monoclonal antibodies and Tnf muteins, demonstrated that the majority of known Tnf activities is mediated by Tnfr1 and can be elicited by exclusive Tnfr1 engagement. In contrast, a direct signaling activity of Tnfr2 was found only in a subset of Tnf activities, including the enhancement of T cell proliferation and the activation of NF-κB and cytotoxicity in cell lines and transfected cells (17-23). These findings prompted the proposition that Tnfr2 mainly has an accessory function to Tnfr1 signaling, either by enhancement and modulation of the Tnfr1 signals in intracellular transduction pathways or by ligand passing (5, 10, 24).

The view of distinct modes of Tnfr1 and Tnfr2 signaling has been further confirmed in studies on receptor-associated intracellular molecules. Traf1 and Traf2, two members of a newly defined family of this class of proteins, were initially found to interact with a specific intracellular region of Tnfr2 but not with Tnfr1 (28). In contrast, Tradd, a member of a second family of such presumptive signal transducers, was identified as a protein specifically associating with the intracellular domain of Tnfr1 (31). More recently, it was shown that Tradd-Traf2 and Tradd-Fas-associated death domain protein interactions define two distinct Tnfr1 signal transduction pathways leading to a bifurcation between Tnfr1-dependent NF-κB activation and apoptosis, respectively (35). The discovery of additional proteins that may engage directly or in complexes cytoplasmic domains of Tnfr1 and Tnfr2, such as Tramp-1, receptor interacting protein, LMP1-associated protein, cellular inhibitor fibroblasts Erk1/Erk2 activity peaked only 15 min after Tnf stimulation, and significant activity was still obvious after 60 min, when compared with unstimulated fibroblasts. Tnfr1<sup>a</sup>/tnfr2<sup>a</sup> fibroblasts showed p90<sup>rb</sup> activation by hIL-1α but not by mTnft stimulation.

**Upstream Kinases of Mapk Signal Cascade—Raf-1 kinase and Mapk kinase (Mek) function as upstream transducing elements of the Mapk (Erk) pathway (61-63). To explore pathways leading to Erk activation, Raf-1, Raf-B, Mekk, and Mek-1 activities were investigated in immunoprecipitation kinase assays with wt fibroblasts.**

**DISCUSSION**

Several reports had provided evidence that members of the 90-kDa S6 kinase family (collectively termed rsk) can be phosphorylated and thereby stimulated by the ubiquitous and simultaneous expression of two distinct Tnf receptors on almost all cells is intriguing (6, 67-70). While their extracellular domains have sequence similarity, their intracellular domains are unrelated, suggesting distinct modes of activation of signal transduction pathways (6, 71). Studies using Tnfr1- and Tnfr2-selective agonists, such as monoclonal antibodies and Tnf muteins, demonstrated that the majority of known Tnf activities is mediated by Tnfr1 and can be elicited by exclusive Tnfr1 engagement. In contrast, a direct signaling activity of Tnfr2 was found only in a subset of Tnf activities, including the enhancement of T cell proliferation and the activation of NF-κB and cytotoxicity in cell lines and transfected cells (17-23). These findings prompted the proposition that Tnfr2 mainly has an accessory function to Tnfr1 signaling, either by enhancement and modulation of the Tnfr1 signals in intracellular transduction pathways or by ligand passing (5, 10, 24).

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NF-κB activation was found to be dependent by TNFR1 as well as TNFR2. This clearly demonstrates the importance of the TNF receptor interaction at the cell surface (9–12). These data are also consistent with the association kinetics of TNF to the two receptors measured with U937 cells where rate constants for the observed initial association of 0.002 and 0.037 min⁻¹ at 0 °C were measured for TNFR1 and TNFR2, respectively, the steady state of TNFR2 association not being reached before 2 h (10). There is an obvious parallel between the kinetics of TNF binding to the TNF receptors and the onset of the activation of the Mapk pathway, and it may be argued that the more rapid onset of the Mapk pathway activation under TNFR2 control reflects the faster on-rate of TNF and TNFR2 binding although there may be further rate-determining steps in the pathway that couples the receptor-proximal events and the Mapk pathway.

Interestingly, the time dependence of kinase activation after TNFR1 and TNFR2 stimulation was distinct. The kinase activations, when triggered through TNFR1, were shifted to a later onset and persisted for more prolonged times when compared with TNFR2 stimulation. To interpret these findings the differential binding kinetics of TNF to TNFR1 and TNFR2 must first be considered. The half-life times of TNF binding from the measurement of exchange rates at the cell surface have been determined as t1/2 > 3 h and t1/2 = 10 min for TNFR1 and TNFR2, respectively (10). These values agree well with exchange rates measured with the respective human dimeric recombinant TNFR1- and TNFR2-IgG heavy chain fusion constructs (i.e. t1/2 = 7 h and t1/2 = 5–10 min, respectively) which due to their dimeric structure with two receptor moieties binding one TNF trimer may be thought to approximate the TNF-TNF receptor interaction at the cell surface (9–12).

To further dissect TNFR1 and TNFR2 functions and signal transduction pathways at the level of transcription factors and the Mapk cascade, presumably downstream of the proteins engaging the cytoplasmic receptor domains such as TRAF and TRADD and of the sphinomyelinase pathways, we have studied TNF responses in primary fibroblast cultures from wt, TNFR1−/−, and TNFR2−/− mice. TNFR1 was found to exclusively mediate early and transient NF-κB activation and fibroblast proliferation. The exclusive control of NF-κB activation through TNFR1 in this system is consistent with previous findings in human umbilical cord vein endothelial cells and HL60 cells, whereas both TNFR1 receptors were found to control NF-κB activation in other cell lines and transfection studies, pointing to cell specificity in this response (14, 29, 72). In contrast to NF-κB, both TNFR1 and TNFR2 independently and synchronously activated AP-1 from 30 min to 3 h after TNF stimulation. The AP-1 activation demonstrated that both TNFR1 and TNFR2 independently are competent to accede major signal pathways. The independent signaling competence of TNFR1 and TNFR2 was further confirmed in the studies of various kinases of the Erk/Mapk signal transduction cascade: Erk1, Erk2, Raf-1, Raf-B, Mek-1, Mekk, and p90Rsk all were found to be activated independently by TNFR1 as well as TNFR2. This clearly demonstrates that the activation of the Mapk pathway alone does not suffice to elicit those TNF functions that are under exclusive TNFR1 control. The activation of the Mapk cascade through both TNFR1 and TNFR2 was transient, the stimulated enzyme activities in general being again down-regulated on a time scale of less than 1 h, whereas other responses such as AP-1 activation were more long lasting. It is noted that the down-regulation of these kinase activities occurred in the continued presence of active TNF concentrations, since no significant decrease of TNF activity within the first few hours could be detected in the culture media in control studies.

Furthermore, the TNF concentration chosen was found in preliminary control studies to be saturating with regard to the Mapk response. The time dependence of kinase activation after TNFR1 and TNFR2 stimulation was distinct. The kinase activations, when triggered through TNFR1, were shifted to a later onset and persisted for more prolonged times when compared with TNFR2 stimulation. To interpret these findings the differential binding kinetics of TNF to TNFR1 and TNFR2 must first be considered. The half-life times of TNF binding from the measurement of exchange rates at the cell surface have been determined as t1/2 > 3 h and t1/2 = 10 min for TNFR1 and TNFR2, respectively (10). These values agree well with exchange rates measured with the respective human dimeric recombinant TNFR1- and TNFR2-IgG heavy chain fusion constructs (i.e. t1/2 = 7 h and t1/2 = 5–10 min, respectively) which due to their dimeric structure with two receptor moieties binding one TNF trimer may be thought to approximate the TNF-TNF receptor interaction at the cell surface (9–12).

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### Table 1: Relative activation factors of Erk1/Erk2, Erk1, and Erk2 from in-solution kinase assays with immunoprecipitates of wt, tnfr1−/−, tnfr2−/−, and tnfr2−/−tnfr2−/− fibroblasts treated with mTNF or mH-1a, using Mbp as substrate

| mTNF (min) | wt | tnfr1−/− | tnfr2−/− | tnfr2−/−tnfr2−/− |
|-----------|----|----------|----------|------------------|
| 0         | 0  | 0        | 0        | 0                |
| 5         | 0  | 0        | 0        | 0                |
| 15        | 0  | 0        | 0        | 0                |
| 60        | 0  | 0        | 0        | 0                |
|            |    | 0        | 0        | 0                |

**Activation factor**

| mH-1a (min) | wt | tnfr1−/− | tnfr2−/− | tnfr2−/−tnfr2−/− |
|-------------|----|----------|----------|------------------|
| 5           | 0  | 0        | 0        | 0                |
| 15          | 0  | 0        | 0        | 0                |
| 60          | 0  | 0        | 0        | 0                |
|            |    | 0        | 0        | 0                |

**Activation factor**

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FIG. 7. Time-dependent activation of p90Rsk in mTNF- and mH-1a-treated primary mouse fibroblasts. Wt, tnfr1−/−, tnfr2−/−, and tnfr2−/−tnfr2−/− fibroblasts in parallel cultures were treated with mTNF (10 ng/ml) for various times or with mH-1a (10 ng/ml, 15 min). Cell lysates (calibrated to 30 μg total protein) were immunoprecipitated with 1 μg of p90Rsk-specific polyclonal antibody, and in-solution kinase assays were performed with immunoprecipitates using Mbp and [γ-32P]ATP. Phosphorylated Mbp substrate was visualized by SDS-PAGE and PhosphorImager analyses under standard conditions. Dried gels were exposed to PhosphorImager screens, and 33P incorporation was quantified by image analysis (Imagequant software package, Molecular Dynamics).

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PhosphorImager counts (PIC), means ± S.D. of two independent immunoprecipitation kinase assays with erk1-CT, erk1-NT, and erk2 antibodies. The counts for each of the kinases and for each independent experiment were normalized to those of time 0 (PICo) by defining the activation factor (AF) at time t by AFt/PICt/PIC0 and the mean ± S.D. of the normalized PIC is presented.
regulating signal progression (73–75). The cells used in the present studies have been rendered quiescent by serum deprivation in order to increase the signal-to-noise ratio of activated kinase responses. While it cannot be ruled out entirely, it is unlikely that quiescence generates a kinase reactivity pattern unrelated to the normal cell condition. It may be envisioned rather that in a cell under standard conditions the signals leading to Mapk activation as documented in the present studies are superimposed as transients on a background of ongoing normal cellular activity in the Mapk pathway.

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REFERENCES

1. Beutler, B. (1992) *Tumor Necrosis Factors*, Raven Press, Ltd., New York
2. Vassalli, P. (1992) *Annu. Rev. Immunol.* 10, 411–452
3. Tracey, K. J., and Cerami, A. (1993) *Annu. Rev. Cell Biol.* 9, 317–343
4. Loetscher, H., Steinmetz, M., and Lesslauer, W. (1991) *Cancer Cells* 3, 201–226
5. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995) *Nature* 372, 560–563
6. Dembic, Z., Loetscher, H., Gubler, U., Pan, Y.-C. E., Lahm, H.-W., Gentz, R., Brokhaus, M., and Lesslauer, W. (1990) *Nature* 345, 560–563
7. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) *Cell* 76, 959–962
8. Bazzoni, F., and Beutler, B. (1993) *J. Immunol.* 151, 421–428
9. Loetscher, H., Angehrn, P., Schlaeger, E. J., Gentz, R., and Lesslauer, W. (1992) *J. Biol. Chem.* 267, 5889–5892
10. Tartaglia, L. A., Benacerraf, B., Fiers, W., and Lesslauer, W. (1993) *Bacterial Endotoxin: Recognition and Effector Mechanisms* (Levin, J., Alving, C. R., Munford, R. S., Stutz, P. L., eds) pp. 455–462, Elsevier Science Publishers, Amsterdam
11. Tartaglia, L. A., Benacerraf, B., Fiers, W., and Lesslauer, W. (1993) *Nature* 367, 661–663
12. Belda, C., Wiegmann, K., Adam, D., Holland, R., Neubohr, M., Herrmann, F., Shaz, B. M. (1995) *EMBO J.* 14, 1156–1165
13. Pages, G., Lenormand, P., Allemand, G., Chambard, J.-C., Meloche, S., and Pouyssegur, J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 8319–8323
14. Sarkadi, L., Rawlinson, L. M., Marshall, J. C., and Kracht, M. (1995) *FEBS Lett.* 334, 189–192
15. Royer, I., Schwenger, P., Li, W., Schlissinger, J., and Vilcek, J. (1993) *J. Biol. Chem.* 268, 18994–18999
16. Dignam, J. D., Lebovitz, R. H., and Roeder, R. G. (1983) *Nucleic Acids Res.* 11, 1475–1489
17. Laemmli, U. K. (1970) *Nature* 227, 680–685
18. Vilcek, J., Palmobella, V., and Hebron, S. (1993) *Biochim. Biophys. Acta* 1191, 258–264
19. George, G., Genta, R., Brokhaus, M., Loetscher, H., and Lesslauer, W. (1992) *J. Immunol.* 149, 911–917
20. Hohmann, H. P., Brokhaus, M., Baeuerlein, P. A., Remy, R., Kolbeck, R., and van Loon, A. P. G. M. (1990) *J. Biol. Chem.* 265, 22409–22417
21. Laemml, R., and Medvedev, A. N., Unostad, U., Bombara, M. P., Ranges, G., Sundan, A., and Espevik, T. (1983) *J. Biol. Chem.* 258, 7775–7781
22. Heller, R. A., Song, K., and Fan, F. (1993) *J. Cell Biol.* 73, 216
23. Heller, R. A., and Kromke, M. (1994) *J. Cell Biol.* 126, 5–9
24. Rothe, J., Loetscher, H., Bluehmann, L., Lang, Y., Kolbeck, P., Koenig, F., Allthage, A., Zinkernagel, R., Steinmets, M., and Bluehmann, H. (1993) *Nature* 364, 798–802

TABLE II

| mTfno (min) | Activation factor<sup>a</sup> |
|-------------|-----------------------------|
|             | wt | tfnfr<sup>1</sup> | tfnfr<sup>2</sup> | tfnfr<sup>1</sup>/tfnfr<sup>2</sup> |
|             | 5  | 15  | 60 | 5  | 15  | 60 | 5  | 15  | 60 |
| p90<sup>6a</sup> | 2.6 | 2.0 | 2.4 | 2.6 | 2.0 | 2.4 | 2.6 | 2.0 | 2.4 |
| Raf-1 | 0.3 | 0.1 | 0.0 | 0.3 | 0.1 | 0.0 | 0.3 | 0.1 | 0.0 |
| Raf-B | 1.0 | 0.2 | 0.0 | 1.0 | 0.2 | 0.0 | 1.0 | 0.2 | 0.0 |
| Mek-1 | 0.4 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 |
| Mekk | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

<sup>a</sup>Phiophosphorimer counts (PIC), mean ± S.D. of three independent immunoprecipitation kinase assays with anti-p90<sup>6a</sup>, anti-Raf-1, anti-Raf-B, anti-Mek-1, and anti-Mekk antibodies. The counts for each of the kinases and for each independent experiment were normalized to those of time 0 (PIC<sub>0</sub>) by defining the activation factor (AF) at time t by AF<sub>t</sub> = PIC<sub>t</sub>/PIC<sub>0</sub> and the mean ± S.D. of the normalized PIC are presented.

26. Erickson, S. L., de Sauvage, F. J., Kikly, K., Carver-Moore, K., Pitts-Mek, S., Gillette, N., Sheehan, K. C. F., Schreider, R. B., Goddell, D. V., and Moore, M. W. (1994) *Nature* 372, 560–563
27. Darnay, B. G., Reddy, S. A. L., and Aggarwal, B. B. (1994) *J. Biol. Chem.* 269, 20299–20304
28. Rothe, M., Wong, S. C., Henzel, W. J., and Goddell, D. V. (1994) *Cell* 78, 499–502
Ashworth, A., Marshall, C. J., and Cowley, S. (1994) EMBO J. 13, 1610–1619
64. Storm, S. M., Cleveland, J. L., and Rapp, U. R. (1990) Oncogene 5, 345–351
65. Traverse, S., and Cohen, P. (1994) FEBS Lett. 350, 13–18
66. Catling, A. D., Reuter, C. W. M., Cox, M. E., Parsons, S. J., and Weber, M. J. (1994) J. Biol. Chem. 269, 30014–30021
67. Loetscher, H., Schlaeger, E. J., Lahm, H.-W., Pan, Y.-C. E., Lesslauer, W., and Brockhaus, M. (1990) J. Biol. Chem. 265, 20131–20138
68. Loetscher, H., Pan, Y.-C. E., Lahm, H.-W., Gents, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. (1990) Cell 61, 351–359
69. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rite, G. C., Wang, G. H. W., Gutman, T., Granger, G. A., Lentz, R., Raab, H., Kehr, W. J., and Goeddel, D. V. (1990) Cell 61, 361–370
70. Smith, C. A., Davis, T., Anderson, D., Solam, M., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990) Science 248, 1019–1023
71. Tartaglia, L. A., and Goeddel, D. V. (1992) J. Biol. Chem. 267, 4304–4307
72. Wiegmans, K., Schuetze, S., Kampen, E., Himmler, A., Machleidt, T., and Kronke, M. (1992) J. Biol. Chem. 267, 24069–24075
73. Wu, J., Lau, L. F., and Sturgill, T. W. (1994) FEBS Lett. 353, 9–12
74. Liu, Y., Gorospe, M., Yang, C., and Holbrook, N. J. (1995) J. Biol. Chem. 270, 8377–8380
75. Chu, Y., Solski, P. A., Khororavi-Far, R., Der, C. J., and Kelly, K. (1996) J. Biol. Chem. 271, 6497–6501