Abstract. The various biological activities of tumor necrosis factor (TNF) are mediated by two receptors, one of 55 kD (TNF-R55) and one of 75 kD (TNF-R75). Although the phenotypic and molecular responses elicited by TNF in different cell types are fairly well characterized, the signaling pathways leading to them are so far only partly understood. To further unravel these processes, we focused on TNF-R55, which is responsible for mediating most of the known TNF effects. Since several studies have demonstrated the importance of receptor clustering and consequently of close association of the intracellular domains for signaling, we addressed the question of whether clustering of the intracellular domains of TNF-R55 (TNF-R55i) needs to occur in structural association with the inner side of the cell membrane, where many signaling mediators are known to reside. Therefore, we investigated whether induced intracellular clustering of only TNF-R55i would be sufficient to initiate and generate a full TNF response, without the need for a full-length receptor molecule or a transmembrane region. Our results provide clear evidence that inducible forced trimerization of either TNF-R55i or only the death domain elicits an efficient TNF response, comprising activation of the nuclear factor κB, induction of interleukin-6, and cell killing.

Tumor necrosis factor (TNF) is a multifunctional cytokine mainly produced by activated macrophages. It was originally identified by its antitumoral activity in vivo and its selective cytotoxic/cytostatic action on some transformed cell lines (Carswell et al., 1975). Since then, it has become clear that TNF exerts a wide variety of biological activities on both transformed and untransformed cells. TNF is an important mediator in inflammation and cellular immune responses and is also believed to play a key role in the pathology of many diseases (for reviews see Vassalli, 1992; Fiers, 1995; Aggarwal and Natrajan, 1996). In its biologically active form, TNF is a trimeric protein consisting of three identical 17-kD subunits (Wingfield et al., 1987; Jones et al., 1989). Two TNF receptors with molecular masses of 55 kD (TNF-R55) and 75 kD (TNF-R75) have been identified, cloned, and characterized for several species (for review see Vandenabeele et al., 1995). They are present on nearly all cell types and both belong to a growing TNF receptor superfamily, which covers more than 10 receptor proteins (for review see Smith et al., 1994). Nearly all family members are type I membrane proteins that show sequence homology only in their extracellular domains, consisting of cysteine-rich pseudorepeats. Their respective cytoplasmic domains, however, generally lack sequence homology among themselves (except for a small homologous region present in the intracellular parts of TNF-R55 [TNF-R55i] and Fas antigen), which suggests major differences in signaling mechanisms. Moreover, these receptors do not contain sequences suggestive of intrinsic catalytic activity. TNF-R55 is the receptor responsible for mediating most of the effects exerted by TNF, including activation of the nuclear factor κB (NF-κB), gene induction, synthesis of prostaglandins, and cytotoxicity. TNF-R55i lacks intrinsic kinase activity and has no SH2 domain. TNF-R55i contains two functional domains: a COOH-terminal region of ~80 amino acids, called death domain (DD), which is responsible for NF-κB activation and signaling cytotoxicity, and an NH2-terminal region involved in induction of NO synthase (Tartaglia et al., 1993) and activation of several kinases and phospholipases (Wiegmann et al., 1994; Belka et al., 1995; Adam et al., 1996b). A region homologous to
the DD is present in the intracellular part of the fas receptor (Itoh and Nagata, 1993). Recently, genes coding for several proteins that can associate with members of the TNF receptor superfamily or are involved in the signaling pathway have been cloned and their products characterized (Rothé et al., 1994; Boldin et al., 1995a, 1996; Chin-naiyan et al., 1995; Hsu et al., 1995; Stanger et al., 1995; Muzio et al., 1996). The majority of these proteins are directly or indirectly associated with the DD of TNF-R55. However, a number of proteins that associate with the DD of TNF-R55 were produced in Escherichia coli (Boulder, CO). In CAT-ELISA, 1/1,000 dilutions of the commercial preparation were used.

TNF signaling is initiated by receptor clustering, either by the trivalent ligand TNF or by cross-linking monoclonal antibodies (Engelmann et al., 1990; Espevik et al., 1990). It has been demonstrated that expression of human TNF-R55 (hTNF-R55) deletion mutants, lacking most of their intracellular domain, in murine cells failed to elicit cytotoxicity after anti-hTNF-R55 antibody treatment. Moreover, expression of these mutant human receptors suppressed endogenous murine TNF-R55 (mTNF-R55) signaling after TNF treatment, probably by formation of receptor clusters combining murine functional with human truncated, nonfunctional TNF-R55 (Brakebusch et al., 1992).

From these and other results, we may conclude that TNF-R55 clustering is necessary and sufficient for initiating the signaling cascade. As many receptor-initiated signaling pathways are directly linked to membrane-associated components (such as G proteins, Grb, Sos, PKC, and Ras), we inquired whether the membrane juxtaposition of TNF-R55i is also important and necessary for signaling. Therefore, we developed a system for the controlled expression in the cytoplasm of L929 cells of a trimerized hTNF-R55i complex, obtained by fusion of the intracellular receptor domain to the cytosolic, trimeric chloramphenicol acetyltransferase (CAT) protein. Our results demonstrate that, while the induced intracellular expression of hTNF-R55i as such in L929sA cells could not initiate TNF effects, induced expression of the trimeric construct could indeed elicit effectively the various TNF-signaling pathways leading to NF-κB activation, gene induction, and cytotoxicity.

Materials and Methods

Cytokines, Antibodies, and Reagents

mTNF and hTNF were produced in Escherichia coli and purified to at least 99% homogeneity in our laboratory. The specific activity of the preparations was tested in a standard cytotoxicity assay. Recombinant human interferon (hIFN-α (A/D), which is also active on murine cells, was a generous gift from Dr. C. Weissmann (University of Zurich, Zurich, Switzerland); it had a specific antiviral activity of 7.9 × 10^4 U/ml as determined on murine cells in an L929 cell/HSV assay. mIFN-γ was produced in CHO cells and had a concentration of 10^3 U/ml.

htr-13, a murine monoclonal antibody directed against hTNF-R55i, was a generous gift from Dr. M. Brockhaus (Hoffmann-La Roche, Basel, Switzerland; Brockhaus et al., 1990). Purified polynuclear rabbit anti-CAT antibody, biotinylated anti-CAT antibody, streptavidin-alkaline phosphatase, and pure CAT enzyme were purchased from 5 Prime-3 Prime, Inc. (Boulder, CO). In CAT-ELISA, 1/1,000 dilutions of the commercial preparations were used.

1-deoxy[dichloroacetyl-1-14C] chloramphenicol (55 mCi/mmol) and [α-35S]-methionine, with and without rifampicin in the growth medium. E. coli 2580/ITpP26 bacteria (Mertens et al., 1995), transformed with pETThTNFR55i, were grown overnight at 28°C in Luria broth, supplemented with triacilin and kanamycin. The next morning, the culture was diluted 50-fold in Luria broth, further grown until an A_600 of 0.5 was obtained, and induced with IPTG (1 mM final concentration). After 45 min of growth at 20°C, the culture was split; rifampicin (200 μg/ml) was added to half of the culture. 45
Construction of an Inducible Eukaryotic hTNF-R55i Expression Vector pMxhTNFR55i

First, a universal eukaryotic expression plasmid was made by introducing an SV-40 DNA fragment, containing the small t-splice sequences and polyadenylation signals, into the multicloning site following the Mr promoter of pSP64Mx, thus creating pSP64Mxpa. Subsequently, the cDNA coding for hTNF-R55i was isolated from pETHTNFR55i as an XbaI fragment and cloned into the same site of the multicloning site of pSP64Mxpa. In this way, a vector for hIFN-α–inducible expression of hTNF-R55i in mammalian cells was created, which was named pMxhTNFR55i.

Construction of Eukaryotic CAT (Fusion) Expression Plasmids

The cDNA coding for CAT was transferred from pBLCAT5 as an SauAI fragment into the multicloning site of pSP64Mxpa, creating pMxCAT. The cDNA of hTNF-R55i was recovered from pMxhTNFR55i and introduced into pMxCAT, in-frame at the 3'-end of the CAT-coding sequence, and separated by an oligonucleotide coding for a flexible spacer peptide Gly-Gly-Ser-Gly. In one construction, pMxCAThTNFR55i, the stop codon of the CAT gene was omitted, which resulted in a translatable fusion protein CAT-R55i. In a parallel construction, pMxCAT(stop)-hTNFR55i, to be used as a control, the CAT termination codon was conserved. In this way, the two vectors are expected to result in almost identical translation products. pMxCAThTNFD was constructed by exchanging the cDNA of the total hTNF-R55i for the cDNA of the DD, the first amino acid of the DD. The cDNA coding for CAT was transferred from pBLCAT5 as an SauAI fragment into the multicloning site following the Mr promoter of pSP64Mx, thus creating pSP64Mxpa. Subsequently, the cDNA coding for hTNF-R55i was isolated from pETHTNFR55i as an XbaI fragment and cloned into the same site of the multicloning site of pSP64Mxpa. In this way, a vector for hIFN-α–inducible expression of hTNF-R55i in mammalian cells was created, which was named pMxhTNFR55i.

Stable Transfection of Eukaryotic Expression Plasmids into L929sA Cells

The plasmids carrying the cDNA sequences for hTNF-R55i and the different CAT fusions were transfected into L929sA cells using the calcium phosphate precipitation method (Graham and van der Eb, 1973) and the neomycin gene, coded for by pSV2neo, as a selection marker. G418-resistant clones were picked up after 10–12 d of selection with 400 μg/ml of G418.

Cytotoxicity Tests

L929sA cells were seeded in 96-microwell plates at 15,000 cells/well on day −1 in 100 μl DMEM growth medium. At day 0, inductions were carried out by adding the appropriate concentration of the agonists in 100 μl medium. After the indicated time period, cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining (Tada et al., 1986).

Alternatively, cells were seeded in bacterial-grade Petri dishes (Sterilin, Feltham, UK) under which conditions they no longer adhered to the plastic surface and remained in suspension. At appropriate time intervals after induction, samples were subjected to a propidium iodide (PI) exclusion assay as described (Grooten et al., 1993).

Northern Blot Analysis

Induction of mRNA expression in parental or transfected L929sA cells was assessed by Northern blot analysis. In brief, cells were grown in 9-cm dishes. At confluency, they were treated with 1,000 IU/ml hIFN-α (A/D) for the indicated time period. Total RNA was isolated with TRIzol reagent (Life Technologies, Paisley, UK), denatured in glyoxal buffer, and separated on a 1.2% agarose gel containing 20 mM phosphate buffer, pH 7. RNA was fixed on Hybond-N+ membranes (Amersham Life Science) using a standard capillary blotting procedure. Membranes were hybridized with an appropriate probe: hTNF-R55i, interleukin-6 (IL-6), or glyceraldehyde-3-phosphate dehydrogenase cDNA fragment, labeled with α-32PdCTP using a Random Primed Labeling Kit (Boehringer Mannheim).

IL-6 Assay

IL-6, secreted in the medium of the expressing cells, was quantified by its growth-stimulating activity on 7TD1 cells, measured by colorimetric determination of the hexosaminidase levels (Laedegren, 1984).

NF-κB Activity

Activation of NF-κB was measured in an electrophoretic mobility shift assay. Confluent monolayers of transfected L929sA cells were treated with hIFN-α or mTNF for varying periods of time. Nuclear protein extracts and binding reactions were performed as described previously (Pates et al., 1993). The core site of the double-stranded oligonucleotide, used in the binding reaction, was the murine IL-6-κB motif -GGGATTTC-. It was 32P-labeled using Klenow enzyme in a terminal fill-in reaction. After purification, 4,000–8,000 cpm of the freshly labeled oligonucleotide (corresponding to ~50–100 pg or 3–5 fmol) was used for the binding assay.

CAT-ELISA

To quantify the amount of CAT protein present in cell lysates, a sandwich immunosorbent assay was optimized in our laboratory (Vanden Berge et al., 1993) based on the original protocol (Boehringer Mannheim).

CAT Enzyme Assay

Confluent monolayers of parental or transfected L929sA cells were treated with 1,000 IU/ml hIFN-α or left untreated. After 6 h, cells were washed with PBS, pelleted, resuspended in 0.25 M Tris-HCl, pH 7.8, and subjected to four freeze–thaw cycles by alternate transfer of the suspensions between a dry ice/ethanol bath and a 37°C water bath (5 min in each). After 15 min of centrifugation at 4°C, supernatants were assayed for enzymatic activity. For immunoprecipitation, htr-13 was added to the cell lysates for 2 h, followed by a 2-h incubation with protein G–Sepharose beads. The enzymatic assay was subsequently carried out with the beads. The assay mixture contained 1 μC of [32P]-deoxychloramphenicol, 20 μg of protein extract or immunoprecipitated lysate, and 10 μl of 9 M acetyl coenzyme A in a total volume of 90 μl. Controls contained CAT enzyme instead of cell extract. All reagents, except coenzyme A, were preincubated together for 5 min at 37°C. After equilibration had been reached at this temperature, the reaction was started by adding coenzyme A. The reaction went on for 2.5 h at 37°C and was stopped with 1 ml of ice-cold ethyl acetate, which was used to extract the chloramphenicol. The organic layer was dried and taken up in 20 μl ethyl acetate. 5 μl of this solution was spotted on silica gel thin-layer plates and resolved with chloroform/methanol (95:5, ascending). Visualization of the separated acetylated form was obtained by incubation in a Storage Phosphor Screen and quantification with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Results

Bacterial Expression of hTNF-R55i cDNA

We converted the cDNA sequence corresponding to hTNF-R55i into an independently translatable cistron by creating an ATG codon at the beginning of the hTNF-R55i–coding sequence, following a previously described strategy (Nakamaye and Eckstein, 1986). In this way, an ATG codon was obtained at position 201 (numbering of the entire mature hTNF-R55 according to Loetscher et al., 1990), i.e.,
three amino acids before the presumed first amino acid of the intracellular domain (Fig. 1 A). At the same time, an extra NcoI site was created. The last amino acid of the transmembrane region of hTNF-R55 is believed to be Leu 203 (Loetscher et al., 1990). (B) Schematic representation of the different CAT fusion proteins. The numbering of the amino acids is according to Loetscher et al. (1990). Gray boxes represent the DD of hTNF-R55.

The hTNF-R55i cDNA gene was recloned in the bacterial expression vector pET11d (Rosenberg et al., 1987). Transformation in K12 strain 2580/pT7pol26 (Mertens et al., 1995) and subsequent induction with IPTG clearly revealed the production of a polypeptide with the correct size (30 kD) after Coomassie brilliant blue staining of an SDS gel. The same result was obtained after metabolic labeling of induced bacteria in the absence or presence of rifampicin. Figure 2 depicts the results of these experiments. The 30-kD protein expressed in Escherichia coli could be immunoprecipitated selectively with htr-13, a monoclonal antibody specific for hTNF-R55i (Fig. 2 b). Since these experiments demonstrated that the recombinant hTNF-R55i cistron gave rise to a polypeptide of the correct size, it was isolated from the bacterial expression vector and recloned in a eukaryotic expression vector, creating the plasmid pMxhTNFR55i.

Induced Expression of hTNF-R55i in the Cytoplasm of L929sA Cells

In pMxhTNFR55i, the coding sequence for hTNF-R55i is under control of the murine Mx promoter, which is selectively inducible by type I IFN and which has been shown to result in tightly controlled heterologous gene expression in VERO cells (Leonart et al., 1990).

Murine fibrosarcoma L929sA cells were transfected with pMxhTNFR55i using the calcium phosphate procedure. Several G418-resistant clones were isolated and tested for TNF effects after induction with 1,000 IU/ml hIFN-α. Since L929sA cells are very sensitive to the cytotoxic effect of TNF, we first tested for the occurrence of cell killing. However, we could not observe cell death in any of the selected clones, even after a long induction period (up to 72 h) with hIFN-α. Therefore, the integration of hTNF-R55i cDNA into the host genome and its transcription in the corresponding mRNA were checked by Southern and Northern analysis, respectively. The results revealed that all of the clones tested had indeed taken up the cDNA and expressed low levels of hTNF-R55i mRNA after induction with hIFN-α. The presence of the corresponding protein, however, could not be demonstrated. Since TNF has been shown to induce IL-6 expression in L929s cells (Defilippi et al., 1987; Vandevoorde et al., 1991), we checked whether this activity could be observed after induced expression of hTNF-R55i. The medium of hIFN-
α-induced transfectants was tested in an IL-6 bioactivity assay; no increase in IL-6 levels as compared to the background level of noninduced transfectants could be detected.

We concluded that expression of hTNF-R55i in L929sA cells under control of the Mx promoter was not sufficient to initiate TNF signalization. In all likelihood, the low induced expression level in the clones obtained did not allow us to demonstrate the presence of the protein and furthermore did not lead to an effective aggregation or clustering required for signaling. The possibility that higher expressing clones were counter-selected because of leakage expression in the noninduced state cannot be excluded.

**Induced Expression of Trimerized hTNF-R55i Elicits Cytotoxicity in L929sA Cells**

In view of the negative results with solitary expression, we developed an inducible system designed to force direct clustering of the hTNF-R55i domains. This consisted of the hTNF-R55i domain fused to CAT, a cytosolic, trimeric protein (Leslie, 1990). The resulting construct pMxCAThTNFR55i (where the hTNF-R55i cDNA is coupled in-frame via a tetrapeptide linker to the coding sequence of CAT) and the control vector pMxCAT (stop)-hTNFR55i (where the continuous reading frame of the fusion product is interrupted by the natural termination codon of the CAT gene; Fig. 1 B) should yield almost identical mRNAs (except for the three nucleotides of the stop codon) but should give rise to totally different protein products, namely the fusion protein CAT-R55i and CAT, respectively.

Both constructs were transfected in L929sA cells. From repeated experiments, it soon became apparent that after selection, the number of clones transfected with pMxCAThTNFR55i was considerably lower than that obtained with pMxCAT(stop)hTNFR55i. This observation indicated a strong counter-selection pressure in the transfection experiment producing CAT-R55i molecules (see below). Several G418-resistant clones were examined for cell killing after addition of 1,000 IU/ml hIFN-α to the medium. In the pMxCAThTNFR55i-transfected cells, at least 50% of the clones tested died within 24 h after administration of hIFN-α, while the G418-resistant cells transfected with the analogous construct pMxCAT(stop)hTNFR55i did not die, and no change in their growth or morphology was observed. As opposed to our previous experiments with the solitary intracellular domain, these results definitely demonstrate that after “trimerization” of hTNF-R55i, the signaling by TNF leading to cell killing could be reproduced by the hIFN-α-inducible system. A particular clone, CAT-R55i cl 2, was used for detailed characterization. The hIFN-α–induced cytotoxicity in this clone (Fig. 3,

**Figure 3.** hIFN-α–induced cytotoxicity in cells expressing CAT and CAT-R55i. CAT cl 15 (A and C) and CAT-R55i cl 2 (B and D) were treated with 1,000 IU/ml hIFN-α. Photographs were taken 8 h (A and B) or 24 h later (C and D). Bar, 100 μm.
B–D) can be compared with the nontoxic response of hIFN-α on a CAT-expressing clone (Fig. 3, A–C). By following the cytotoxicity as a function of time, either by staining of surviving cells with MTT (Fig. 4) or by PI uptake by dying cells (Fig. 5), we observed that 4 to 5 h after administration of hIFN-α, cell death in CAT-R55i–expressing cl 2 became already apparent (Fig. 4 A), while the same treatment on CAT cl 15 (Fig. 4 B) or parental L929A cells had no cytotoxic effect. In addition, Northern blot analysis revealed that mRNA of CAT(stop)R55i and CAT-R55i became detectable 2 h after induction (Fig. 6A). The much lower level of transcript in the latter cells as compared to the former will be discussed in a later section. The observed transcription/translation kinetics correlate well with the expression pattern of other Mx-controlled genes (Lleonart et al., 1990). As a matter of fact, the time kinetics of cell killing after treatment of CAT-R55i cl 2 with hIFN-α are very comparable with the kinetics obtained after treatment of the same cells or parental L929sA cells with TNF (Fig. 4, C and D). In addition, several other treatments that augment or decrease cytotoxicity induced by TNF in L929s cells were also examined in CAT-R55i cl 2 after hIFN-α treatment. The combined hIFN-α/LiCl treatment resulted in a synergistic enhancement of the cytotoxicity in CAT-R55i–expressing cells (Fig. 4 E), as seen with a combined TNF/LiCl treatment of parental L929s cells (Beyaert et al., 1989). Treatment of CAT-R55i cells with hIFN-α in combination with dexamethasone leads to partial reduction of the cytotoxicity (results not shown), as does a combined TNF/dexamethasone treatment on parental L929s cells (Suffys et al., 1987).

IFN-γ induces many of the same effects as type I IFNs, but cannot, for example, induce the Mx gene. Indeed, mIFN-γ could not mimic the effects induced by hIFN-α (Fig. 4 A), and this provides further proof that the expression of CAT-R55i, leading to cytotoxicity, was an Mx promoter-driven effect (Fig. 4, A and B). Finally, the cytotoxicity after a combined treatment with hIFN-α and ActD, as compared to an hIFN-α treatment alone, further showed that cell killing depended on the expression of CAT-R55i fusion protein (Fig. 4 F). In contrast, TNF/ActD treatment of parental L929sA cells resulted in an augmented cytotoxicity, most probably because protective proteins cannot be synthesized.

Figure 4. Kinetics of cell death by trimerized TNF-R55i. Cytotoxicity on parental L929sA cells (D), CAT cl 15 (B), and CAT-R55i cl 2 (A, C, E, and F) was measured at different time points, following different induction conditions, by MTT conversion. Inductions were carried out using the following concentrations: 1,000 IU/ml hIFN-α (■), 1,000 IU/ml mIFN-γ (□), 10 mM LiCl (●), 10 mM LiCl + 1,000 IU/ml hIFN-α (○), 1 μg/ml ActD (▲), 1 μg/ml ActD + 1,000 IU/ml hIFN-α (△), 500 IU/ml mTNF (×), or 500 IU/ml hTNF (∗). The percentage of cell survival of treated cells was compared to the untreated cell population (●).
In summary, the hIFN-α-driven signal for cell killing is entirely comparable, both in a qualitative and a quantitative way, to the cytotoxicity elicited by TNF itself. This means that the intracellular expression of trimerized hTNF-R55i polypeptide is by itself sufficient for generating cytotoxic mechanisms within a cell, without the need for direct linkage to the transmembrane domains of the TNF receptor.

**Induced Expression of Trimerized hTNF-R55i Results in NF-κB Activation and IL-6 Gene Expression**

Since TNF activates NF-κB and induces IL-6 expression in L929s cells (Patestos et al., 1993), we investigated whether the induced expression of trimerized hTNF-R55i could also elicit these TNF effects in CAT-R55i-transfected clones. To check for NF-κB activation, gel-shift analysis was performed as previously described (Patestos et al., 1993). Selected transfectants expressing either CAT-R55i (cl 2) or CAT (cl 15) were induced with hIFN-α; cell lysates were made after different periods of induction, ranging from 30 min to 4 h. Activated NF-κB complex started to appear after 2.5 h and became prominent after 4 h of hIFN-α treatment in cells expressing CAT-R55i, but not in transfectants producing CAT protein (Fig. 7).

To test for IL-6 gene expression, medium of hIFN-α-induced transfectants expressing CAT-R55i or CAT was collected at several time points after induction and analyzed for IL-6 bioactivity. CAT-R55i-expressing cells showed elevated IL-6 expression levels as compared to noninduced cells, hIFN-α-treated, parental L929sA cells, and hIFN-α-treated CAT-expressing cells. IL-6 expression was already detectable 5 to 6 h after administration of hIFN-α. Northern blot analysis with total RNA samples extracted at different induction periods confirmed these results (Fig. 6 b).

Taken together, expression of trimerized hTNF-R55i molecules in L929sA cells elicited a full TNF response, including cell killing and gene induction effects.
Expressing clones (Fig. 6). Considering the much smaller mRNA abundance in cl 2 as compared to cl 15 and the control cells could easily be demonstrated. The lower, constitutively binding band has recently been characterized (Plaisance et al., 1997).

Transfection with pMxCAThTNFR55i Is Subject to Severe Counter-Selection, and as a Consequence, Trimerized hTNF-R55i Is Only Expressed in Low Quantities in Surviving Clones

To demonstrate the presence of induced proteins in transfected cells, various approaches were tried. Although the CAT protein produced by induced pMxCAT(stop)-hTNFR55i-transfected cl 15 could easily be demonstrated and quantified by CAT-ELISA (30 to 50 ng/mg protein) or visualized by immunofluorescence, CAT-R55i fusion protein of induced pMxCAThTNFR55i-transfected cl 2 could not be revealed unambiguously with any of these techniques. Furthermore, attempts to immunoprecipitate the fusion protein also failed, although the CAT protein in the control cells could easily be demonstrated.

Therefore, mRNA expression levels of the previously selected, inducible CAT-R55i-expressing cl 2 and CAT-expressing cl 15 were checked after induction with hIFN-α by Northern blot analysis using a hTNF-R55i cDNA fragment as a common probe. The results clearly reveal the low mRNA abundance in cl 2 as compared to cl 15 and suggest that, although the former is biologically active, there must have been selection for very low CAT-R55i-expressing clones (Fig. 6a). Considering the much smaller number of transfectants with pMxCAThTNFR55i, clones with a higher expression level probably did not survive the 2-wk selection procedure in G418-containing medium because a small leakage of the Mx promoter, generating continuously minute amounts of CAT-R55i protein, may have killed the positive cells. In contrast, inducible expression of a nonlethal protein, such as the CAT protein itself, did not lead to counter-selection, as cells easily tolerated a certain degree of leakiness in promoter control.

Yet, we were able to demonstrate and to quantify the CAT-R55i fusion protein in the transfected cells by making use of the enzymatic activity of the CAT protein in front of the receptor domain. The results of this assay, performed on total protein extracts, clearly demonstrated inducible enzymatic CAT activity in CAT-R55i cl 2 and of course in cl 15 (Fig. 8A). In agreement with the very different levels of expression revealed by Northern blot analysis, the level of enzymatic activity in cl 2 was much lower than the quantities in cl 15 cells. This again indicates that only low CAT-R55i-expressing cells survived the transfection/selection procedure, i.e., presumably only those with no leakage of the Mx promoter.

To ascertain that the enzymatic activity observed in CAT-R55i cl 2 extract corresponded to intact CAT-R55i protein and, for example, not to a proteolytically derived CAT protein, we immunoprecipitated lysates from cl 2, cl 15, or parental L929sA cells with htr-13, an antibody that specifically recognizes hTNF-R55i; these immunoprecipitates were used for an enzymatic CAT assay. CAT enzymatic activity was clearly demonstrated with immunoprecipitates from cl 2 after induction, while immunoprecipitates from cl 15 or parental L929sA cells were negative (Fig. 8A). These results unambiguously demonstrate that the enzymatic activity derived from cl 2 was effectively generated by an intact CAT-R55i fusion protein.

The possibility that additional mechanisms may have contributed to the occurrence of only limited amounts of the lethal fusion protein, even after induction with hIFN-α, cannot be excluded. Indeed, several groups have reported on the probable existence of protective mechanisms within the cell designed to restrict the number of TNF-R55 molecules per cell, as overexpression of TNF-R55 is lethal (Brakebusch et al., 1992; Tartaglia and Goeddel, 1992; Boldin et al., 1995). Degradation by proteasomes could be such a mechanism (Darnay et al., 1994). In agreement with this hypothesis, addition of the proteasome inhibitor PSI (Figueiredo-Pereira et al., 1994; Traenckner et al., 1994) to hIFN-α-induced CAT-R55i-expressing cells resulted in an 1.8-fold higher expression level of CAT-R55i as measured by the CAT assay (Fig. 8B) and a more rapid cell killing. This suggests that (partial) prevention of CAT-R55i protein degradation synergistically augmented the cytotoxicity. Nevertheless, even under these conditions we were unable to directly demonstrate the presence of CAT-R55i fusion protein by CAT-ELISA or immunoprecipitation.

Induced Expression of Trimerized CAT-DD Fusion Protein Results in IL-6 Synthesis and Cytotoxicity in L929s Cells

Since the DD of hTNF-R55 was reported to be sufficient for induction of cytotoxicity, we investigated whether forced trimerization of only the DD of hTNF-R55 by fusion to the CAT gene (CAT-DD) would also induce cytotoxicity. A first expression vector pMxCAThTNFDD was constructed such that the sequence coding for the DD was linked almost directly to the COOH terminus of the CAT gene (Fig. 1B). This construct was transfected into L929sA cells, in parallel with the pMxCAThTNFR55i and the pMxCAT(stop)hTNFR55i constructs as a reference. Comparison of the number of antibiotic-resistant clones obtained indicated again an evident counter-selection for cells transfected with pMxCAThTNFR55i, but not for cells transfected with pMxCAThTNFDD or pMxCAT(stop)---

Figure 7. NF-κB activation as measured by an electrophoretic mobility shift assay. (a) NF-κB activation in CAT-R55i cl 2 after treatment with 1,000 IU/ml mTNF (control) or hIFN-α for periods varying from 90 to 240 min. (b) Results of similar treatments on CAT cl 15. The lower, constitutively binding band has recently been characterized (Plaisance et al., 1997).
hTNFR55i. This was a first indication that the CAT-DD protein was not as active in cell killing as was the CAT-R55i fusion protein. Indeed, after induction with hIFN-α of CAT-DD clones, almost no cell killing could be observed. Since the DD was demonstrated to be necessary and sufficient for cell killing, the lack of functionality in this CAT-DD construct could be due to structural constraints. Indeed, the COOH termini of the CAT subunits are ~55 Å from each other (Leslie, 1990). Hence, the trimeric DDs may have been separated too far to allow functional clustering. In the CAT-R55i chimeric protein, however, this physical constraint was probably avoided by the upstream polypeptide region in front of the DD. Therefore, we constructed the pMxCATlinkDD vector, which has a 15 amino acid linker, (Gly-Gly-Gly-Gly-Ser)₃, inserted between the two protein domains (Fig. 1B). After transfection of this pMxCATlinkDD construct in L929sA cells, a strong counter-selection was again observed, as previously seen with the pMxCAThTNFR55i construct. G418-resistant clones were isolated and tested for inducible cell killing. After induction with hIFN-α, 60% of the clones died within 24 h with kinetics comparable to the cell death induced by CAT-R55i. The results obtained with two clonal lines (cl 4 and cl 12) are shown in Fig. 9A. IFN-α induction of this trimeric fusion protein also activated NF-κB and induced IL-6 gene expression (Fig. 9B); note that cl 12 died so quickly that only small amounts of IL-6 were synthesized.

From these results it is clear that the membrane-proximal part of hTNF-R55i has no essential signaling function in cytotoxicity, NF-κB activation, and IL-6 gene induction.

**Discussion**

Many hormone and cytokine receptors are clustered by their respective ligands as an essential and initial step for subsequent intracellular signaling. In the case of TNF signaling, receptor clustering is also crucial. This was first demonstrated by several groups using agonistic antibodies against the extracellular domain of the TNF receptors to mimic TNF actions (Engelmann et al., 1990; Espevik et al., 1990). Moreover, TNF is only biologically active in its trimeric form, and the extracellular domain of the receptor binds in the cleft between two subunits (Van Ostade et al., 1991; Banner et al., 1993), which means that TNF can bind and thereby cluster three receptor molecules (Loetscher et al., 1991; Pennica et al., 1992). This brings the intracellular receptor domains in close proximity to each other to allow signaling. It has previously been reported that expression of receptor molecules or their intracellular receptor domains was sufficient to simulate TNF effects (Boldin et al., 1995b). In that study, it was assumed, but not proven, that clustering of the TNF receptor domains occurred upon overexpression of the individual molecules. In the present paper, we show that expression of the intracellular domain of the TNF-R55 as such is not sufficient to elicit TNF effects and clearly demonstrate that only the "forced clustering" of these receptor domains leads to various characteristic TNF effects, without the need for the extracellular or transmembrane portion of the receptor. The apparently different results obtained by Boldin et al. (1995a) and ourselves are most probably related to the expression levels that can be obtained in the cell system.
used. As the HeLa cells used in the former study were much less sensitive to the cytotoxic action of TNF than the L929sA cells, counter-selection during the stable transfection procedure may not have been so strong in the HeLa cells. Hence, after subsequent induction, the higher expressing clones might have been able to produce sufficient levels of TNF-R55i to allow self-association and signaling. We also have tried to express the hTNF-R55i using the tetracycline-controlled system in L929sA cells, and we obtained similar results as with the Mx promoter–driven, inducible expression. This further proves that not the expression system as such, but rather the TNF sensitivity of the cell system used is responsible for the apparent difference in experimental outcome. Moreover, we effectively demonstrate that intracellular domain clustering is necessary and sufficient for receptor signaling.

Whether trimerization is really necessary, or whether dimerization might be sufficient to initiate the signaling cascade, is still a controversial issue and is not answered by our results. Several groups reported effective signaling with chimeric TNF receptors containing an extracellular domain known to form bimolecular complexes after binding of the appropriate ligand, e.g., PDGF or EPO (Adam et al., 1995; Bazzoni and Beutler, 1995; Bazzoni et al., 1995). On the other hand, natural TNF is a trimeric molecule, and moreover, receptor-specific, multivalent IgM antibodies are far more potent in mimicking TNF actions than agonistic, bivalent IgG antibodies (Espevik et al., 1990). Finally, it was also reported that an EPO/TNF-R75i chimera, which can only be dimerized, is deficient in signaling (Declercq et al., 1995). It might therefore be of interest to test forced dimerization of cytoplasmic receptor domains in a similar system, e.g., by expressing the TNF-R55i as a fusion with a specific dimerizing domain.

We have obtained inducible, forced trimerization by fusion of hTNF-R55i to the bacterial CAT protein. CAT is a trimeric enzyme of identical subunits of ~25 kD, oriented in a parallel fashion, such that their COOH-terminal ends are ~55 Å apart. Linkage of hTNF-R55i to the CAT COOH-terminal end automatically places the former domains in close proximity to each other, which allows clustering and subsequent signaling. In this respect, it is remarkable that the fusion protein CAT-DD, where the DD is linked directly to the CAT COOH-terminal end, could not elicit TNF effects. Considering the three-dimensional structure of the CAT protein, this inability to initiate signaling may be explained by the insufficient accessibility of the respective DDs to each other due to the spatial configuration of the trimeric CAT. Support for this notion is given by the finding that the signaling capacity of the fusion protein could indeed be restored by adding extra amino acids between the CAT and the DD polypeptide chains, as is the case in the CATlinkDD fusion. This linker allows a greater flexibility for the DD tails to interact, resulting in full signaling capacity.

Many receptor signaling systems that have been studied in detail involve associated factors and/or signal transducers that are constitutively or conditionally associated with the inner side of the cell membrane. This topologically constrained, structural interaction between receptor and signal transducer allows optimal functioning. However, in the system here described, this is not the case, as the fusion proteins are expressed as cytoplasmic proteins, and no obvious signals for membrane anchorage are known in the intracellular receptor domain. Therefore, the fact that signaling by the trimeric CAT-R55i is as effective as ligand-induced hTNF-R55 clustering means that the exact juxtaposition of the hTNF-R55i trimer relative to the membrane is not essential for function. It is known that the CAT protein is soluble in the cytosol; however, because of the low expression level of CAT-R55i, we still have to ascertain the localization of the latter polypeptide in the cell. We can of course not exclude the possibility that associating factors link it to the membrane, but even so it still would be in a structurally quite different constellation as compared to the natural, TNF-induced TNF-R55 clusters.

Remarkably, since CATlinkDD fusion proteins expressed in L929sA are as efficient in gene induction and cell killing as the total intracellular receptor domain fusion
proteins, the intracellular region upstream of the DD does not seem to play a role in signal transduction leading to IL-6 gene induction and cytotoxicity. Nevertheless, besides a purely structural role in creating the proper setting for the DDs to interact as evidenced in this study, the upstream region has been shown to be implicated in activation of neutral sphingomyelinase (Wiegmann et al., 1994; Adam et al., 1996b; Adam-Klages et al., 1996), several kinases and phospholipases, and induction of NO synthase (iNOS) (Tartaglia et al., 1993). By Northern analysis, we could demonstrate indeed that CATlinkDD-expressing cells did not induce iNOS (even after additional stimulation with IFN-γ), while CAT-R55i–expressing cells did. Therefore, the additional signaling effected by the membrane-proximal domain and responsible for the various activities of TNF, other than IL-6 gene expression (and other, similarly regulated genes) and cell killing, is now being studied by induced expression of mutated fusion constructs.

Our results clearly show that minute amounts of CAT-R55i are fatal to a sensitive cell. This was already clear from the strong counter-selection observed during the transfection/selection procedure with pMxCAThTNFR55i as compared to pMxCAT(stop)hTNFR55i. Moreover, the expression level of the fusion product in the G418-resistant clones obtained was so low that we could not demonstrate the generated protein by standard techniques, but it still was sufficient to cause biological effects, including NF-κB activation, gene induction, and cytotoxicity. It seems likely therefore that there are protective mechanisms that strictly control the number of TNF-R55 molecules per cell, since spontaneous clustering or overexpression of TNF-R55 is lethal to the cell. Several groups already reported the existence of such “survival” mechanisms, e.g., degradation by proteasomes may well be involved in the elimination of excess TNF-R55. Darnay et al. (1994) reported that deletion of the nucleotides encoding the 39 amino acids proximal to the transmembrane region minimized the degradation of TNF-R55i, expressed as a fusion with glutathione-S-transferase. This degradation-sensitive area is located next to the binding region of a TNF-R55i–associating protein that shows homology to a proteasomal subunit (Boldin et al., 1995b). Indeed, the short half-life of the TNF receptors is consistent with a turnover involving degradation in proteasomes. Results obtained with the proteasome inhibitor PSI are in agreement with this hypothesis. Inhibition of CAT-R55i breakdown by addition of PSI resulted in higher expression levels, leading to an increased cytotoxicity. Hence, TNF-R55 degradation by proteasomes might well occur as a restricting mechanism inside the cell, insuring that expression levels of the TNF receptors are kept constitutively low, thus preventing unwanted activation and signaling by spontaneous association.

In conclusion, by using the strictly controlled Mx promoter system, we were able to obtain cells producing a structural equivalent of the activated hTNF-R55. After induction, synthesis of very low amounts of the trimeric hTNF-R55i was sufficient to elicit typical TNF responses, including gene induction and cytotoxicity. Both responses were also obtained after forced clustering of only the receptor DDs (CATlinkDD). Induction of IL-6 requires at least two signaling pathways, one involving activation of NF-κB and possibly mediated by TNF-R55i DD → TRADD → TRAF2 interactions (Hsu et al., 1996), and the other activation of p38 MAP kinase (Beyaert et al., 1996). The pathway leading to cytotoxicity may be mediated by TNF-R55i DD → TRADD → FADD interactions (Hsu et al., 1996; Chinnaian et al., 1996; Boldin et al., 1996). The CAT fusion system is exceptionally suitable for further dissection and identification of various functions encoded by the hTNF-R55i and subdomains thereof, and may allow the physical and biochemical characterization of activated hTNF-R55i complexes as such and their association properties with other signal-transducing and/or regulatory proteins.

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