A Novel Cytoplasmic Domain of the p55 Tumor Necrosis Factor Receptor Initiates the Neutral Sphingomyelinase Pathway*

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The human p55 tumor necrosis factor (TNF) receptor (TR55) initiates at least two independent signaling cascades. The acidic sphingomyelinase (A-SMase) pathway involves a phosphatidylcholine-specific phospholipase C, an endosomal A-SMase, and controls expression of multiple TNF-responsive genes through induction of transcription factors such as NF-κB. The neutral sphingomyelinase (N-SMase) pathway comprises a membrane-bound N-SMase, proline-directed protein kinases, as well as phospholipase A₂, and appears critical for the inflammatory responses induced by TNF. While the domain of TR55 that induces A-SMase is probably identical to the death domain, the exact location and extent of a putative N-SMase activation domain are still unknown. Structure-function analysis of TR55 deletion mutants revealed a novel region of 11 amino acids at position 309–319 that is both necessary and sufficient for activation of N-SMase. The N-SMase activation domain is distinct from the death domain and incapable of induction of A-SMase, NF-κB, and cytotoxicity. Taken together, our results suggest that a functionally independent region of TR55 is responsible for selectively initiating the N-SMase pathway that couples to an important inflammatory signaling cascade.

TR55, a novel cytoplasmic domain of 11 amino acids located within the death domain, mediates TNF-induced activation of the protein kinase Raf (23), possibly involving CAP kinase (24) and thereby linking TR55 to the MAP kinase cascade.

The domain of TR55 activating the A-SMase pathway strikingly corresponds to the so-called death domain responsible for mediating the cytocidal effects of TNF (9, 11, 25), and proteins have been described that bind directly to TR55 within this region (TRADD, Ref. 26; TRAK, Ref. 27). Conversely, an activation domain for the N-SMase pathway has not yet been identified. So far, structure-function analysis of TR55 has revealed that the N-SMase pathway must be signaled for by a domain N-terminal of amino acid 345 (11). Intriguingly, N-SMase has been proposed to operate at the outer leaflet of the plasma membrane (28), leading to the hypothesis that activation of N-SMase may even require extracytoplasmic parts of TR55.

By analysis of a set of TR55 deletion mutants stably expressed in murine 702/3 pre-B cells, we show that the N-SMase activation domain (NSD) consists of a cytoplasmic region of the receptor that comprises only 11 amino acids directly adjacent to the N terminus of the death domain. The NSD extends from amino acids 309–319 and is both necessary and sufficient to mediate TNF-dependent N-SMase activation. The identification of the NSD represents a prerequisite for the isolation of novel TR55-associated proteins that are components of the N-SMase pathway.

MATERIALS AND METHODS

Plasmids and Reagents—pEF-BOS-TR55 was obtained by cloning a Sail-XbaI fragment of pADB-TR55 (7) containing the complete coding region of the human p55 TNF receptor into the XbaI site of pEF-BOS (Ref. 29; kindly provided by Dr. S. Nagata) following treatment with Klenow polymerase. The TR55-specific mouse monoclonal antibody H398 (30) was obtained from Dr. Schärich (Stuttgart). Highly purified murine and human recombinant TNF-α was provided by Dr. G. Adolf (Boehringer Ingelheim, Vienna). Human recombinant IL-1 was purchased from Genzyme.

Construction of TR55 Deletion Mutants—The complete coding sequence of hTR55 was isolated from pADB-TR55 as a Sail-XbaI fragment and cloned into pAlter-1 (Promega). Using an oligonucleotide-directed in vitro mutagenesis kit (Promega), stop codons were introduced at positions 205 (TAT → TAA, oligonucleotide 5'-TTCG-
TAGGGTACATTAACC-3') and 320 (CAG → TAG, oligonucleotide 5'-GCTAGGCTCTATGCGCATG-3') to generate TR55Δ205 and TR55Δ320. Internal deletions spanning either amino acids 308–340 (oligonucleotide 5'-CTCTGCGCTGAGCCAGGCGCTG-3') or 212–308 (oligonucleotide 5'-CTCTGCGCTGAGCCAGGCGCTG-3') of TR55 were introduced to obtain TR55Δ308–340 and TR55Δ212–308. Sequencing of the complete mutant cDNAs verified that no additional mutations had occurred. For TR55Δ320, in vitro transcription-translation ensured that the mutant cDNA was translated into a protein of the appropriate size. The mutant cDNAs were recovered from pALTER-1 as SalI-HindII (TR55Δ205), SalI-XbaI (TR55Δ308–340), or SalI-EcoRI fragments (TR55Δ320, TR55Δ212–308), respectively, and cloned into the XbaI site of the expression vector pEF-BOS after treatment with Klenow polymerase (generating a stop codon at position 346 in the case of TR55Δ346). Mutant TR55Δ244 was generated by deleting the SalI-HindII fragment of wild type TR55 into pEF-BOS introducing an in-frame stop codon immediately 3' of the truncation. All expression constructs were sequenced again to ensure correct orientation and presence of the desired mutation. Transient expression of the constructs in COS-1 cells followed by radioligand binding assays verified that the truncated receptors were properly expressed at the cell surface.

Cell Culture and Transfections—COS-1 cells and the murine pre-B cell line 70Z/3 were originally obtained from ATCC. Cell lines were maintained in a mixture of Click's/RPMI 1640 (50/50 volume %) supplemented with 10% fetal bovine serum, 10 mM glutamine, and 50 μg/ml each of streptomycin and penicillin in a humidified incubator containing 5% CO2.

For transient expression experiments, 1 × 105 COS-1 cells were electroporated at 960 microfarads/280 V with 20 μg of the appropriate pEF-BOS-TR55 plasmid. Cells were seeded onto 10-cm dishes, supplied with fresh medium after 24 h, and harvested after 72 h. Stably transfected 70Z/3 cells were obtained by cotransfection of the corresponding pEF-BOS-TR55 construct with BMGNeo (31) using electroporation and subsequent selection with 1 mg/ml Geneticin (Life Technologies, Inc.) and homogenized in 200 μl of 0.2% Triton X-100. The amount of radioactive phosphocholine produced from [3H]-labeled sphingomyelin was performed according to Quintern and Sandhoff (33) with some modifications (11). Briefly, cells were treated with TNF-α or TNF-α and incubated for 2 h to allow metabolization of MTT to MTT-formazan. MTT-formazan was solubilized with isopropl alcohol-HCl (24:1) and colorimetrically determined at 570 nm in a microplate reader (Dynatech).

Neutral Sphingomyelinase Domain of TR55

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described (32). Electrophoretic mobility shift assays were performed by incubating 6 μg of nuclear extract with 4 μg of poly(dI-dC) (Pharmacia Biotech Inc.) in binding buffer (5 mM HEPES (pH 7.8), 5 mM MgCl2, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol) in a total volume of 20 μl for 20 min at room temperature. After incubation was continued for 2 h to allow metabolization of MTT to MTT-formazan. MTT-formazan was solubilized with isopropl alcohol-HCl (24:1) and colorimetrically determined at 570 nm in a microplate reader (Dynatech).
RESULTS

The N-SMase Activation Domain Is Located in the Cytoplasmic Portion of the p55 TNF Receptor—To clarify whether N-SMase was possibly activated by the extracellular domain of TR55, we generated a C-terminal deletion mutant of TR55 (TR55Δ205, Fig. 1) that completely lacks the cytoplasmic domain. TR55Δ205 was transfected into the murine pre-B cell line 70Z/3 that does not express endogenous TR55 but is capable of displaying TNF-specific responses after transfection of cDNA encoding human TR55 (7). Geneticin-resistant cells were selected for expression of the mutant receptor by two rounds of staining using the monoclonal antibody H398 directed against the extracellular portion of hTR55 followed by selection of stained cells in a magnetic column. The selected pool of transfectants was analyzed for cell surface expression of the mutant receptor by radioligand binding assays and additional flow cytometry analyses (data not shown). Average receptor numbers per cell as well as ligand binding affinity of the truncated receptor was determined by Scatchard analysis. While the binding affinity of the truncated receptor for TNF was comparable with that of wild type TR55, the average receptor number per cell was distinctly larger than in cells expressing wild type receptors (Fig. 1). This is probably due to the absence of toxic effects that have been described for highly expressed intact cytoplasmic domains of TR55 (34).

TR55Δ205 transfectants were assayed for activation of N-SMase after treatment of cells with TNF or IL-1, which was used as a positive control for N-SMase activation because 70Z/3 cells express endogenous IL-1 receptors. While exposure to IL-1 elicited elevated N-SMase activity in 70Z/3TR55Δ205 cells, no changes of N-SMase activity were seen when the cells were treated with TNF (Fig. 2), suggesting that activation of the N-SMase pathway requires sequences from the cytoplasmic rather than the extracellular portion of TR55.

The N-SMase Activation Domain Consists of a Stretch of 11 Amino Acids between Positions 309 and 319 of the p55 TNF Receptor—Fig. 2. TNF-mediated activation of N-SMase requires amino acids 309-319 of the p55 TNF receptor. 70Z/3 cells expressing wild type TR55 or deletion mutants of TR55 were stimulated with 100 ng/ml TNF for the indicated times (b). Extracts containing the cytosol/membrane fraction were prepared and assayed for N-SMase activity using the substrate [14C]sphingomyelin as described. Treatment of cells with 100 ng/ml IL-1 (c) served as an internal positive control. N-SMase activities are expressed as percent of control. Basal levels of phosphocholine production were 96–165 pmol·mg⁻¹·h⁻¹. The values shown represent the means from triplicate determinations; error bars indicate the respective standard deviations. One representative from multiple experiments (n ≥ 3 for each deletion mutant) is shown.
cessively smaller deletions of the TR55 cytoplasmic domain (Fig. 1). When the transfectants were tested for N-SMase activity, neither 70Z/3TR55Δ244 nor 70Z/3TR55Δ308–340 cells showed an increase after treatment with TNF (Fig. 2). Therefore, amino acids 308–340 of TR55 must contain determinants required for successful activation of the N-SMase pathway.

Two additional deletion mutants proved that a domain between amino acids 308 and 340 was not only necessary but also sufficient to activate the N-SMase pathway. Mutant TR55Δ212–308/346 contains exactly the fragment from amino acid 309 to 345 but lacks almost all of the remaining cytoplasmic domain. The mutant TR55Δ320 was generated to examine whether the death domain (which extends C-terminal of position 326, Ref. 25) was involved in N-SMase activation. As shown in Fig. 2, both TR55Δ212–308/346 and TR55Δ320 mediated TNF-dependent induction of N-SMase with the same kinetics as observed with IL-1. In summary, these results indicate that amino acids 309–319 are sufficient for activation of N-SMase and that the death domain is not involved in activation of this enzyme.

The N-SMase Activation Domain Does Not Contribute to Induction of A-SMase, Cytotoxicity, or NF-κB—The death domain, located in close vicinity of the NBD between amino acids 326 and 413, is responsible for induction of cell death by TR55 (9, 25). The same region is also required for activation of A-SMase and NF-κB (11). When the generated 70Z/3 transfectants (all devoid of an intact death domain) were tested for their ability to signal for any of the above-mentioned responses through the NSD, neither transfectant was able to mediate TNF functions (Figs. 3–5). These data indicate that the NSD by itself does not mediate TNF-induced activation of A-SMase, NF-κB, or cytotoxic response.

DISCUSSION

N- and A-SMase are activated independently and by different cytoplasmic domains of TR55. Each SMase couples to a select signaling pathway with a distinct set of downstream enzymes (11). A similar functional dichotomy of SMase signaling has recently been described for the Fas/APO-1 receptor, another member of the TNF/NGF receptor family (35). For Fas/APO-1 mutants, it has been shown that the inability to activate PC-PLC and A-SMase correlates to ablation of the cytotoxic signal (35), suggesting that the A-SMase pathway is initiated by the death domain. The recent discovery of proteins binding directly or indirectly to the death domain (26, 27, 36, 37) promises a rapid advance in elucidation of the A-SMase/apoptosis pathway.

In contrast, no accurate knowledge is currently available of how TR55 initiates the N-SMase pathway. This pathway includes important cellular responses such as activation of PLA₂ followed by production of proinflammatory metabolites. Equally important, the N-SMase pathway is potentially linked...
to the network of receptor tyrosine kinase signaling. It has recently been shown that TNF-dependent phosphorylation and activation of the c-raf-1 kinase is still functional in 70Z/3TR55Δ345 transfectants but not in 70Z/3TR55Δ205 cells that lack the NSD, implicating N-SMase in c-raf-1 kinase activation (23). The link of the N-SMase pathway to the Ras/Raf pathway is most likely accomplished by CAP kinase, as TNF-dependent phosphorylation of c-raf-1 kinase by CAP kinase as well as complex formation of c-raf-1 kinase and CAP kinase have been reported following by increased activity of c-raf-1 kinase toward MAP kinaseextracellular signal-regulated kinase kinase (MEK) (24). In line with the lack of information on the N-SMase activation mechanism, no receptor-associated protein has been isolated so far that could be attributed to the N-SMase pathway.

In an effort to characterize the region(s) of TR55 that initiates the N-SMase pathway, we have generated, expressed, and analyzed a set of deletion mutants that all affect receptor sequences N-terminal of the death domain. The initial possibility that TR55 might contribute to N-SMase activation through its extracellular domain was ruled out by the observation that a receptor that completely lacks the cytoplasmic domain is unable to signal through N-SMase. This clearly indicates that the cytoplasmic portion of TR55 carries information critical for induction of the N-SMase pathway. By analysis of further deletion mutants of TR55, a region of 11 amino acids (309–319) could be defined that apparently is both required and sufficient for the N-SMase pathway.

The delineation of the N-SMase activation domain to positions 309–319 excludes a contribution by the adjacent death domain, whose N-terminus is located between position 326 and 340 (25). This fits nicely into a model where different domains of TR55 initiate independent signaling pathways, most likely by binding different sets of associated proteins. The functional independence of the N-SMase pathway is further underscored by the fact that mutants with an inactive death domain were also unable to signal through A-SMase and NF-κB regardless whether a functional NSD was present or not. In addition, no effect of the NSD on cytotoxicity was seen. This provides evidence that N-SMase and supposedly also enzymes secondary to N-SMase like MAP kinases and PLA2, (reviewed in Ref. 20) by themselves are not sufficient for induction of cell death in 70Z/3 cells. At this point, however, we cannot rule out a possible contribution of the N-SMase pathway to the induction of apoptosis. In Jurkat cells, Fas/APO-1 ligation has been shown to induce Ras via the sphingomyelin (N-SMase) pathway, leading to subsequent apoptosis (38), which would imply a participation of N-SMase in the induction of programmed cell death. However, the activation of Ras via the action of ceramide by itself appeared not to be sufficient for Fas/APO-1-mediated apoptosis (38), which is in line with our findings that the NSD on its own is unable to signal cell death. The possibility remains that the NSD may be required for an apoptotic response in concert with the death domain.

Although both TR55 and Fas/APO-1 are capable of inducing the N-SMase pathway, there are no obvious homologies between the NSD and Fas/APO-1 sequence, indicating that both receptors have their own specific activation domains for N-SMase.

In summary, the results of our study indicate that the N-SMase pathway is activated by a domain both functionally and spatially distinct from the death domain. It is thus plausible to assume that a distinct, yet unknown factor may bind to the NSD initiating this signaling cascade. So far, two proteins have been described that bind to TR55 outside the death domain; however, one of them has a binding site that does not match the NSD (protein 55.11, binding site between residues 243 and 308; Ref. 39) while the amino acid sequences that mediate binding of the other protein (TRAP-1; Ref. 40) are diffusely distributed outside the death domain and therefore do not seem to be specific for the NSD. The identification of proteins that bind specifically to the newly defined NSD is a current subject of investigation.

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