The 55-kDa receptor for tumor necrosis factor (TR55) triggers multiple signaling cascades initiated by adapter proteins like TRADD and FAN. By use of the primary amine monodansylcadaverine (MDC), we addressed the functional role of tumor necrosis factor (TNF) receptor internalization for intracellular signal distribution. We show that MDC does not prevent the interaction of the p55 TNF receptor (TR55) with FAN and TRADD. Furthermore, the activation of plasmamembrane-associated neutral sphingomyelinase activation as well as the stimulation of proline-directed protein kinases were not affected in MDC-treated cells. In contrast, activation of signaling enzymes that are linked to the “death domain” of TR55, like acid sphingomyelinase and c-Jun-N-terminal protein kinase as well as TNF signaling of apoptosis in U937 and L929 cells, are blocked in the presence of MDC. The results of our study suggest a role of TR55 internalization for the activation of select TR55 death domain signaling pathways including those leading to apoptosis.

Tumor necrosis factor (TNF), originally defined by its antitumoral activity, is now recognized as a pleiotropic cytokine exerting a wide variety of immunoregulatory activities (for review, see Refs. 1–3). TNF action is mediated by two types of cell surface receptors of 55 kDa (TR55) and 75 kDa (TR75) molecular masses, respectively. Both receptors mediate distinct TNF responses (4–6). The majority of activities of soluble TNF appears to be mediated by TR55 (7–11). Like other cytokine receptors, the cytoplasmic domain of both TNF receptors lacks intrinsic enzymatic activities. The activation of intracellular signaling enzyme systems is initiated by a selective interplay between the cytoplasmic domain of the TNF receptors and a number of recently identified TNF receptor-associated proteins (see Ref. 12 for review). The adapter protein TRADD associates with the so-called death domain of TR55, recruiting FADD, RIP, and TRAF-2 (13–16). FADD mediates the activation of a protease termed FLICE/MACH (17, 18), representing one of the first steps in induction of the apoptotic pathway. RIP mediates activation of the transcription factor NF-kB associated with anti-apoptotic regulatory functions (19). TRAF-2 links TR55 to activation of the N-terminal c-Jun kinase (JNK) cascade (20). The TR55-associated protein FAN (21) binds to a domain designated neutral sphingomyelinase activation domain (NSD) that is N-terminally adjacent to the death domain (22). FAN mediates TNF-induced activation of neutral sphingomyelinase (N-SMase) (21).

Results from numerous studies have revealed that TNF signaling further involves activation of downstream enzyme systems at multiple subcellular compartments such as the plasmamembrane, endosomes, mitochondria, the cytosol, and the nucleus (for review, see Refs. 11, 23, and 24). Membrane-associated enzyme systems transmitting TR55 signals include plasmamembrane-bound phospholipases such as phosphatidylinositol-specific phospholipase C (25), which generates the lipid second messenger molecule 1,2-diacylglycerol and a N-SMase (9), producing ceramide by sphingomyelin hydrolysis. Ceramide generated at the plasmamembrane triggers activation of a 97-kDa ceramide-activated protein kinase (26), recently suggested to be identical with the “kinase suppressor of ras” (27). Ceramic-activated protein kinase belongs to a family of proline-directed protein kinases (PDPK) (9), including members of the mitogen-activated protein kinases (28). TNF signaling further involves intracellular membrane compartments like caveolae and endosomes harboring an acid SMase (A-SMase) (9, 29, 30). In mitochondria, TNF induces reactive oxygen species that are generated at the level of the oxidative phosphorylation complex III (31). The induction of the mitochondrial permeability transition has been linked to the TNF cytotoxic pathway (32). In addition, cytosolic protein kinase cascades including PKC and JNK have been identified to transmit TNF signals (33–35).

The question of how TNF signals are targeted to the different intracellular compartments is of fundamental biological significance. Intriguingly, rapidly diffusible ions like Ca^{2+} seem not to be involved in TNF signaling. Rather, the two lipid second messenger molecules 1,2-diacylglycerol and ceramide are likely to reside within membranes because of their hydrophobic nature. Thus the mechanisms of intracellular TNF signal trafficking remain obscure.

In the present study we investigated the effects of inhibition of TNF receptor endocytosis on TNF signal transduction. Because structural motifs within the TNF receptor required for endocytosis are currently unknown, genetic approaches to block TNF receptor internalization are not available at present. We here employed the primary amine monodansylcadaverine (MDC) or K^{+} depletion to inhibit TNF receptor internalization.
MDC is an inhibitor of transglutaminase, a membrane-bound enzyme that actively participates in internalization of various receptor systems (36–44).

We show here that MDC as well as K⁺-depletion block TNF endocytosis, which is related to inhibition of TR55 death domain signaling like TNF-dependent activation of endosomal A-SMase, JNK, and TNF-mediated apoptosis. In contrast, TNF-dependent stimulation of plasmamembrane-associated N-SMase and PDP kinase are not affected by MDC or K⁺ deprivation.

MATERIALS AND METHODS

Cell Culture and Reagents—U937, Jurkat, and HeLa cells were obtained from the American Type Culture Collection, Manassas, VA, and maintained in a mixture of Click’s/RPMI 1640 (50%, 50% v/v) supplemented with 5% fetal calf serum, 10 mM glutamine, and 50 μg/ml each streptomycin and penicillin in a humidified incubator containing 5% CO₂. The human embryonic kidney cell line HEK 293 was kindly provided by Dr. M. Schmidt, Essen, Germany and maintained in high glucose Dulbecco’s modified Eagle’s medium (ICN), 10% fetal calf serum, 10 mM glutamine, and 50 μg/ml each streptomycin and penicillin. The cDNA for glutathione S-transferase (GST)-Jun (1–166) was provided by Dr. P. Angel (DKFZ, Heidelberg, Germany). Highly purified recombinant human TNF (3 × 10⁷ units/ml) was provided by Dr. G. Adolf, Bender Research Institute Vienna, Austria. Monodansylcadaverine was obtained from Sigma, and C₂-hexanoylsphinogosine was purchased from Biomol Feinchemikalien GmbH, Hamburg, Germany. Anti-CD95 (Fas/APO-1) was obtained from Calbiochem-Novabiochem GmbH, Bad Soden, Germany. A pRK5 expression plasmid encoding TRADD was kindly provided by Dr. D. V. Goeddel, Tularik Inc., S. San Francisco, CA. The monoclonal anti-TR55 antibody htr9 was a gift from Dr. W. Lesslauer and Dr. H. Loetscher, Hoffmann-La Roche, Basel, Switzerland.

Measurements of TNF Receptor-mediated Internalization—One million U937 cells were incubated for 1 h at 0 °C with 1 ng of ¹²⁵I-labeled human recombinant TNF (NEN Life Science Products, specific activity 2160 kBq/μg) to saturate cell surface TNF receptors. After washing the cells three times in cold PBS, the temperature was shifted to 37 °C for 1 h to allow receptor internalization. To determine the amount of internalized ¹²⁵I-TNF receptor complexes, noninternalized ligand was removed by passing cells through a pH 3 gradient at 500 g consisting of (a) 0.5 ml of culture medium supplemented with 20% Ficoll and (b) 3 ml of 100 mM NaCl, 50 mM glycine/HCl, pH 3, supplemented with 10% Ficoll, and 0.5 ml of culture medium containing 5% Ficoll. To determine the total amount of cell-associated ¹²⁵I-TNF, a second aliquot of cells was passed through a gradient in which the second layer (b) was replaced by PBS, pH 7.3, containing 10% Ficoll. Radioactivity in the cell pellets was determined by liquid scintillation counting. To measure nonspecific binding, a parallel experiment was performed in which a 200-fold excess of unlabeled TNF was added to the cells. Specific binding was calculated by subtracting nonspecific from total binding, and the amount of ¹²⁵I-TNF internalized was calculated as the percent of

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**Fig. 1.** A, inhibition of TNF receptor internalization by K⁺-depletion, MDC treatment, or low temperature. U937 cells were either left untreated or incubated with 100 μM MDC for 1 h, depleted (depl.) of intracellular K⁺, or kept at 14 °C. Cells were then incubated for 2 h with ¹²⁵I-TNF (10 ng/ml) at 4 °C. Afterward, cells were shifted to 37 °C, and TNF receptor internalization was determined as described under “Materials and Methods.” The mean values from three experiments performed in triplicates (± SD) are shown. B, MDC dose-dependent inhibition of TNF receptor internalization. U937 cells were preincubated with the indicated concentrations of MDC, and TNF receptor internalization was determined as in A (n = 3, ± S.D.).

**Fig. 2.** MDC blocks uptake of biotinylated TNF in HeLa cells. HeLa cells were grown on glass coverslips and either left untreated (a–d) or were incubated with 100 μM MDC (e–g) for 1 h at 37 °C. Cells were then shifted to 4 °C, and 100 ng/ml biotin-TNF was added. After 2 h, the cells were washed with PBS, and 1 aliquot was fixed with cold methanol as the control of surface staining (a). The remaining cells were shifted to 37 °C, and biotin-TNF bound to the TNF receptor was allowed to internalize for 10 min (b and e), 30 min (c and f), and 60 min (d and g). Cells were fixed and stained with avidin-FITC, and fluorescence was analyzed using fluorescence microscopy (magnification 100×).
specific binding determined at pH 7.3.

Fluorescence Microscopy of TNF-Biotin/Avidin-FITC-labeled Cells—HeLa cells were grown on glass coverslips in Click’s RPMI medium and either left untreated or were incubated with 100 mM MDC for 1 h at 37 °C. Cells were then shifted to 4 °C, and 100 ng/ml biotinylated TNF (human recombinant TNF-α, biotin conjugate, Fluorokine, R&D Systems, Wiesbaden) was added. After 2 h, cells were washed with PBS to remove unbound TNF, one set of cells was fixed with ethanol as a control of surface staining, and the other cells were shifted to 37 °C, and biotin-TNF bound to the TNF receptor was allowed to internalize for 10, 30, and 60 min. Cells were fixed with cold methanol, avidin-FITC (1:40 final dilution) was added for 1 h at 37 °C, and fluorescence was documented using a fluorescence microscope (Zeiss). The biological activity of biotinylated TNF was identical to nonmodified TNF as estimated in MTT cytotoxicity assays, indicating that biotinylation of recombinant TNF did not alter its receptor binding properties.

Inhibition of TNF Receptor Internalization—Low temperature effects were assayed by incubation of cells at 14 °C for 1 h. Depletion of intracellular K⁺ was performed as described (45). Briefly, cells were washed twice in buffer A (100 mM NaCl, 50 mM Hepes, pH 7.4) and subjected for 5 min to hypotonic medium (medium/water 1:1) followed by a 10 min incubation in buffer A. All treatments were performed at 37 °C. The cells were then resuspended in buffer B (100 mM NaCl, 1 mM CaCl₂, 50 mM Hepes, pH 7.4) and kept at 37 °C for an additional 30 min. The effect of MDC was estimated by treating cells with 100 μM MDC for 1 h in serum-free medium supplemented with 2% bovine serum albumin.

Assays for Neutral and Acid Sphingomyelinase—Micellar SMase assays using exogenous radiolabeled sphingomyelin as substrate was performed according to a method previously described (9, 46). Briefly, U937 cells (3 × 10⁶/ml) in triplicates were homogenized, and 50 μg of protein from the cellular lysates were examined for SMase activity using a micellar assay system with [N-methyl-¹⁴C]sphingomyelin (0.2 μCi/ml, specific activity 56.6 μCi/mmol, Amersham Pharmacia Biotech) as a substrate. [¹⁴C]Phosphorylcholine, produced from [¹⁴C]sphingomyelin, was extracted from the aqueous phase, identified by TLC, and routinely determined by liquid scintillation counting.

JNK Assay—Cell extracts were prepared by homogenization in lysis buffer (consisting of 25 mM HEPES, pH 7.55, 100 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.25 mM EDTA, 10 mM NaF, 0.1% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/μl each of leupeptin, pepstatin, and aprotinin) followed by centrifugation for 30 min at 20,000 × g. For assessment of JNK activity, 100 μg of supernatant was immunoprecipitated with 2 μg of affinity-purified polyclonal anti-JNK-1 antibody (C-17) obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Immune complexes were recovered by protein G-Sepharose (Amersham Pharmacia Biotech), washed extensively, and assayed for in vitro kinase activity using 5 μg of GST-Jun 1–166 as a substrate. Phosphorylation was performed for 20 min at 30 °C in 20-μl assays consisting of 50 mM HEPES, pH 7.55,
RESULTS

Inhibition of TNF Receptor Internalization—TR55 receptors are rapidly internalized upon binding of the ligand via the classical receptor-mediated endocytic pathway, involving clathrin-coated pit formation (48). Notably, only TR55 is internalized upon TNF binding, whereas TR75 is shed from the cell surface (49, 50). To investigate the functional role of TR55 endocytosis for intracellular signal transduction and processing of the apoptotic signal, TNF receptor internalization was blocked by the transglutaminase inhibitor MDC or by disrupting clathrin-coated pit formation by depleting cells from intracellular K+.

In a first approach, the inhibitory effects of MDC on TNF receptor endocytosis in U937 cells were analyzed by measuring endocytosis of 125I-labeled TNF and compared with effects of K+ depletion or “freezing” membranes at 14 °C. As shown in Fig. 1A, pretreatment of U937 cells with 100 μM MDC, K+ depletion, or low temperature resulted in an inhibition of TNF-induced receptor internalization by 58.5, 64.4, or 74%, respectively. These values correspond well to those previously obtained with endothelial cells (41) and human skin fibroblasts (51).

The inhibitory effect of MDC on TNF receptor internalization was dose-dependent (Fig. 1B), with half-maximal inhibition at 30 μM MDC. This value is consistent with the half-maximal MDC concentration required for inhibition of transglutaminase, an enzyme crucially involved in coated pit formation, the initial step in receptor endocytosis (37).

To demonstrate the effect of MDC on intracellular TNF receptor distribution by an independent method, we subsequently employed biotinylated TNF-avidin-FITC to stain TNF receptors on HeLa cells and to follow the internalization of the ligand upon incubation at 37 °C. Analysis of TNF binding on HeLa cells by indirect fluorescence microscopy revealed a uniform surface staining of TNF-biotin/avidin FITC complexes at 4 °C (Fig. 2a). Shifting the incubation temperature to 37 °C for 10 min resulted in a pattern of condensed fluorescence, typical of multimerized, clustered receptors most likely located in early endosomal vesicles (Fig. 2b). After 30 min at 37 °C, the vesicular staining appeared concentrated in larger intracellular compartments (Fig. 2c), indicating accumulation of TNF in late endolysosomal vesicles.

60 min, a perinuclear staining was observed colocalizing with lysosomal compartments (Fig. 2d). These kinetics of intracellular staining indicate uptake of receptor-bound TNF and transport of the ligand to lysosomes within 1 h, an observation that is in line with electron microscopic evaluations (48). In contrast, preincubation of the cells with MDC completely blocked the intracellular translocation of TNF in endolysosomal or perinuclear lysosomal compartments as revealed by the uniform surface staining with TNF-biotin/avidin FITC at 10, 30, and 60 min (Fig. 2, e–g). These observations suggested that TNF receptor internalization and subsequent intracellular trafficking was prevented by MDC.

Activation of A-SMase but Not N-SMase Is Blocked by MDC and after K+ Depletion—Activation of acid and neutral SMases are early TNF-responsive events (9) regulated by the death...
domain-associated protein TRADD (52) and the neutral sphingomyelinase activation domain (NSD)-associated protein FAN (21), respectively. As shown in Fig. 3A, TNF stimulation of A-SMase was completely blocked after pretreatment of cells with MDC. The effect of MDC on A-SMase activation did not result from direct inhibition of the enzyme, because this agent did not inhibit A-SMase when added directly to A-SMase assays (data not shown). The stimulation of A-SMase was also inhibited when cells were deprived of K\(^+\) to block TNF receptor endocytosis. Thus, MDC-mediated inhibition of A-SMase is likely secondary to the blockade of TNF receptor internalization.

In contrast, preincubation of U937 cells with MDC did not prevent TNF-induced activation of N-SMase (Fig. 3B). Blocking TNF receptor internalization by K\(^+\) depletion also did not prevent N-SMase activation. These data indicate that activation of the plasmamembrane-associated N-SMase occurs independent of TNF receptor internalization.

**Differential Requirements of Cytosolic Protein Kinases for TNF Receptor Internalization**—TNF induces activation of PDPK including a ceramide-activated protein kinase (53) and mitogen-stimulated protein kinases (54). The stimulation of these protein kinases is possibly secondary to ceramide generated by N-SMase (9, 54). As shown in Fig. 4, TNF induction of PDP kinase activity was not inhibited by MDC. In contrast, pretreatment with MDC resulted in a marked inhibition of TNF-induced activation of JNK (Fig. 5, b and c). Notably, treatment of cells with exogenous C\(_6\)-ceramide overcame the MDC-imposed blockade of JNK activation (Fig. 5, e and f). Similar effects of MDC on PDP kinase and JNK were also observed with K\(^+\)-depleted cells (Figs. 4 and 5, c and f). Taken together, these findings suggest that the cytosolic protein kinases JNK and PDPK are activated by separate pathways that are either dependent or independent of TNF receptor internalization.

**MDC Does Not Block the Association of FAN and TRADD with TR55**—We next examined whether the differential inhibitory effects of MDC on TNF receptor death domain signaling could be explained by selective interference with the association of death domain-binding protein TRADD and the adaptor protein FAN to TR55. For this purpose, HEK 239 cells were transfected with the FLAG-tagged full-length FAN or TRADD fusion constructs, and cellular lysates were subjected to immu-
noprecipitation using the anti-TR55 antibody htr-9. The co-immunoprecipitating FAN and TRADD-proteins were detected by Western blotting using anti-FLAG antibody M5. As shown in Fig. 6, MDC treatment did not reduce the association of FAN or TRADD with TR55. Thus MDC does not seem to selectively affect the stability of TR55/TRADD complexes, indicating that the specific action of MDC on TR55 death domain signaling cannot be explained at the level of TR55-associated proteins.

**TNF-mediated Cell Death Is Blocked by MDC**—The induction of programmed cell death is one important hallmark of TNF, CD95 ligand as well as ceramide action. To study the effects of the inhibition of receptor internalization on cell death, we performed pretreatment of cells with MDC, which did not cause significant toxic side effects when applied at prolonged times. In contrast, K⁺ depletion of cells for 24 h proved to be toxic for U937 cells (data not shown) and, therefore, could not be employed to study long term effects of TNF such as the induction of cell death.

As shown in Fig. 7, TNF and exogenous C₆-ceramide induced the death of U937 cells after 24 h. TNF-mediated cytotoxicity was prevented by MDC pretreatment in a dose-dependent manner, with half-maximal protection between 25 and 50 μM MDC, corresponding to the dose-dependent MDC inhibition of TNF receptor internalization (Fig. 1B). At 100 μM MDC, the fraction of TNF-induced dead cells estimated by MTT assays was reduced from 63 to 24%, corresponding to a 62% inhibition (Fig. 7). In contrast, the cytotoxic effect of exogenous C₆-ceramide taken up by the cells by pinocytosis was not affected by MDC. The protective effect of MDC on TNF-induced cytotoxicity was observed up to 48 h after TR55 triggering (43.5% inhibition at 100 μM MDC, data not shown), indicating that inhibition of TNF receptor internalization by MDC provides significant long term protection against TNF-induced cell death.

In dose-response experiments, we evaluated whether pre-treatment with MDC was effective in inhibiting death of U937 cells at different concentrations of TNF, ceramide, and anti-CD95 antibody. Fig. 8 shows the dose-dependent cytotoxicity induced by TNF, ceramide, and CD95 triggering. MDC was protective against TNF-induced cell death (Fig. 8A), whereas ceramide- and CD95-induced cell death were not affected by MDC (Fig. 8, B and C). These results suggest that the protective effect of MDC appears to be selective for TNF-mediated cell death.

In morphological analyses, TNF-induced chromatin condensation in U937 cells, typical for apoptosis, is shown (Fig. 9b). Incubation with 100 μM MDC before TNF treatment significantly reduced the fraction of apoptotic cells from 65 to 19%, corresponding to a 70.8% inhibition of TNF-induced apoptosis (Fig. 9, f and i). Apoptosis was also induced by exogenous C₆-ceramide (Fig. 9c) that, again, was not inhibitable by MDC.

**FIG. 6. MDC does not inhibit association of FAN or TRADD with TR55.** HEK 293 cells were transiently transfected with 20 μg of either pFLAG.CMV2-FAN (A) or pRK5.FLAG-TRADD (B) and cultured for 18 h. Cells were left untreated or were treated with 100 μg/ml MDC for 1 h. Cellular lysates were prepared and directly applied to SDS-polyacrylamide gel electrophoresis for control of FAN and TRADD expression. Immunoprecipitation (IP) was performed using the anti-TR55 antibody htr-9. The presence of FAN and TRADD was analyzed by Western blotting using anti-FLAG antibody M5 and the ECL immunodetection system.

**FIG. 7. TNF-mediated cytotoxicity is blocked by MDC and can be reconstituted by exogenous ceramide.** U937 cells were left untreated (C, open bars) or incubated with either 30 ng/ml TNF (black bars) or 25 μM C₆-ceramide (Cer, gray bars) after pretreatment with the indicated concentrations of MDC. Cell viability was assessed by MTT assays after 24 h. The values represent the mean of a representative experiment performed in quadruplicates (±S.D.).
The protective effect of MDC against TNF-induced apoptosis was also observed in a different cell line, L929, indicating that the role of TNF receptor internalization for signaling cell death is not restricted to U937 cells (data not shown). As demonstrated in Fig. 9 (d, h, and i), induction of apoptosis in Jurkat cells by anti-CD95 antibody could not be blocked by MDC pretreatment. Similar results were obtained by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis (data not shown). Together, these findings suggest that the anti-apoptotic effect of MDC observed in TNF-treated cells is specific for cell death mediated by TR55.

**DISCUSSION**

In the present paper the role of receptor internalization for TNF signaling was investigated. Intriguingly, inhibition of TR55 internalization by MDC or K⁺ depletion abolished select signaling events while leaving others unaffected. In particular, TNF-induced activation of A-SMase, JNK, and the induction of apoptosis were sensitive to MDC. In contrast, MDC did not inhibit TNF-induced activation of N-SMase and PDP kinases. Similar results were obtained with K⁺-depletion, which inhibited TNF receptor internalization and, like MDC, selectively blocked TNF-induced activation of A-SMase and JNK but not N-SMase or PDP kinases.

MDC has been extensively used to block endocytosis and trafficking of various ligand-receptor systems. MDC is known as a potent competitive inhibitor of transglutaminase (36, 37, 58, 59). The enzymatic action of transglutaminase involves cross-linking of proteins by forming an isopeptide bond between a lysine residue of one protein and a glutamine residue of another protein during coated pit formation. MDC has been shown to block endocytosis of α₂-macroglobulin and many
polypeptide hormones and cytokines like epidermal growth factor, hepatocyte growth factor, insulin-like growth factor-I, and IL-8 (37, 38, 40, 43, 44, 58, 60). MDC-mediated inhibition of transglutaminase has also been implicated in the blocking of endocytosis of vesicular stomatitis virus, Semliki Forest virus, and other types of endocytosis occurring through clathrin-coated vesicles (61, 62). MDC does not affect the number or affinity of TNF receptors expressed on the cell surface (41).2

MDC blocked TNF receptor internalization at concentrations similar to the concentrations required for inhibition of transglutaminase in lysates from Chinese hamster ovary cells (37), suggesting that the inhibition of this enzyme is related to the MDC effects on TNF receptor endocytosis. MDC concentrations exceeding 100 μM did not further increase the inhibitory effect on TNF receptor internalization in U937 cells. These findings are consistent with a previous study by Davies and co-workers, reporting on MDC-mediated inhibition of α2-macroglobulin receptor clustering (37).

The differential requirement of TR55 internalization for the activation of A-SMase and N-SMase are in concordance with a previous report by Hofmeister et al. in which the authors used an independent approach to study the functional consequences of IL-1 receptor internalization (63). An IL-1 receptor type I-positive EL4 thymoma cell line was employed, which is defective in the IL-1R accessory protein (IL-1RAcP) required for IL-1 receptor internalization. In this IL-1R internalization-defective cell line, IL-1 induction of N-SMase appeared normal, whereas the activation of A-SMase was completely impaired.

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2 S. Schütze, unpublished observations.
Transfection with IL-1RARp cDNA restored IL-1 receptor internalization as well as IL-1-induced A-SMase stimulation, indicating the requirement of IL-1R internalization for A-SMase activation.

Our findings on the differential requirement of receptor internalization for activation of A- and N-SMase may help to explain previous data by Andrea et al. (51). These authors could not detect a requirement of TNF or IL-1 receptor internalization for sponging meloin turnover. Here we show that it is only N-SMase that is insensitive to MDC or K+ depletion, whereas activation of A-SMase depends on TNF receptor internalization. Because the overall enzymatic activity of A-SMase exceeds that of N-SMase by factors of 3–10, depending on the cell line investigated (Fig. 4 and Refs. 9 and 65), the remaining N-SMase activity might have escaped the authors’ detection system.

A role for receptor clustering and internalization for activation of JNK has been also suggested by Rosette and Karin (64), who report that exposure of HeLa cells to UV light or osmotic depletion, and Pastorino et al. (32). It should be emphasized that mechanism by which TR75 signals cytotoxicity is different from disrupting the TRADD/TR55 signaling complex.

The importance of TNF receptor internalization for signaling cascade. TNF receptor endocytosis may play a previously unrecognized role in relaying TNF signals to intracellular compartments, where TNF receptor binding membranes can be sensed as “signaling rafts.”

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