Receptor-mediated Endocytosis Is Not Required for Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-induced Apoptosis*1

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is selectively toxic to tumor compared with normal cells. Other members of the TNF family of death ligands (TNF, CD95L) engage their respective receptors (TNF-R1 and CD95), resulting in internalization of receptor and ligand and recruitment of adaptor proteins to the caspase activation platform known as the death-inducing signaling complex (DISC). Recently, TNF-R1 and CD95 have been shown to induce apoptosis with an absolute requirement for internalization of their corresponding receptors in the formation of a DISC. We show that TRAIL and its receptors are rapidly endocytosed in a time- and concentration-dependent manner. Blockade of receptor internalization with hyperosmotic sucrose did not inhibit TRAIL-induced apoptosis but, rather, amplified the apoptotic signaling of TRAIL. Plate-bound and soluble TRAIL induced similar levels of apoptosis. Together these results suggest that neither ligand nor receptor internalization is required for TRAIL-induced apoptosis. Internalization of TRAIL is mediated primarily by clathrin-dependent endocytosis and also by clathrin-independent pathways. Inhibition of clathrin-dependent internalization by overexpression of dominant negative forms of dynamin or AP180 did not inhibit TRAIL-induced apoptosis. Consistent with the finding that neither internalization of TRAIL nor its receptors is required for transmission of its apoptotic signal, recruitment of FADD (Fas-associated death domain) and procaspase-8 to form the TRAIL-associated DISC occurred at 4 °C, independent of endocytosis. Our findings demonstrate that TRAIL and TRAIL receptor 1/2, unlike TNF-TNF-R1 or CD95L-CD95, do not require internalization for formation of the DISC, activation of caspase-8, or transmission of an apoptotic signal in BJAB type I cells.

Induction of apoptosis occurs primarily by two main pathways, the Bcl-2 family regulated pathway (also known as the intrinsic or mitochondrial pathway) and the extrinsic pathway (1–4). Caspase-9, the apical caspase in the Bcl-2 family regulated pathway, is activated after perturbation of mitochondria resulting from cellular stress, growth factor withdrawal, or cytotoxic insults. Caspase-8 is the apical caspase in the extrinsic pathway and is activated after ligation of death receptors by members of the TNF family, including TNF, CD95L (FasL/Apo-1L), and TNF-related apoptosis-inducing ligand (TRAIL). Ligation of cell surface death receptors, such as CD95, TNF receptor 1 (TNF-R1), and TRAIL-R1 (DR4) or TRAIL-R2 (DR5), by their cognate ligands or agonistic antibodies, results in receptor aggregation and recruitment of the adaptor protein MORT1/FADD (Fas-associated death domain) (1, 2, 4, 5). FADD then recruits the initiator caspase-8, which is activated within the death-inducing signaling complex (DISC) (6, 7). Currently there is much interest in exploring the therapeutic potential of TRAIL because it induces apoptosis selectively in many cancer cells but not in most normal cells (1).

Since the recognition of the DISC it has generally been considered that activation of the extrinsic pathway occurred almost exclusively at the plasma membrane. Although endocytosis of TNF and its associated receptors has long been recognized (8–10), it was not considered to be important in signaling to death. However, independent studies in our laboratory and others recently demonstrated that caspase-8 and FADD are not recruited to a TNF-induced plasma membrane-bound receptor signaling complex but, instead, are activated elsewhere within the cell (11–14). It has been proposed that an initial membrane-associated complex comprising TNF, TNF-R1, TRADD (TNFR1-associated death domain protein), TNF receptor-associated factor 2, and RIP (receptor interacting protein) is formed that dissociates after endocytosis, releasing TRADD or possibly RIP to bind through their death domains to FADD, which in turn recruits and activates caspase-8 (12). Induction of apoptosis by CD95 seems to occur by two main routes, 1) in type I cells where it involves formation of large amounts of DISC accompanied by rapid receptor internalization and does not require a mitochondrial amplification loop or pathway and 2) in type II cells, which form a DISC more slowly and less extensively and require amplification through cross-talk with mitochondria (15, 16). Recently a requirement for

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3 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; TMRE, tetramethylrhodamine ethyl ester; z-VAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp(Ome) fluoromethyl ketone; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; WT, wild type; RIP, receptor-interacting protein.
receptor internalization for CD95 ligand-mediated DISC amplification and caspase activation in type I cells has also been proposed (17). It was noted that recruitment of DISC components occurred primarily after internalization of the receptor into an endosomal compartment and also that inhibition of internalization inhibited CD95 DISC formation and apoptosis in type I cells (17). Thus, there appears to be a major role(s) for internalization of both TNF-R1 and CD95 in apoptosis induction. We, therefore, wished to explore a possible role for internalization of TRAIL and its receptors in TRAIL-induced apoptosis.

In this study we show that TRAIL is rapidly internalized primarily by clathrin-dependent endocytosis but also by clathrin-independent endocytosis. However internalization of TRAIL or its receptors is not required to initiate a full apoptotic signal, unlike requirements previously described for TNF-R1 and CD95 (14, 17). Furthermore, the TRAIL DISC is formed in the complete absence of internalization and is fully competent to initiate the apoptotic program after exposure to TRAIL.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The Epstein-Barr virus-negative BJAB Burkitt lymphoma B cell line and TRAIL-R2/DR5-deficient BJAB cells (18) were generously provided by Dr. A. Thorburn (University of Colorado Health Sciences Center, Aurora, CO). BJAB cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM GlutaMAX. Human cervical carcinoma HeLa cells inducibly expressing either hemagglutinin-tagged wild type dynamin-1 or a dominant negative K44A mutant form of dynamin-1 under control of a tetracycline promoter (tet-off system) (19, 20) were provided by Dr. R. C. Bleackley (University of Alberta, Edmonton, Canada) with permission from Dr. S. Schmid (Scripps Research Institute, La Jolla, CA). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g of glucose/liter and supplemented with 10% fetal bovine serum and 2 mM GlutaMAX. A dominant negative dynamin-2 K44A mutant construct and a C-terminal AP180 construct (AP180C) were generous gifts from Dr. B. J. Nichols (MRC Laboratory of Molecular Biology, Cambridge, UK). Nucleotide sequences of the insert regions within these constructs were independently verified by automated DNA sequencing. Rabbit polyclonal DR4 (catalog no. 1139) and DR5 (catalog no. 2019) antisera were purchased from Axxora (Nottingham, UK). Mouse monoclonal antibodies to FADD (catalog no. 610040), dynamin (catalog no. 610245), and AP180 (catalog no. 610469) were from BD Transduction Laboratories. Mouse anti-tubulin monoclonal antibody (catalog no. CP06) was from Oncogene Research (Merck). Caspase-3 and -8 antibodies were used as described (21). His6- and T7-tagged TRAIL was expressed and purified as previously described (22). Alexa Fluor 647 (AF647) succinimidyl ester, Alexa Fluor 633 (AF633)-conjugated transferrin, Alexa Fluor 488 (AF488)-conjugated cholera toxin subunit B, streptavidin Alexa Fluor 568 (AF568), and tetramethylrhodamine ethyl ester (TMRE) were purchased from Invitrogen. A nucleofector and cell line nucleofector kits were obtained from Amaxa (Cologne, Germany). Ultra-pure sucrose was from Sigma-Aldrich. The broad spectrum caspase inhibitor, benzylxoxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (z-VAD-fmk) was purchased from MP Biomedicals (Cambridge, UK). Annexin V-fluorescein isothiocyanate was purified and utilized as previously described (23).

**Preparation of Alexa Fluor 647-labeled TRAIL Conjugate**—Recombinant dual His6- and T7-tagged human TRAIL, purified on nickel-nitrioltriacetic acid beads, was washed extensively with ice-cold PBS and conjugated by adding the succinimidyl ester derivative of AF647 carboxylic acid followed by incubation on ice for 30 min in the dark. Unbound fluorochrome was removed with a series of PBS washes. Conjugated ligand was eluted using PBS containing 0.1 M EDTA. No difference was observed in the efficacy of AF647-labeled compared with unlabeled TRAIL to induce apoptosis in BJAB cells (data not shown).

**Assay for TRAIL and Transferrin Uptake by FACS**—To measure uptake of TRAIL and transferrin, 0.25–0.5 × 10⁶ cells in complete RPMI 1640 medium were either incubated at 4 °C in the presence or absence of AF647-conjugated TRAIL (1 μg/ml unless indicated otherwise), AF633-conjugated transferrin (0.25 μg/ml), or 37 °C in the presence of ligand for the indicated times. Samples were rapidly chilled on ice to inhibit endocytosis and briefly centrifuged at 4 °C. After washing twice in pre-chilled wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂), cell surface-bound ligand was removed by resuspension in pre-chilled acid wash solution (0.2 M NaCl, 0.2 M acetic acid) for 5 min on ice. Cells were subsequently washed 3 times in wash buffer and resuspended in cold PBS containing 2% (w/v) fetal bovine serum before immediate quantification of ligand internalization by FACS using a FACSCalibur flow cytometer. Data were analyzed using CellQuest software (BD Instruments). For experiments using HeLa cells, subconfluent cultures grown in the presence of G418 (0.4 mg/ml) and tetracycline (2 μg/ml) were treated with trypsin/EDTA and replated in 6-well plates (0.75–1 × 10⁶ cells/well) in the absence of G418 and puromycin ~48 h before use. HeLa cells were ≤80% confluent. Internalization of TRAIL and transferrin in HeLa cells was determined as described, except that after acid stripping of cell surface-bound ligand and subsequent washes, adherent HeLa cells were trypsinized and washed again before FACS analysis. Induction of WT dynamin or dominant negative K44A dynamin was confirmed by immunoblotting (data not shown).

**Analysis of Internalization by Confocal Microscopy**—BJAB cells were pre-cooled to 4 °C for 1 h and then exposed to biotin-labeled TRAIL for 45 min, washed extensively, and treated with streptavidin-AF568 at 4 °C for 45 min. Cells were washed and either fixed in 4% paraformaldehyde, or TRAIL was allowed to internalize at 37°C for the indicated times up to 30 min. After internalization, cells were adhered to poly-L-lysine-coated slides and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were counterstained with the DNA dye Hoechst 33342, and Z-stack images were collected on a Zeiss LSM510 with Axiovert 200 microscope and analyzed with laser scanning microscopy and ImageJ software. HeLa cells inducibly expressing wild type dynamin-1 or the dominant negative K44A mutant dynamin-1 were plated on glass coverslips, and internalization of biotinylated TRAIL (500 ng/ml) or transferrin directly conjugated with AF633 (5 μg/ml) was measured...
in the same manner as BJAB cells. HeLa cells were counterstained after fixation with Hoechst 33342, and the plasma membrane marker cholera toxin B was directly conjugated to AF488.

Reversible Biotinylation of Cell Surface Proteins and Receptor Internalization Assay—BJAB cells were chilled on ice and washed 3 times with ice-cold PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ (PBS-MC). Cell surface proteins were labeled with EZ-link®sulfo-NHS-SS-biotin according to the manufacturer’s instructions (Pierce). Cells were washed with PBS-MC and resuspended in ice-cold culture media containing TRAIL (1 μg/10⁶ cells) and further incubated on ice for 45 min to allow ligand binding. Cells were washed with PBS-MC to remove unbound ligand and resuspended in medium at either 0 or 37 °C. Cells were incubated at 37 °C for the indicated times and chilled on ice to stop internalization. After 3 washes with ice-cold PBS-MC, cells were resuspended in cold 2-mercaptoethanesulfonic acid (MESNA) buffer containing 20 mM MESNA, 50 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM MgCl₂, and 1 mM CaCl₂ and incubated on ice for 45 min to remove cell surface biotin moieties. 2-Mercaptoethanesulfonic acid was quenched by resuspending cells with PBS-MC containing 10 mM iodoacetamide. Cell lysates were prepared in DISC buffer containing 20 mM MESNA, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). Internalized biotinylated proteins were pulled down with immobilized NeutrAvidin™ protein (Pierce). Western blotting was used to detect relevant proteins.

Inhibition of Receptor-mediated Endocytosis by Hypertonic Medium—To inhibit receptor internalization, BJAB cells were preincubated for 30 min in culture medium containing 0.25 M sucrose (600 mosmol) before PBS-MC washes and reversible biotinylation. After washing, cells were resuspended in medium containing sucrose (0.25 M) and further incubated for 30 min at 37 °C to study internalization. A sucrose concentration was selected based upon its ability to completely block receptor internalization while inducing minimum levels of apoptosis within the short periods of cell culture in BJAB cells.

Assessment of Apoptosis—Apoptosis in BJAB cells was assessed by loss of mitochondrial membrane potential (ΔΨm). Briefly, BJAB cells (1 × 10⁶/ml) were incubated with TMRE (50 ng/ml) at 37 °C for 10 min. To measure apoptosis induction by phosphatidylserine externalization, cells (1 × 10⁶/ml) were stained with annexin V-fluorescein isothiocyanate and incubated at room temperature for 30 min. Cells were stained with propidium iodide (500 ng/ml) and analyzed by FACS using a FACSCalibur flow cytometer, and the data were analyzed using CellQuest software.

Analysis of DISC Formation—BJAB cells (2.5 × 10⁵/treatment) were treated with biotinylated TRAIL for up to 60 min at 37 °C or on ice. Alternatively, cells were pre-cooled on ice for 60 min, incubated with biotinylated TRAIL for 45 min on ice, washed extensively, and either lysed or warmed to 37 °C for up to 60 min. After treatment, cells were washed, lysed, and biotinylated TRAIL and the associated proteins were pulled down using streptavidin-Sepharose beads, and DISC proteins were analyzed by Western blotting as previously described (24).

Transient Transfection of BJAB Cells by Nucleofection—BJAB cells (2.5 × 10⁵ cells/sample) were resuspended in Nucleofector solution V (100 μl) containing 2 μg of plasmid DNA and 1 μg of pmaxGFP (as a marker for transfected cells). Nucleofection of cells was carried out using Program E-23, diluted into 4.9 ml of complete RPMI, and placed in a humidified incubator at 37 °C for 4 h. FACS analysis was performed by gating on the green fluorescent protein-positive cell population and analyzing either uptake of AF647-labeled TRAIL or AF633-labeled transferrin or alternatively loss of ΔΨm using TMRE.

Apoptosis Induced by Immobilized TRAIL—Cells were seeded at a concentration of 2–5 × 10⁵/ml in complete RPMI 1640 for 12–24 h before treatment. Immediately before treatment cells were washed and resuspended in fresh media. BJAB cells were used at a concentration of 5 × 10⁵/ml. Soluble TRAIL was used at a concentration of 250 ng/ml unless stated otherwise. For experiments involving plate-bound TRAIL, 96-well plates (Cellstar®) were coated with 1 μg/ml soluble TRAIL (in PBS) at 4 °C for 12–24 h. Wells were washed 3 times with PBS (3 × 200 μl) immediately before the addition of cells. The concentration of TRAIL used to coat each well was determined by titration of plate-bound TRAIL against the titration of soluble TRAIL. The degree of apoptosis due to 1 μg/ml plate-bound TRAIL was found to be equivalent to 250 ng/ml soluble TRAIL over the duration of these experiments. Apoptosis was assessed by measuring loss of ΔΨm using TMRE.

RESULTS

Internalization of TRAIL in BJAB Cells—Internalization of TRAIL was assessed in BJAB cells exposed to AF647-conjugated TRAIL. Cells were incubated with fluorochrome-labeled TRAIL either on ice to block endocytosis or at 37 °C for increasing times to permit internalization. After the indicated times, cell surface-associated ligand was stripped by acid washing, and internalized TRAIL was detected by FACS. Increasing the temperature to 37 °C resulted in a rapid internalization of TRAIL with uptake first detected at 5 min and proceeding for up to 60 min (Fig. 1A). Using this method, concentrations of TRAIL as low as 50 ng/ml resulted in uptake of detectable TRAIL (supplemental Fig. 1). To confirm the specificity of the method and assess the involvement of TRAIL-R2 in the endocytosis of TRAIL, we compared the levels of TRAIL internalization in parental versus TRAIL-R2 (DR5)-deficient BJAB cells (18) using uptake of transferrin as a control. Levels of TRAIL-R1 were unaltered in the TRAIL-R2-deficient BJAB cells (18) (data not shown). Uptake of TRAIL was substantially, but not completely reduced in TRAIL-R2-deficient compared with wild type BJAB cells, whereas uptake of transferrin was unaffected (supplemental Fig. 2, B and C), consistent with the notion that internalization of TRAIL is mediated via both TRAIL-R1 and TRAIL-R2. Uptake of transferrin at 37 °C, used as a control, was almost completely blocked by hyperosmotic medium containing sucrose (0.25 M) (Fig. 1B). Hypertonic sucrose is known to block endocytosis of ligands and receptors (25, 26). Importantly, uptake of TRAIL was completely blocked by hypertonic medium (Fig. 1B). Internalization of biotinylated TRAIL was also measured by confocal microscopy in wild type BJAB cells (Fig. 1C). Cells loaded at 4 °C showed a clear labeling of TRAIL on the cell surface, and no TRAIL appeared to be internalized (Fig. 1C, t = 0 min). Upon warming the cells to 37 °C, internal-
**Internalization-independent Killing by TRAIL**

**A**

![Graph showing internalization of TRAIL](image)

**B**

![Image showing internalization with and without sucrose](image)

**C**

![Images showing internalization over time](image)

**FIGURE 1. Internalization of TRAIL is rapid and is inhibited by hyperosmotic sucrose.** A, BJAB cells were suspended in complete RPMI medium at a density of $1 \times 10^5$ cells/ml and incubated for the indicated times at 4 or 37 °C in the presence or absence of TRAIL-AF647 (1 μg/ml). After acid washes, as described under “Experimental Procedures,” fluorescence was detected using the FL4 channel of a FACS Calibur, and data were analyzed using CellQuest software. The left panel shows a histogram for selected times during a kinetic analysis of TRAIL internalization, and the right panel shows the mean fluorescence intensity values at each time over a 0–60 min period. B, BJAB cells, suspended in complete RPMI medium at a density of $1 \times 10^5$ cells/ml, were preincubated for 30 min at 4 or 37 °C either in the presence or absence of 0.25 M sucrose before incubation in the presence or absence of TRAIL-AF647 (1 μg/ml) for an additional 30 min or, alternatively, transferrin-AF633 (0.25 μg/ml) for an additional 5 min. Assays were performed as described in panel A. C, BJAB cells were incubated in the presence of biotinylated TRAIL and streptavidin-568 (red) for 45 min at 4 °C, washed extensively then shifted to 37 °C for the indicated times, fixed, and analyzed by confocal microscopy. Cells were counterstained with Hoechst 33342 (blue). The white bar represents 5 μm.

Internalization of TRAIL was observed at 5 min. Internalization was increased at 15 min with marked staining of small vesicles, perhaps representing early endosomes, and was more complete by 30 min (Fig. 1C), with increased vesicular staining concentrated in larger intracellular compartments possibly representing late endolysosomal vesicles (27).

**Ligand Internalization Is Not Required for TRAIL-mediated Cell Death**—To address the possible role of TRAIL internalization in the induction of apoptosis, soluble TRAIL was adhered to a solid matrix by overnight incubation in 96-well tissue culture plates at 4 °C and washed extensively in PBS before the introduction of BJAB cells to the coated wells. No appreciable difference in the kinetics or magnitude of apoptosis induction between plate-bound and soluble TRAIL was observed (Fig. 2A). Thus, our data demonstrate that ligand internalization is not an obligatory requirement to initiate an apoptotic stimulus in response to TRAIL, but they do not exclude the possibility that the receptor(s) is internalized as part of the death process.

Because internalization of TRAIL was not required for cell death, we next wished to determine the minimum duration of exposure of the cell to ligand in order to induce apoptosis. To accomplish this, BJAB cells were incubated in the presence of plate-bound TRAIL for 0.5–4 h followed by removal to a fresh well in the absence of any TRAIL and incubated further for a cumulative incubation time of 4 h (Fig. 2B). Exposure to plate-bound TRAIL for only 30 min was sufficient to induce some apoptosis, whereas exposure for ~2 h was sufficient to induce maximum levels of apoptosis (Fig. 2B). Taken together, these data demonstrate that TRAIL need not be internalized to induce apoptosis, and interactions between ligand and receptor that occur within the first 2 h are sufficient to effectively initiate maximal apoptosis.

In this and subsequent experiments we utilized the loss of mitochondrial membrane potential ($\Delta \Psi m$) as a measure of cell death by apoptosis. To confirm that this was a valid measure of TRAIL-induced apoptosis in BJAB cells, we measured the time-dependent loss of $\Delta \Psi m$ and correlated it with the increases in both phosphatidylserine externalization and the appearance of a sub-G1 hypodiploid peak, both commonly used measures of apoptosis. The time-dependent loss in $\Delta \Psi m$ correlated well with the increases in both phosphatidylserine externalization and the hypodiploid peak (Table 1). The appearance of the hypodiploid peak was somewhat delayed compared with the loss of $\Delta \Psi m$ or increase in phosphatidylserine externalization compatible with DNA fragmentation occurring at a later stage of apoptosis. Low concentrations of z-VAD-fmk (10 μM), a broad spectrum caspase inhibitor, completely inhibited the induction of cell death assessed by these three different criteria (Table 1). Taken together these data demonstrate that TRAIL causes a time-dependent induction of apoptosis in BJAB cells, and the magnitude of this induction can be reliably assessed by measuring loss of $\Delta \Psi m$.

**Receptor Internalization after Ligation by TRAIL**—Although internalization of TRAIL was not necessary to induce apoptosis, it was possible that internalization of surface receptor(s) was required. To assess receptor internalization, extracellular...
absence of ligand (Fig. 3A, lanes 2–5), compatible with low levels of spontaneous receptor endocytosis (28). Treatment with TRAIL caused a marked stimulation of internalization of both TRAIL-R1 and -R2 (Fig. 3A, lanes 6–9). The increased internalization of these receptors was associated with increased recruitment of both FADD and caspase-8 (Fig. 3A, lanes 7–9). Caspase-8 was also processed to its p43/p41 forms and p18 forms (Fig. 3A, lanes 7–9, and data not shown). Incubation of BJAB cells with hyperosmotic medium containing 0.25 M sucrose (∼600 mosmol) resulted in an almost complete block of internalization of TRAIL-R1 and -R2 (Fig. 3A, lane 10). Because of the lack of internalized receptors, no FADD and only small amounts of caspase-8 were detected (Fig. 3A, lane 10).

Receptor Internalization Is Not Required for TRAIL-induced Apoptosis—To assess the importance of receptor internalization in TRAIL-induced apoptosis, BJAB cells were incubated in either normal or hyperosmotic medium in the presence of TRAIL (1 μg/10⁶ cells), and apoptosis was assessed by loss of ΔΨm. Surprisingly, a marked time-dependent increase in apoptosis was observed in cells exposed to TRAIL under hyperosmotic conditions (Fig. 3B). After 4 h, hyperosmotic stress alone did not induce any apoptosis (Fig. 3B, filled square with an asterisk). These results strongly suggest that receptor internalization is not required for TRAIL-induced apoptosis and may even suggest that internalization could dampen the apoptotic signal.

Under some circumstances hyperosmotic stress alone may induce apoptosis by activation of the intrinsic or extrinsic pathway (29, 30). To determine whether hyperosmotic stress was sensitizing to TRAIL or vice versa, BJAB cells were incubated with a low concentration of z-VAD-fmk (10 μM). At this concentration, z-VAD-fmk selectively blocks death receptor-mediated apoptosis rather than apoptosis induced by perturbation of mitochondria and activation of the intrinsic pathway (23). z-VAD-fmk (10 μM) completely blocked the increased apoptosis observed after incubation of cells in hyperosmotic medium with TRAIL (Fig. 3B), demonstrating that hyperosmotic medium was sensitizing the cells to TRAIL-induced apoptosis. Examination of TRAIL-induced caspase processing revealed an increase in caspase-8 activation after 1 h in cells incubated with hyperosmotic medium, which correlated with an increase in caspase-3 processing (Fig. 3C, compare lanes 1 and 3). No induction of apoptosis was observed in cells incubated for 4 h in hyperosmotic medium alone (Fig. 3B), in agreement with an absence of processing of caspase-8 or -3 (Fig. 3C, compare lanes 5 and 9). After 4 h in hyperosmotic medium, TRAIL induced almost complete processing of caspase-8 and -3, and the processing of caspase-8 was accompanied by the appearance of a small amount of the p18 large subunit (Fig. 3C, lane 11). This TRAIL-induced processing of both caspase-8 and -3 was almost completely inhibited by z-VAD-fmk (10 μM), as evidenced by the maintenance of almost all the intact caspase-8 and -3 zymogens (Fig. 3C, lane 12). Although hyperosmotic medium inhibited ligand-mediated TRAIL-R1 and R2 internalization, induction of apoptosis and activation of caspases were not inhibited, further suggesting that TRAIL receptor internalization is not necessary for cell death signal transduction in TRAIL-induced apoptosis.

**TABLE 1**

| Time (h) | ΔΨm | % Cells | Sub-G1 |
|---------|------|--------|--------|
|         |      |        |        |
| 0       | 9.8 ± 2.4 | 9.3 ± 2.4 | 5.5 ± 0.6 |
| 0.5     | 10 ± 2.3  | 10 ± 2.2  | 5.5 ± 0.9  |
| 1       | 20 ± 1.1  | 27 ± 2.6  | 14 ± 2.5  |
| 2       | 39 ± 2.2  | 52 ± 7.0  | 37 ± 3.8  |
| 4       | 52 ± 2.9  | 58 ± 6.3  | 41 ± 1.5  |
| z-VAD-fmk| 9.5 ± 2.0 | 8.5 ± 2.0  | 5.0 ± 0.7  |
Receptor Internalization Is Not Required for DISC Formation

Because our data strongly suggested that internalization of TRAIL-R1 and -R2 was not required for TRAIL-induced apoptosis, we wished to determine the role, if any, of receptor internalization in DISC formation. BJAB cells were treated with biotinylated TRAIL either preloaded onto chilled cells at 4 °C to synchronize the cells for internalization and subsequently warmed to 37 °C or maintained throughout at 37 °C for the indicated times. Cells were then washed and lysed, streptavidin-coupled agarose beads were used to bind labeled ligand, and DISC proteins were analyzed by Western blotting. Exposure of cells to TRAIL at 37 °C resulted in formation of a DISC within 5 min, and binding of TRAIL-R1 and TRAIL-R2 together with the recruitment of FADD and procaspase-8 observed at this early time point (Fig. 4A, lane 2). The two caspase-8 bands most likely represent the two major isoforms, namely caspase-8a and -8b. Both binding of TRAIL-R1 and TRAIL-R2 and recruitment of FADD and caspase-8 were already marked at 5 min and were maintained for 15 min but showed a slight decrease at 30 min (Fig. 4A, lanes 3–4). Caspase-8 was partially processed to its p43/41 forms, and a small amount of its p18 catalytically active large subunit was also detected (Fig. 4A, lane 4).

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detectable (Fig. 4A, lanes 3 and 4). At 60 min, although significant amounts of TRAIL-R1 and -R2 were still bound to biotinylated TRAIL, less FADD and caspase-8 were associated with the receptors (Fig. 4A, lane 5), presumably because they had dissociated from the receptor(s), due possibly to the internalization of the TRAIL/TRAIL receptor complex into a cellular compartment where the pH results in dissociation of the complex.

Significant changes in the DISC composition were observed when BJAB cells were initially preloaded with TRAIL at 4 °C followed by washing away excess unbound TRAIL and then transferring the cells to prewarmed medium at 37 °C. Interestingly, at 4 °C TRAIL bound extensively to both TRAIL-R1 and TRAIL-R2 accompanied by substantial recruitment of FADD and the proforms of caspase-8 to the DISC (Fig. 4A, lane 6). After transfer to 37 °C, the levels of FADD remained roughly constant, whereas small time-dependent changes were observed in the levels of bound TRAIL-R1 and TRAIL-R2 (Fig. 4A, lanes 7–10), possibly due to different dissociation rates of the ligand with TRAIL-R1 and TRAIL-R2 at the different temperatures (31). At 5 min, caspase-8 was already processed into its p43/41 forms together with a substantial amount of the p18 large subunit (Fig. 4A lane 7). At 15 min, the proform of caspase 8 was almost entirely processed with a corresponding further increase in the amount of p18 (Fig. 4A, lane 8), and by 30 min the proform was entirely processed into its p43/41 and active p18 subunits (Fig. 4A, lane 9). These results clearly demonstrate that all caspase-8 initially recruited to the DISC was processed rapidly to its p43/41 and active p18 forms and did not support the idea that all caspase-8 initially recruited to the DISC was processed to its p43/41 and p18 forms (Fig. 4A, lanes 7–10), possibly due to different dissociation rates of the ligand with TRAIL-R1 and TRAIL-R2 at the different temperatures (31). At 5 min, caspase-8 was already processed into its p43/41 forms together with a substantial amount of the p18 large subunit (Fig. 4A lane 7). At 15 min, the proform of caspase 8 was almost entirely processed with a corresponding further increase in the amount of p18 (Fig. 4A, lane 8), and by 30 min the proform was entirely processed into its p43/41 and active p18 subunits (Fig. 4A, lane 9). These results clearly demonstrate that all caspase-8 initially recruited to the DISC was processed rapidly to its p43/41 and active p18 forms and did not support the idea that any additional caspase-8 above that initially associated with the DISC at 5 min was recruited during the remainder of the incubation.

Further analysis of the intriguing finding of FADD and caspase-8 recruitment to the DISC at 4 °C revealed a time-dependent binding of TRAIL-R1 and TRAIL-R2 that was accompanied by substantial recruitment of FADD and caspase-8 (Fig. 4B). Within 5 min of exposure to TRAIL at 4 °C, a small amount of binding of both TRAIL-R1 and TRAIL-R2 was observed accompanied by recruitment of small amounts of FADD and caspase-8 to the DISC (Fig. 4B, lane 4). After 10 min of exposure, recruitment of all DISC proteins reached maximum levels and did not decrease within the 45-min duration of the experiment. After 30 min a small amount of processed caspase-8 was observed. Because internalization of receptors is completely inhibited at 4 °C, these data demonstrate unequivocally that internalization of TRAIL and/or its receptors is not required for the formation of a functional DISC complex.

Clathrin-mediated Endocytosis Is Not Required for TRAIL-induced Cell Death—While this manuscript was in preparation, it was reported that TRAIL-R2 was internalized by clathrin-mediated endocytosis (28). Therefore, we tested the possible involvement of clathrin-dependent internalization in the regulation of TRAIL-mediated apoptosis. BJAB cells were transiently transfected with either a GTPase-inactive, dominant negative dynamin-2 K44A mutant or, alternatively, the C-terminal fragment of AP180 (AP180C), which interferes with the recruitment and subsequent assembly of clathrin coats at the plasma membrane (32). To facilitate analysis of both TRAIL-induced apoptosis and ligand internalization in the transfected cell population, cells were co-transfected with a green fluorescent protein expression vector. Western blot analysis confirmed expression of the transfected plasmids (Fig. 5A). Uptake of transferrin was strongly blocked by overexpression of either dominant negative dynamin-2 or AP180C (Fig. 5B). Under these conditions internalization of TRAIL was also significantly inhibited by overexpression of AP180C or dominant negative dynamin 2 (Fig. 5C). Experiments were performed at short times after transfection (4 h) to avoid cell death that occurred at later times after transfection of either dominant negative dynamin-2 or AP180C constructs (data not shown). Transfection of cells with either plasmid did not change the susceptibility of BJAB cells to TRAIL-induced apoptosis (Fig. 5D), indicating that clathrin-mediated ligand internalization is not required for TRAIL-induced apoptosis in BJAB cells.

Blockade of Clathrin-mediated Endocytosis Does Not Inhibit TRAIL-induced Apoptosis in HeLa Cells—Because blockade of clathrin-mediated endocytosis using transient transfection of plasmids that inhibit clathrin-mediated endocytosis did not appear to inhibit TRAIL-induced apoptosis in BJAB cells, we wished to confirm this in a well-characterized cellular model with greater control of clathrin-mediated endocytosis. To accomplish this we used a model of HeLa cells stably expressing a dominant negative K44A dynamin-1 mutant under the control of a tetracycline-inducible promoter (19, 20). HeLa cells expressing WT dynamin internalized transferrin at 37 °C but not at 4 °C, whereas internalization of transferrin in HeLa cells expressing the K44A dynamin mutant was markedly inhibited at either temperature (Fig. 6A). Similarly, uptake of transferrin was strongly perturbed in the dominant negative dynamin-1 compared with WT dynamin-expressing HeLa cells using a FACS-based internalization assay (supplemental Fig. 3C). These results demonstrated that clathrin-mediated endocytosis was clearly blocked in the K44A mutant dynamin-expressing cells, confirming that the wild type and mutant dynamin-expressing cells behaved as shown previously. Next we investigated the effects of the dominant negative K44A dynamin on TRAIL internalization (Fig. 6B). No internalization of TRAIL was apparent when either WT or mutant cells remained at 4 °C (Fig. 6B). TRAIL was internalized within 15 min of release to 37 °C in HeLa cells expressing WT dynamin with little detectable TRAIL being retained at the plasma membrane (Fig. 6B). TRAIL was also internalized in the HeLa cells expressing the K44A dynamin mutant, although more TRAIL appeared to remain at the cell surface, suggesting that TRAIL was internalized either more slowly or alternatively via a clathrin-independent mechanism in the mutant cells (Fig. 6B). Although a significant decrease in TRAIL uptake in the dominant negative dynamin-expression HeLa cells compared with WT dynamin-expressing HeLa cells was observed, some TRAIL appeared to be internalized (supplemental Fig. 3B). Taken together our results support the notion that TRAIL may be internalized by clathrin-mediated endocytosis, but when this pathway is blocked it can also be internalized by clathrin-independent endocytosis.
Because TRAIL internalization was partially blocked in HeLa cells expressing the K44A dynamin mutant, we investigated the role of clathrin-dependent endocytosis in TRAIL-induced apoptosis. Apoptosis was assessed by measuring the percentage of annexin V-positive cells by flow cytometry. Control levels of apoptosis in the wild type and K44A dynamin mutant cells were 5 ± 1 and 8 ± 1% (mean ± S.E., n = 3), respectively. After exposure for 4 h of either HeLa cells expressing WT or dominant negative K44A mutant dynamin to TRAIL (500 ng/ml), the levels of apoptosis were 74 ± 6 and 73 ± 5%, respectively (supplemental Fig. 3A). Thus, TRAIL induced similarly high levels of apoptosis in both types of HeLa cells, indicating that clathrin-dependent endocytosis is not required for TRAIL-induced apoptosis.

DISCUSSION

Our studies have examined a possible requirement for ligand- and receptor-mediated internalization in TRAIL-induced apoptosis using a variety of experimental approaches. We demonstrate that there is no defined requirement for endocytosis of ligand or receptor during TRAIL-induced apoptosis. First, we show that TRAIL-induced recruitment of both FADD and procaspase-8 to its receptors to form the TRAIL DISC occurs at 4 °C, a condition when endocytosis is completely blocked (Fig. 4). These observations demonstrate that initial assembly of the TRAIL DISC occurs in the absence of internalization. Second, complete blockade of endocytosis by overexpression of a dominant negative K44A mutant form of dynamin (Fig. 5). Clathrin-dependent endocytosis is not required for TRAIL-induced apoptosis. A, BJAB cells were transiently transfected by nucleofection as described under "Experimental Procedures." After 4 h, whole cell lysates were prepared from 0.5 × 10⁶ cells using ice-cold radioimmune precipitation assay buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a complete protease inhibitor mixture, 1 mM Na₃VO₄, and 10 mM NaF and equal quantities of protein resolved by SDS-PAGE. After transfer to nitrocellulose, blots were probed with either anti-dynamin-1 (1:1000), anti-AP180 (1:250), or anti-tubulin (1:2000). B and C, BJAB cells were transfected by nucleofection as described above in A and then incubated for 4 h at 37 °C. Cells were subsequently either cooled at 4 °C or maintained at 37 °C for 30 min and then incubated in the presence or absence of TRAIL-AF647 (1 μg/ml) for an additional 30 min or transferrin-AF633 (Tfn-AF633) (0.25 μg/ml) for an additional 5 min. Internalization assays were performed as described in the legend to Fig. 1. D, BJAB cells were transfected by nucleofection as described above in panel A. After 4 h at 37 °C, cells were treated with 0 (open bars), 0.25 (gray shading), or 1 μg/ml (block shading) TRAIL for an additional 4 h at 37 °C. TMRE (40 nM) was added for the final 10 min, and ΔVm was assessed. Data shown are the mean ± S.D. from three separate experiments.
After incubation at 37 °C, HeLa cells were fixed and counterstained with Hoechst 33342 (101 m) for 45 min at 4 °C, washed extensively, and released up to 37 °C for 30 min. APRIL 27, 2007 • VOLUME 282 • NUMBER 17

TRAIL-induced apoptosis (33, 34). BJAB cells are type I cells in their susceptibility to CD95 (15, 16), it has also more recently been used to classify differential susceptibility of cells to TRAIL-induced apoptosis (33, 34). BJAB cells are type I cells in terms of TRAIL sensitivity and, interestingly, were used in the original studies describing the formation of the native TRAIL DISC (35–37). Detectable levels of both FADD and procaspase-8 were recruited to TRAIL receptors at 4 °C as early as 5 min after the addition of ligand to BJAB cells, with large amounts of TRAIL DISC formed 10 min after ligand addition. Thus, formation of the TRAIL DISC in BJAB cells occurs without the necessity of either receptor-mediated endocytosis or of any enzymic activity. Moreover, these findings imply a clear difference in the requirement for receptor-mediated internalization for DISC assembly between TRAIL receptor and Fas signaling pathways (17, 28). Whether the requirement for receptor-mediated endocytosis in CD95L-induced, but not TRAIL-induced apoptosis, is related to the selective localization of CD95 but not TRAIL receptors to lipid rafts requires additional studies (38–40). Assembly of the TRAIL DISC at 4 °C raises the question as to whether a portion of FADD weakly pre-associates with TRAIL receptors in the absence of ligand, and triggering of the cell surface receptors with TRAIL induces a conformational change that results in much tighter binding of FADD to TRAIL-R1 and -R2; hence, FADD and, subsequently, caspase-8 are detected in the DISC at 4 °C (Fig. 4). Weak pre-association of receptors and proximal signaling molecules in the absence of ligand has been previously reported for interaction of Janus-activated kinases with type I and II interferon receptors (41, 42).

Recent studies have highlighted a requirement for receptor internalization in death receptor-induced apoptosis for both TNF-R1 and Fas/CD95 (14, 17). More specifically, clathrin-dependent endocytosis was required for Fas-mediated apoptosis, and Fas engagement promoted assembly of a CD95 DISC within an early endosomal compartment in type I cells (17). Paradoxically, both CD95L and TRAIL induce rapid caspase-mediated cleavage of a key adaptor protein, AP2α, which regulates clathrin-coated vesicle formation and cargo selection at the plasma membrane (28, 43). Importantly, treatment with TRAIL blocked clathrin-dependent endocytosis (28). This underscores the possibility that additional non-clathrin-dependent endocytic pathways may contribute to internalization of TRAIL and its receptors under conditions where clathrin-dependent endocytosis is inhibited. Indeed, we observed an incomplete blockade in uptake of TRAIL in both dominant negative dynamin-expressing HeLa cells and BJAB cells transiently transfected with dominant negative dynamin or AP180C (Figs. 5 and 6), consistent with the possibility that other clathrin-independent pathways, such as the recently characterized flotillin-1-dependent endocytic pathway, may contribute to TRAIL receptor-mediated endocytosis (44). Indeed, overexpression of either dominant-negative dynamin-2 or AP180C for longer times (12 h or more) consistently increased flotillin-1 levels (data not shown), so highlighting the possibility that compensatory endocytic pathways could be up-regulated after blockade of one internalization pathway. It is unlikely that caveolin-dependent endocytosis contributes significantly to the internalization of TRAIL receptors, at least in BJAB cells, as these cells do not express caveolin-1.

4 A. Craxton, X.-M. Sun, and G. M. Cohen, unpublished data.
Internalization-independent Killing by TRAIL

TRAIL and its receptors (TRAIL-R1 and TRAIL-R2) were rapidly internalized in the type I cell line, BJAB (Figs. 1 and 3), with internalization of both ligand and its receptors being detected as early as 5 min after TRAIL treatment. Although neither ligand- nor receptor-mediated internalization or, more specifically, clathrin-dependent endocytosis appeared to be required for TRAIL-induced apoptosis, ligand and/or receptor internalization may play roles in non-apoptotic TRAIL-mediated signaling events such as mitogen-activated protein kinase or NF-κB activation (45). In support of this idea, recent studies have shown that TRAIL promotes formation of a secondary signaling complex after initial assembly of the TRAIL DISC (45). In addition to containing the DISC components FADD and caspase-8, this secondary signaling platform also contains RIP, TNF receptor-associated factor 2, and IKKγ/NEMO, which are involved in the activation of c-Jun NH2-terminal kinase/p38, mitogen-activated protein kinase, and NF-κB, respectively. Further studies are necessary to establish the subcellular location(s) of these TRAIL receptor-deficient secondary signaling complexes and whether receptor-mediated endocytosis is required for their assembly.

Although we and other investigators have shown that TRAIL and its receptors are readily endocytosed, the fate of internalized TRAIL receptors remains less clear. An earlier report showed that TRAIL-R1 and TRAIL-R2 were localized predominantly on the plasma membrane and trans-Golgi network of untreated melanoma cells (46). Importantly, although TRAIL induced an overall decrease in cell surface TRAIL-R1 and TRAIL-R2, apparent recycling of these receptors to the cell surface also occurred (46). Hence, it is possible that trafficking of intracellular TRAIL receptors to the plasma membrane may serve as a mechanism to sustain or perhaps amplify TRAIL-induced apoptosis, particularly in cells that have a high proportion of intracellular relative to cell surface TRAIL receptors.

In addition to inhibiting endocytosis of TRAIL and its receptors (Figs. 1 and 3), we observed that hyperosmolarity augmented TRAIL-induced caspase-3 and -8 processing and apoptosis under conditions where hyperosmolarity alone induced no detectable apoptosis (Fig. 3, B and C). Importantly, low concentrations of z-VAD-fmk completely blocked the enhanced apoptosis seen after treatment of cells with TRAIL in the presence of hypertonic compared with normal medium (Fig. 3B), confirming that hyperosmotic medium was sensitizing the cells to TRAIL-induced apoptosis rather than vice versa. Although we do not exclude the possibility that other mechanisms, such as activation of Src family kinases or stress-activated protein kinases or generation of reactive oxygen species, may play roles in the mechanism by which hyperosmolarity sensitizes cells to TRAIL-induced apoptosis, our data raise the possibility that complete blockade of receptor-mediated endocytosis may serve to enhance apoptosis by facilitating increased formation of a cell surface TRAIL DISC.

In summary, our studies show that TRAIL and its receptors are rapidly internalized predominantly via clathrin-dependent but also by clathrin-independent mechanisms. Endocytosis and, in particular clathrin-mediated endocytic processes, are not required for apoptosis induced by TRAIL. This is in contrast to other death receptor ligands TNF and CD95L. Further, formation of the TRAIL DISC in BJAB type I cells can occur in the complete absence of receptor internalization.

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