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*J Immunol* 1999; 162:1042-1048; ;
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TNF Recruits TRADD to the Plasma Membrane But Not the trans-Golgi Network, the Principal Subcellular Location of TNF-R1

Sally J. Jones,* Elizabeth C. Ledgerwood,† Johannes B. Prins,*† Jenny Galbraith,* David R. Johnson,‡ Jordan S. Pober,‡ and John R. Bradley*‡*

The subcellular localization of TNF-R1 to the Golgi apparatus, initially observed in endothelial cells, has been confirmed using transfection of bovine aortic endothelial cells with a human TNF-R1 expression plasmid. The subcellular interactions of TNF-R1 and the TRADD (TNFR-associated death domain protein) adaptor protein have been analyzed in the human monocyte cell line U937 and the human endothelial cell line ECV304 by confocal immunofluorescence microscopy and by Western blot analysis of fractionated cell extracts. In untreated cells, in which TNF-R1 is found on the cell surface but principally localizes to the trans-Golgi network, TRADD is concentrated in the cis- or medial-Golgi region, but separates from the Golgi during cell fractionation. Coimmunoprecipitation studies have shown that TRADD binds to TNF-R1 within 1 min of TNF treatment in a cell fraction containing plasma membrane. This association is followed by a gradual dissociation, which is prevented if receptor-mediated endocytosis is inhibited by hypertonic medium. In contrast, no association is detected between TRADD and TNF-R1 in the Golgi in response to exogenous TNF at any time examined. These results suggest that although TNF-R1 is predominantly a Golgi-associated protein and TRADD also localizes to the Golgi region, exogenous TNF causes TRADD to bind to TNF-R1 only at the plasma membrane. The Journal of Immunology, 1999, 162: 1042–1048.

Materials and Methods

Materials

Medium 199, RPMI 1640 medium, FCS, L-glutamine, penicillin/streptomycin, trypsin-EDTA, brefeldin-A (BFA), sucrose, Tris/NaCl, EDTA, Triton X-100, aprotinin, PMSF, leupeptin, and pepstatin A were all purchased from Sigma-Aldrich (Poole, U.K.). Human rTNF was provided by Autogen Bioclear (P30001A; Pottern, U.K.). The mouse monoclonal anti-human TNF-R1 was obtained from Genzyme Diagnostic (1995-01; Cambridge, MA), goat polyclonal anti-human TNF-R1 Abs were from R&D Systems (AB-225-PB; Minneapolis, MN), mouse monoclonal anti-p58 was from Sigma (G2404; St. Louis, MO) and mouse monoclonal anti-TRADD Ab was from Transduction Laboratories (T50320; Lexington, KY). The rabbit polyclonal Ab to TGN46 was a kind gift from Dr. S. Ponnambalam at the University of Dundee. Secondary FITC-conjugated Abs were from Sigma and Texas Red-conjugated Abs from Dako (Carpinteria, CA). Horseradish peroxidase (HRP) -conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA) and HRP-conjugated porcine anti-rabbit from Dako. Protein G Sepharose 4 fast flow was from Pharmacia (Piscataway, NJ).

Received for publication June 23, 1998. Accepted for publication September 30, 1998.

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1 This work was supported by the National Kidney Research Fund (U.K.), the Wellcome Trust, and the National Institutes of Health. J.R.B. is a National Kidney Research Fund (U.K.) Senior Fellow. J.B.P. is a Wellcome Trust International Fellow.

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3 Abbreviations used in this paper: TRADD, TNFR-associated death domain protein; TRAF, TNFR-associated factors; TGN, trans-Golgi network; HRP, horseradish peroxidase; BAEc, bovine aortic endothelial cells; BFA, brefeldin A; PNS, post nuclear supernatant; MTOC, microtubule organizing center; ER, endoplasmic reticulum.

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Cell culture
ECV304 cells, a spontaneously immortalized cell line of human umbilical vein origin (22) (a kind gift from I. Fritz, Babraham Institute, Cambridge, U.K.), were cultured in medium 199 supplemented with 10% FCS, 1-glutamine, and antibiotics. U937 cells, a human monocyte cell line, were cultured in RPMI 1640 medium supplemented with 10% FCS, 1-glutamine, and antibiotics.

Immunofluorescence microscopy
ECV304 cells were seeded onto sterile glass coverslips in 24-well plates (Costar, High Wycombe, U.K.) 16–24 h before fixation. Cells were washed in PBS, fixed in 2% paraformaldehyde for 2 min, and permeabilized with 0.1% Triton X-100 for 2 min. U937 cells were washed in PBS, fixed as above, cytospun at 600 rpm for 15 s, and permeabilized as above. Cells were incubated with primary Abs diluted in 1% BSA/PBS for 1 h at room temperature. After they were washed, the cells were incubated with secondary conjugated Abs for 1 h at room temperature. Coverslips or slides were mounted in Citifluor and examined using a Nikon Optiphot-II microscope (Nikon, Kingston upon Thames, U.K.) coupled to a Bio-Rad (MRC1000; Hemel Hempstead, U.K.) confocal attachment and COMOS software (BioRad).

Transfection of human TNF-R1 and TNF-R2 into bovine aortic endothelial cells (BAEC)
Expression constructs encoding the TNFR (TNF-R1, pcDNA3, and TNF-R2, pcDNA3) were generated by ligating NotI fragments containing the coding sequences from an expression vector generously provided by Immunex (Seattle, WA) into NotI-digested pcDNA3.1 (Invitrogen, San Diego, CA). BAEC were cultured in DMEM supplemented with 10% FCS, 5 mM HEPES, 1-glutamine (1:100) sodium phosphate, penicillin, and streptomycin (all from Life Technologies, Gaithersburg, MD). Subconfluent BAEC in 35 mm tissue culture dishes (C-6) were transfected with the TNFR expression constructs (3 mg/0.2 ml Optimem with 6 ml lipofectamine/0.2 ml according to the manufacturer’s directions (Life Technologies). After culture overnight, cells were transferred to 10-cm-plates and transfectedants were selected with 0.5 mg ml-1 G418 (Life Technologies).

Subcellular fractionation
ECV304 cells (detached using PBS containing 0.05% EDTA) and U937 cells were washed three times in PBS (600 × g for 5 min). All subsequent steps were performed at 4°C. The washed cell pellets were resuspended (1 × 10^6 cells ml^-1) in homogenization buffer (10 mM Tris-HCl (pH 7.4), containing 3% (w/v) sucrose and proteinase inhibitors 1 mM EDTA, 10 μg ml^-1 aprotinin, 1 μg ml^-1 leupeptin, 1 μg ml^-1 pepstatin A and 1 mM PMSF) and allowed to swell in the hypotonic buffer for 20 min. The cells were then homogenized in buffer containing 0.25 M sucrose using 20–30 strokes in a Dounce homogenizer. The homogenate was centrifuged (1300 × g for 5 min) to obtain a nuclear-cell debris pellet and postnuclear supernatant (PNS). The PNS was further centrifuged (10,000 × g 15 min) and the postmitochondrial/hyposommal (100K supernatant) was further centrifuged (100,000 × g 1 h) to yield a cytosolic fraction (100K supernatant) and a microsomal pellet (100K pellet). The 100K pellet was resuspended in 1.5 M sucrose and overlaid with 1.5 M, 0.9 M, 0.6 M, and 0.25 M sucrose. The discontinuous sucrose gradient was centrifuged at 100,000 × g 2 h. The individual interfaces were removed, diluted in 0.25 M sucrose, and centrifuged (100,000 × g 1 h). The pellets were resuspended in 500 μl of 0.25 M sucrose and assayed for alkaline phosphodiesterase activity (plasma membrane) and α-mannosidase II activity (Golgi marker) as described by Storrie and Madden (23) and the protein concentrations were determined using the Bradford assay (24). Samples were used immediately or stored at −20°C.

SDS-PAGE and Western blot analysis
Equivalent amounts of fractionated protein (5–25 μg) were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were blocked in buffer containing Tris-HCl (pH 7.4), 5% dried milk powder, and 0.05% Tween 20. After blocking, the membrane was immunoblotted with mouse monoclonal anti-TNF-R1 (1:1500), mouse monoclonal anti-p58i (1:500), mouse monoclonal anti-TRADD (1:500), or rabbit anti-TGN46 (1:5000) for 2 h at room temperature or overnight at 4°C. The bound Abs were visualised by chemiluminescence (Kirkegaard & Perry Laboratory, Gaithersburg, MD).

Immunoprecipitation
U937 cells (5 × 10^5 ml^-1) were washed three times in PBS (600 × g for 5 min) and incubated in the presence or absence of TNF (100 ng/ml^-1) for variable time periods. The cells were washed once in PBS (600 × g for 5 min) and either lysed immediately in 500 μl of lysis buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 1% Triton X-100, and proteinase inhibitors) for 1 h on ice or chased for variable time periods before lysis. In the meantime, prewarmed Protein G Sepharose beads (40 μl) were incubated with 20 μl of goat anti-TNF-R1 Ab in 500 μl of lysis buffer using an upright rotator (1 h, 4°C). The U937 lysate was centrifuged (10,000 × g 15 min at 4°C) and the supernatant incubated with the Protein G/Ab complex for 3 h at 4°C. The Sepharose beads were then washed four times with lysis buffer and twice with lysis buffer lacking Triton X-100. The precipitates were resuspended in nonreducing Laemmli sample buffer, boiled, resolved by SDS-PAGE, and transferred onto nitrocellulose. After blocking, the membrane was immunoblotted with the mouse monoclonal anti-TRADD Ab (1:500) for either 2 h at room temperature or overnight at 4°C and visualized with HRP-conjugated donkey anti-mouse IgG using the chemiluminescence detection system.

Coimmunoprecipitation
FIGURE 1. Transfection of human TNF-R1 and TNF-R2 into BAECs. BAECs transfected with human TNF-R1 exhibit a Golgi immunofluorescence pattern (A) when stained with a mouse monoclonal anti-human TNF-R1 Ab, whereas BAEC transfected with human TNF-R2 demonstrate diffuse cytoplasmic and surface membrane staining (B). Staining of nontransfected cells was not observed with either Ab.

Results
Subcellular localization of TNF-R1
In our previous report (13), we showed that in HUVEC, TNF-R1 is principally localized in the Golgi apparatus. Although several different Abs revealed Golgi staining, not all Abs reacted with all cells. Therefore, we transfected expression plasmids for TNF-R1 and TNF-R2 to confirm that there was specific recognition of TNF-R1 in the Golgi. BAECs transfected with human TNF-R1 exhibited a compact juxtanuclear staining pattern (Fig. 1A) characteristic of Golgi-associated proteins (25) when stained with monoclonal anti-human TNF-R1 Ab (R&D Systems). This Ab did not stain untransfected BAEC. In contrast, BAEC transfected with
human TNF-R2 demonstrated diffuse cytoplasmic and surface membrane staining with anti-human TNF-R2 Abs (Fig. 1B). Because untransfected BAECs do not stain with the Ab to human TNF-R1, the Golgi-associated staining can be unequivocally attributed to TNF-R1 localization to this subcellular compartment.

To further analyze the location and function of TNF-R1, we extended our studies to two other cell types, the ECV304 human endothelial and the U937 human monocyte cell lines that provide convenient sources of material for subcellular fractionation and coimmunoprecipitation studies (Fig. 2). Both ECV304 and U937 cells exhibit a staining pattern characteristic of Golgi-associated proteins when labeled with a mouse mAb to TNF-R1 (Figs. 3 and 4). We extended this morphological approach by immunoblotting TNF-R1 in fractionated cells. In both ECV304 (Fig. 2, A and B) and U937 cells (Fig. 2C) TNF-R1 was enriched in the 0.9/1.15 M sucrose interface together with p58, a medial-Golgi marker, and TGN46, a trans-Golgi marker. In Fig. 2B, the TGN46 polyclonal Ab recognizes a close set of bands of approximately 100 to 120 kDa attributable to TGN46 being highly glycosylated (26). α Mannosidase II specific activity was 7-fold higher in this interface than the PNS. The enrichment of TNF-R1 in this Golgi-containing fraction provides biochemical evidence for the Golgi association of TNF-R1, complementing the previous immunocytochemical approaches.

**Sub-Golgi localization of TNF-R1**

To investigate with which Golgi subcompartment (cis-, medial-, trans-Golgi cisternae or TGN) the receptor is associated, cells were cultured in the presence or absence of BFA and again analyzed by confocal microscopy. This fungal metabolite causes the different Golgi compartments to redistribute in a characteristic manner. In many cell types, the TGN collapses around the microtubule organizing center (MTOC) giving a spotlike appearance (27), whereas other Golgi compartments
redistribute into the endoplasmic reticulum (ER) giving a cytoplasmic staining pattern (28). After treating ECV304 cells for 4 h with BFA, the immunofluorescence pattern associated with TNF-R1 and TGN46 was that of a compact center of staining at the nuclear membrane, representative of a collapse to the MTOC, whereas the immunofluorescence pattern associated with p58 was now dispersed throughout the cytoplasm consistent with the redistribution of p58 into the ER (Fig. 3). In all cases, the collapse to the MTOC and the redistribution to the ER was rapid, occurring within 10 min. Merged images following costaining with the rabbit polyclonal anti-TGN46 Ab and the mouse monoclonal anti-TNF-R1 Ab demonstrated colocalization (Fig. 4), although anti-TNF-R1 Abs additionally exhibited weak cytoplasmic staining. Identical staining patterns were also observed in U937 cells (Fig. 5). We conclude that most TNF-R1 resides in the TGN, but smaller receptor pools also exist.

Subcellular localization of TRADD

The principal signal transduction pathways initiated by ligand binding to TNF-R1 are mediated by the recruitment of TRADD. To determine whether the TGN was involved in TNF signaling, we investigated whether TRADD also demonstrated a Golgi distribution. By indirect immunofluorescence using U937 cells (Fig. 5) and ECV304 cells (data not shown), the monoclonal anti-TRADD Ab exhibited a compact perinuclear stain characteristic of Golgi-associated proteins and similar to that observed with anti-TNF-R1 Abs on cells cultured both in the presence and absence of TNF. After BFA treatment, the immunofluorescence pattern associated with anti-TRADD Abs dispersed throughout the cytoplasm (Fig. 5) suggesting redistribution to the ER. This pattern suggests a medial- or cis-Golgi association, unrelated to the distribution of TNF-R1. In contrast to the staining pattern, Western blot analysis of subcellular fractions derived from cells cultured in the absence of TNF suggested that TRADD was a cytosolic protein (Fig. 6A), whereas under the same conditions TNF-R1 was associated with membrane containing fractions (Fig. 6B). These data suggest that in untreated cells, TRADD is loosely associated with the Golgi but not the TGN in which TNF-R1 is localized.

TNF recruitment of TRADD to TNF-R1

In U937 cells, TRADD associates with TNF-R1 in a TNF-dependent process (11). Therefore, we used U937 cells to examine whether TRADD could be recruited to TNF-R1 in the Golgi. First, we examined the ability to communoprecipitate TRADD with TNF-R1. TRADD coimmunoprecipitated with TNF-R1 in TNF-treated, but not mock-treated, cells from the whole cell lysate and 10K pellet, but poorly from the 100K membrane pellet that contains TGN-derived membranes (Fig. 7). Because plasma membranes are found to a greater extent in the 10K pellet, these results are consistent with the conclusion that TRADD communoprecipitated in the 10K pellet is associated with plasma membrane TNF-R1. The failure to find TRADD in immunoprecipitates from the 100K membrane pellet of TNF-treated cells, suggests that the Golgi complex is not involved in signaling in the response to exogenous TNF.

After ligand binding, TNF-R1 is internalized (29) probably via endosomes (present in the 100K pellet) and then lysosomes (present in 10K pellet). Thus our failure to find TRADD associated with TNF-R1 in the 100K pellet not only indicates that TRADD does not associate with TNF-R1 in the Golgi, but also suggests that
TRADD molecules, recruited to TNF-R1 in the plasma membrane, may dissociate as TNF-R1 moves to endosomes.

To examine this possibility the kinetics of TNF-dependent TRADD coimmunoprecipitation at both 4°C (inhibits receptor-mediated endocytosis) and room temperature was determined. The recruitment of TRADD to TNF-R1 in U937 cells is rapid, occurring within 1 min even at 4°C (Fig. 8A). Therefore, we pulsed U937 cells with TNF for 1 min at 4°C and chased in PBS for 1 or 60 min at 20°C, prepared the 10K and 100K pellets, and examined the ability to coimmunoprecipitate TRADD from each of these subcellular fractions. TRADD was coimmunoprecipitated from the 10K pellet after 1 min, but not after 60 min, whereas TRADD was not coimmunoprecipitated from the 100K pellet at either time point (Fig. 8B). These data are consistent with the conclusion that TRADD blotted in the 10K pellet was attributable to the presence of plasma membrane TNF-R1.

To determine more directly whether TRADD was dissociating from TNF-R1 after internalization, the effect of an inhibitor of endocytosis was studied. Incubation of cells in hypertonic medium has been shown to greatly reduce receptor-mediated endocytosis (5, 6). Therefore, we pulsed cells for 1 min with TNF at 4°C, chased for various time periods in either PBS or 0.45 M sucrose, and investigated the ability to coimmunoprecipitate TRADD with TNF-R1. In PBS, TRADD dissociates from the receptor in between 30 and 60 min, but if receptor-mediated endocytosis is inhibited by hypertonic medium, TRADD remains associated with the receptor for at least 60 min (Fig. 8B).

**FIGURE 6.** Recognition of TRADD and TNF-R1 in different subcellular fractions. ECV304 cells were homogenized and fractionated, and 15 μg of protein from the PNS, 10K, and 100K pellet and supernatants were separated by SDS-PAGE. A, Anti-TRADD Ab specifically recognized a band of approximately 34 kDa in the cytosolic (10K and 100K supernatants), but not in the membrane fractions (10K and 100K pellets). B, In contrast, anti-TNF-R1 Ab recognized a band of approximately 60 kDa in membrane fractions, but not in cytosolic fractions.

**FIGURE 7.** Subcellular localization of TNF-R1 and TRADD association after TNF treatment. U937 cells were incubated in the presence or absence of TNF for 30 min. TNF-R1 was immunoprecipitated from either the cell lysate, 10K or 100K pellets, and the immunoprecipitates (IP) were electrophoresed and immunoblotted with mouse monoclonal anti-TRADD Abs. TRADD is only specifically recognized as a 34 kDa-band in the cell lysate and 10K pellet of TNF-treated cells.

**FIGURE 8.** A, The kinetics of TNF-R1 and TRADD association at 4°C and 20°C. U937 cells were treated with TNF for up to 30 min as indicated. The cells were lysed and TNF-R1 was immunoprecipitated from the cell lysate with the goat polyclonal anti-TNF-R1 Ab. The immunoprecipitates (IP) were electrophoresed and immunoblotted with mouse monoclonal anti-TRADD Abs. B, Subcellular localization of TNF-R1 and TRADD association after a pulse and chase study. U937 cells were incubated with TNF for 1 min and chased in PBS for 1 and 60 min. TNF-R1 was immunoprecipitated from either 10K or 100K pellets and supernatants. The immunoprecipitates (IP) were electrophoresed and immunoblotted with mouse monoclonal anti-TRADD Abs. The 34 kDa TRADD band is only recognized in the 10K pellet after a 1 min chase time point.
hibited by hypertonic medium, TRADD remains associated with the receptor for at least 150 min (Fig. 9).

Discussion

This study advances the findings of our previous report describing the association of TNF-R1 with the Golgi apparatus in HUVEC (15) using confocal immunofluorescence microscopy. In the present study, we have confirmed that transfected TNF-R1 localizes to the Golgi and have defined the localization more precisely as the TGN. We also extended the observation to two human cell lines, the ECV304 and U937 cells, so that cell fractionation could be employed to support the morphological studies. We have analyzed the intracellular distribution of TRADD, the initial adaptor molecule involved in TNF-R1 signaling, and performed coimmunoprecipitation studies to determine the function of TGN associated TNF-R1. These findings suggest that TNF-R1 in the TGN is not responsive to exogenous TNF and that internalization of plasma membrane TNF-R1 may terminate signal transduction.

We have studied the localization of TNF-R1 in different cell types with diverse biologic responses to TNF. Cultured human endothelial cells are resistant to TNF-induced cell death, but respond to this cytokine by activating a number of proinflammatory genes (30). In other cell types, TNF is cytotoxic and U937 cells are reported to be sensitive to TNF-induced apoptosis (31). As TNF-R1 is predominantly localized in the Golgi of both endothelial cells and U937 cells, this cellular distribution is unlikely to be important in determining these different responses to TNF.

Our findings indicate that in U937 cells exogenous, TNF causes TRADD to rapidly associate with TNF-R1 at the plasma membrane. It is believed that trimeric TNF induces trimeric clusters of TNF-R1 and the death domains of the clustered receptor create a high affinity binding site for TRADD. When TNF is released from its receptor, the receptor clusters dissociate causing TRADD to be released. As most of the signaling events are completed within minutes of the exposure of cells to TNF, the inability to communoprecipitate TRADD from Golgi-enriched subcellular fractions (100K pellet) within 30 min suggests that exogenous TNF does not induce TRADD recruitment to the Golgi. Our observations do not exclude the possibility that endogenously synthesized TNF may interact with the Golgi-associated TNF-R1. Expression of a transfected-TNF gene in TNF-sensitive tumor cell lines confers resistance to TNF-mediated cell lysis (32), which is correlated with down-modulation of cell surface TNFR (33). Secreted TNF is processed through the Golgi and released by a BFA-sensitive mechanism (34). Further studies will determine whether endogenous TNF can modify cellular response to TNF through interaction with a Golgi receptor. Interestingly, the indirect immunofluorescence studies demonstrated that, in cells which had not been treated with TNF, TRADD was associated with a different Golgi subcompartment to TNF-R1. The mechanism by which the cytosolic protein TRADD associates with the Golgi is unknown, but their different localizations within the Golgi may prevent constitutively synthesized TRADD and TNF-R1 interacting.

The significance of the TGN association of TNF-R1 remains unknown. The TGN packages newly synthesized proteins into transport vesicles that are directed to pre-lysosomal/lysosomal compartments, secretory granules, or the plasma membrane (14, 15, 16, 17) and it participates in retrieving and reusing plasma membrane components internalized by endocytosis such as the mannose-6-phosphate and transferrin receptors (35, 36). As TNF-R1 after TNF binding is also rapidly endocytosed, the internalized receptor ligand complex may recycle to the TGN or other intracellular compartments and act locally to generate TNF-driven signals. Alternatively, the TGN-associated receptor may act as a pool from which the receptor is delivered in a regulated manner to other sites.

Acknowledgements

We thank Paul Luzio and Barbara Reave for helpful discussions.

References


