Reactive Oxygen Species Are Downstream Products of TRAF-mediated Signal Transduction*

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Members of the TNFR (tumor necrosis factor receptor) superfamily are involved in regulating activation and differentiation of cells as well as cell survival and programmed cell death/apoptosis. Multimerization of TNFRs can lead to recruitment of TRAFs (TNFR-associated factors) by the receptors resulting in activation of kinases and transcription factors, such as c-Jun N-terminal kinase and nuclear factor κB (NF-κB). Signal transduction triggered by TNF-α also induces an increase in intracellular reactive oxygen species (ROS). ROS have been suggested to play a role in NF-κB activation, which is thought to promote cell survival. However, oxidation of proteins and lipids by ROS can also result in apoptosis. The processes generating intracellular ROS and the mechanism(s) regulating the cellular redox status have not been fully elucidated. We investigated whether TRAFs play a role in controlling intracellular ROS levels. Our results indicate that recruitment of TRAFs to the plasma membrane of human embryonic kidney (HEK) 293 cells is crucial for activation of signaling pathways, which regulate ROS production in mitochondria. TRAF-mediated changes in ROS levels enhanced NF-κB activation but were not dependent on NF-κB-inducing kinase. Consistent with its anti-apoptotic function, Bcl-xL interfered with TRAF-mediated ROS generation but not NF-κB activation. Taken together, our results suggest a novel role of TRAFs in signal transduction pathways triggered by TNFR-related proteins, which balance cell survival and apoptosis by regulating the electron transport in mitochondria.

The tumor necrosis factor receptor (TNFR) superfamily contains more than 20 cell surface receptors, which can be divided into two subfamilies based on the presence or absence of a cytoplasmic death domain. TNFR family members have been shown to regulate proliferation, differentiation, and survival of cells (for review, see Refs. 1 and 2). TNF-R (CD120a, TNFR p55, TNFR p60) and Fas (CD95, Apo-1) are two death domain-containing TNFR-related proteins, which after engagement by their ligands or cross-linking with antibodies trigger apoptotic pathways by recruitment of the intracellular adapter proteins TRADD (TNFR-associated death domain protein) and/or FADD/Mort-1 (Fas-associated death domain protein/mediator of receptor-induced toxicity) (3, 4). Receptor-induced multimerization of FADD leads to caspase activation, which causes degradation of specific target proteins and, thereby, damage of the cell integrity (5, 6). Members of the TNFR family lacking a death domain including TNF-R-II, CD30, Ox40, and 4-1BB cannot interact with TRADD or FADD but do recruit TNFR-associated factor (TRAF) 2 to the plasma membrane (7–10). The adapter protein TRAF2 is involved in signaling pathways, which regulate cell survival (for review, see Ref. 11). Effector molecules of TRAF-mediated signaling include protein kinases such as NF-κB-inducing kinase (NIK) or c-Jun N-terminal kinase (JNK), which can activate the transcription factors nuclear factor κB (NF-κB) and activator protein 1 (AP1). Both transcription factors can antagonize apoptotic processes by increasing transcription of pro-survival genes (12–14). Previous studies have shown that TNF-α alters the redox status of cells (15–17). TNF-α homo- and heterotrimers are ligands of several members of the TNFR superfamily, which activate NF-κB (18). Various other stimuli that result in NF-κB activation can also increase intracellular ROS levels, and Schreck and colleagues (16) have suggested that NF-κB activation may require elevated levels of ROS. However, little is known about the molecular mechanisms of signal transduction pathways linking cell surface receptors to sites of intracellular ROS generation. Both the requirement of intracellular ROS for activation of downstream target proteins and the mechanisms of ROS generation are subjects of ongoing research.

This study addresses the role of TRAF proteins in regulating the increase of intracellular ROS levels upon receptor cross-linking. Treatment of cells with TNF-α and overexpression of constitutively active forms of TNFR-related proteins both resulted in increased intracellular ROS levels. Signaling triggered by TNF-R-I, which does not recruit TRAFs directly to its cytoplasmic domain, can cause increases levels of intracellular ROS in the absence of exogenous TRAFs. In contrast, TNFR-related proteins including TNF-R-II, CD30, 4-1BB, and Ox40, which have been shown to trigger TRAF-mediated signal transduction cascades, depend on soluble TRAF molecules in the cytoplasm to trigger ROS production. Studies with chemical inhibitors of ROS production and with cells either lacking a functional respiratory chain (ρ− cells) or expressing Bcl-xL suggest that TNFR-related receptors regulate the cellular redox
status by signal transduction pathways, which involve the electron transport chain in mitochondria.

EXPERIMENTAL PROCEDURES

Cell Line—For all experiments the human embryonic kidney cell line HEK293 (ATCC) or HEK293 \( \beta \) cells, which contain mitochondria but lack an intact respiratory chain, were used. HEK293 \( \beta \) cells were generated by incubating wild-type HEK293 cells in medium containing ethidium bromide (25 mg/ml), sodium pyruvate (1 mM), and uridine (50 \( \mu \)g/ml) (19).

Transfections—Cells were transfected by Ca\(\textsubscript{3}(\text{PO}_4)\text{2}\) precipitation of the appropriate plasmids according to standard protocols (20). In brief, 5 \( \times \) 10\(^5\) cells/well were seeded on six-well plates, and 6 h before transfection medium was changed. If not otherwise described, cells were co-transfected with 65 ng of expression plasmids (pcDNA3) containing the coding sequence of TRAF2 and of various chimeric constructs consisting of the extracellular and transmembrane domain of CD28 and the cytoplasmic domain of TNFR-related proteins. For co-expression of NIK, 60 ng of pcDNA3 encoding NIK was used (kind gift of Dr. David Wallach). For co-expression of A20, 60 ng of pcDNA3(FLAG-A20) was used (kind gift of Dr. Richard Ulevitch). Eight hours after transfection, the medium was changed. Twenty-four hours after transfection, surface expression of CD28-chimeric proteins was analyzed by FACS using phycoerythrin-conjugated monoclonal antibody 37.51 specific for mouse CD28 (PharMingen) or the cells were lysed in reporter lysis buffer (Promega). After lysis, soluble and insoluble fractions of the lysates were separated by centrifugation in a microcentrifuge at 6,000 rpm for 5 min at 4 °C. The soluble fractions were used to analyze NF-\(\kappa\)B activity by luciferase assay and ROS levels by fluorometry. TRAF2 levels were analyzed in both detergent-soluble and -insoluble fractions by Western blot.

Luciferase Assay—To determine NF-\(\kappa\)B activity in cells, luciferase assays were performed as described previously (9). Cells were co-transfected with 65 ng of either a NF-\(\kappa\)B-responsive luciferase reporter plasmid containing two canonical \( \kappa\)B sites (pGL2[2xNF-\(\kappa\)B]) (21) or a control plasmid lacking the \( \kappa\)B sites (pGL2), 65 ng of \( \beta \)-galactosidase expression plasmid (as internal transfection efficiency control), receptor and TRAF expression constructs. The total amount of DNA was kept constant at 400 ng by adding pcDNA3 plasmid. Twenty-four hours after transfection, cells were harvested, washed once in phosphate-buffered saline, and incubated for 15 min at room temperature in reporter lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). After precipitation of the insoluble components of the lysates by centrifugation, luciferase assays were performed with aliquots of the lysates and analyzed by luminesmetry according to the manufacturer’s protocol (Promega). \( \beta \)-Galactosidase reactions were performed with the same lysates, and luciferase data were normalized to account for variations in transfection efficiency. NF-\(\kappa\)B activation was calculated by dividing the relative light units measured after transfection with pGL2[2xNF-\(\kappa\)B] by relative light units measured after transfection with pGL2. Transfections were done in triplicate, and the data shown are representative of at least three experiments.

Western Blot Assays and Separation of Subcellular Compartment—Eight hours after transfection of HEK293 cells or HEK293 \( \beta \) cells, the medium was changed, and 24 h after transfection the cells were lysed in reporter lysis buffer (Promega) or in lysis buffer containing 0.5% Triton X-100, 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 8.5 \( \mu \)g/ml aprotinin, 5.5 \( \mu \)g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 mM Na\(_2\)VO\(_4\). Soluble and insoluble fractions of the lysates were separated as described above. The pellets were washed three times in lysis buffer. Twenty-five percent of the soluble fractions and the complete corresponding insoluble fractions were separated by 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred to polyvinylidene difluoride membrane, and TRAF2 in the distinct samples was quantitated by Western blotting. For TRAF2 detection, a 1:5,000 dilution of a TRAF2-specific polyclonal rabbit Ab (C-20, Santa Cruz) was used as primary antibody. Primary Ab binding was detected using a 1:10,000 dilution of HRP-conjugated anti-rabbit Ig (Santa Cruz), followed by chemiluminescence according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

Measurement of ROS—Intracellular ROS generation was assessed using 2,7’-dichlorofluorescein diacetate (Molecular Probes). Cells were plated on Petri dishes and incubated with 10 \( \mu \)M dichlorofluorescein diacetate (DCFH-DA) under various conditions described in the text and figure legends. ROS in the cells cause oxidation of DCFH-DA, yielding the fluorescent product 2’,7’-dichlorofluorescein (DCF). The DCF fluorescence was measured either in intact cells using a FACS-Calibur (Becton Dickinson) or after lysing cells in lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) in the supernatant of cell lysates, which had been centrifuged to remove debris, using a spectrofluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to values obtained from mock-treated controls.

Electromobility Shift Assays—Nuclear extracts were prepared from cells by a modified extraction procedure (22). In brief, cells were lysed in reporter lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid) by TNF-\(\alpha\) treatment and analyzed by electro-mobility shift assay with an anti-\(\beta\)-NF-\(\kappa\)B serum (Chemicon) or anti-\(\kappa\)B sites antibodies (pGL2[2xNF-\(\kappa\)B]) (21) or a control plasmid lacking the \( \kappa\)B sites (pGL2). Analysis of reporter DNA binding activity was determined by upward shift analysis with an anti-\(\kappa\)B sites antibody and the p65 subunit of NF-\(\kappa\)B. All reaction mixtures were electrophoresed on 4% non-denaturing polyacrylamide gel. Subsequently, the gels were dried and autoradiographed.

RESULTS AND DISCUSSION

Signaling Triggered by TNFR Family Members Causes Increased Levels of Intracellular ROS—TNF-\(\alpha\) has been shown previously to cause increased ROS levels in cells expressing TNFR at their cell surface (16). To investigate the effects of TNF-\(\alpha\) on the redox status of HEK293 cells, cells incubated with soluble human TNF-\(\alpha\) and DCDF-DA were analyzed by FACS. DCFH-DA is a membrane-permeable fluorescent dye, which can be used to analyze intracellular ROS levels. DCFH becomes trapped inside cells after cleavage of its acetyl groups by cellular esterases. DCFH-DA has been shown to become oxidized by nitric oxide and hydrogen peroxide but is not directly oxidized by either superoxide or free hydroxyl radicals (23, 24). As shown previously, oxidation of DCFH-DA to DCF results in ligand-independent multimerization of TNFR-II and TRAF2, but not TRAF2 alone, also led to increased intracellular ROS levels in response to TNF-\(\alpha\) (Fig. 1A). To accurately quantify the changes in ROS levels caused by TNF-\(\alpha\), HEK293 cells were incubated with DCFH-DA, lysed, and analyzed by fluorometry. Compatible with the responses detected by FACS analysis, intracellular ROS levels determined by fluorometry were significantly elevated in the presence of TNF-\(\alpha\) (Fig. 1B). In all subsequent experiments, ROS levels of cell lysates were analyzed by fluorometry and changes in comparison to mock-treated samples are indicated.

The higher affinity of soluble TNF-\(\alpha\) for TNFR-I compared with TNFR-II and lack of detectable TNFR-II expression on HEK293 cells suggested a role of TNFR-I in regulating ROS production in HEK293 cells. To test this hypothesis, HEK293 cells were co-transfected with expression constructs encoding human TNF-\(\alpha\)-II and TRAF2. Overexpression of TNFR-II and TRAF2 results in ligand-independent multimerization of TNFR-II on the cell surface and induces TRAF-mediated signaling events (8, 27). As seen after TNF-\(\alpha\)-induced multimerization of endogenous TNFR, signaling induced by overexpressed TNFR-II and TRAF2, but not TRAF2 alone, also led to...
increased levels of intracellular ROS (Fig. 1C). Incubation of HEK293 cells expressing endogenous TNFR-I and overexpressing TNFR-II with TNF-α resulted in an additional increase of intracellular ROS levels (data not shown).

In contrast to TNFR-I, TNFR-II lacks a death domain in its cytoplasmic domain, and signaling triggered by TNFR-II is thought to promote cell activation and survival (28). Other members of the TNFR subfamily lacking a death domain include CD30, Ox40 (CD134), and 4-1BB (CDw137) (1, 2). To compare downstream events triggered by these TNFR-related proteins and to avoid differences in receptor-induced signaling caused by different affinities for their ligands, we utilized chimeric receptor molecules as described previously (9, 27, 29). In brief, we constructed chimeric receptors containing the extracellular and transmembrane domain of mouse CD28 and the cytoplasmic domains of TNFR-related molecules, such as CD30, 4-1BB, and Ox40. CD28 is expressed as dimer on the cell surface linked by a cysteine-cysteine bond in the extracellular domain of the monomeric receptor chains. As seen after engagement of full-length TNFR by their respective ligands, forced dimerization of the cytoplasmic tails of CD30, Ox40, and 4-1BB by fusion to the extracellular and transmembrane domain of CD28 results in signaling events, such as NF-κB activation (9, 29). Direct comparison of the cell surface expression levels of the distinct receptors by FACS using an antibody specific for the extracellular domain of CD28 showed similar transfection efficiencies and expression levels of all receptors independent of TRAF2 expression (Fig. 2A). As we have shown previously, signaling triggered by TNFR-related molecules causes intracellular redistribution of TRAF2, which leads to accumulation of TRAF2 in a detergent-insoluble fraction of cellular lysates. Despite similar expression levels, the distinct chimeric receptors caused significant quantitative differences in TRAF2 levels in detergent-soluble and -insoluble cellular fractions (27). To test if these constitutively active chimeric forms of these TNFR-related molecules have an effect on intracellular ROS levels, HEK293 cells were transfected with expression constructs encoding TRAF2 and either a tailless CD28 mutant as control or a chimeric CD28 molecule containing the cytoplasmic domains of CD30, 4-1BB, or Ox40. Although expression of TRAF2 in the absence of receptor signaling had no detectable effect on the cellular redox status, TRAF2-mediated signal transduction triggered by the cytoplasmic domains of CD28-CD30, CD28-Ox40, or CD28–4-1BB resulted in significantly increased intracellular ROS levels (Fig. 2B). Taken together, these findings suggest a role of ROS in TRAF-mediated signal transduction pathways downstream of TNFR-related proteins.

**TRAF Recruitment to TNFR-related Molecules Induces an Increase of Intracellular ROS Levels**—The cytoplasmic domains of TNFR-II, CD30, 4-1BB, and Ox40 lack a death domain and, therefore, cannot interact with TRADD. However, all of these receptors can recruit TRAF proteins to their cytoplasmic domains (9, 10, 30, 31). The HEK293 cells used for all our experiments did not contain any detectable levels of endogenous TRAF2 (Fig. 5D, lanes 1 and 3). TRAF2 overexpression in the absence of receptor signaling was not sufficient to induce an increase in ROS levels (Fig. 2, B and C) or to trigger NF-κB activation (Fig. 3B). Similar to results obtained with full-length TNFR-II (Fig. 1C), co-expression of TRAF2 and chimeric mutants of CD28-CD30, CD28-Ox40, or CD28–4-1BB caused significantly increased ROS levels in cells (Fig. 2, B and C). Although sufficient to trigger NF-κB activation (9, 29), expression of constitutively active CD28 chimera of CD30, Ox40, or 4-1BB in the absence of exogenous TRAF2 did not result in detectable changes of intracellular ROS levels (Fig. 2, B and C). Although engagement of TNFR-I by TNF-α resulted in significantly increased ROS levels in the absence of exogenous TRAF in these cells (Fig. 1B), changes of ROS levels by constitutively active CD28-CD30, CD28–4-1BB, and CD28-Ox40 were only detectable in the presence of exogenous TRAF2 (Fig. 2, B and C). The molecular differences responsible for this finding are currently under further investigation.

To test if the recruitment of TRAF proteins by TNFR-related proteins is required for changes of the cellular redox potential triggered by these receptors, an N-terminal deletion mutant of TRAF2 (TRAF2DN) was co-expressed. TRAF2DN lacks the RING finger structure and functions as dominant negative TNF molecule, which interferes with TRAF-mediated signaling events in vitro and in vivo (8, 9, 29, 32). Expression of TRAF2DN alone or in the presence of chimeric TNFR-related proteins had no effect on intracellular ROS levels (data not shown). Consistent with the inhibitory effect of TRAF2DN on NF-κB activation triggered by TNFR-related molecules, co-expression of TRAF2DN, TRAF2, and chimeric CD28-CD30, CD28–4-1BB, or CD28-Ox40 receptors resulted in a significantly decreased induction of intracellular ROS levels (Fig. 2B). Therefore, regulation of the cellular redox status by TNFR-related proteins is mediated, at least in part, by TRAF proteins and depends on the N-terminal Ring finger domain of...
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Fig. 2. TNFR-related proteins trigger TRAF-mediated increase of cellular ROS levels. HEK293 cells were transfected with expression constructs encoding TRAF2 and either a tailless mutant of CD28 (CD28-delta) or constitutively active chimeric proteins consisting of the extracellular and transmembrane domain of CD28 fused in frame to the cytoplasmic domain of the indicated TNFR-related molecules (9, 29). A, 24 h after transfection, cells expressing the indicated chimeric receptors alone or with human TRAF2 were harvested, washed twice, and stained with an antibody specific for mouse CD28 (37.51, PharMingen), which was conjugated with phycoerythrin. To analyze differences in transfection efficiencies (percentage of CD28-positive cells) and surface expression levels (relative fluorescence intensity) of the distinct chimeric receptor molecules, fluorescence of the cells was analyzed using a Becton Dickinson FACScalibur. B, cells transfected with the indicated chimeric receptors in the absence of exogenous TRAF (open circles), in the presence of cotransfected human TRAF2 (black circles), or TRAF2 and a dominant-negative N-terminal deletion mutant of TRAF2 (gray circles), were analyzed by fluorometry as described in legend of Fig. 1E. As controls for the individual groups, cells transfected with human TRAF2, TRAF2 and TRAF2DN, or empty pcDNA3 were left either untreated (base line) or were treated with 2.5 μg/ml antimycin A, which inhibits complex III of the respiratory chain causing increased cellular ROS levels. Shown are individual results of duplicate samples of one experiment representative of three. Analysis of variance was performed, and statistically significant differences are indicated (***, p < 0.05). C, cells were transfected as described in B. Shown are the average and the standard error of the mean of results from two independent experiments. The third experiment resulted in identical qualitative differences between the distinct experimental groups but showed significant differences in the absolute numbers of ROS levels. Although TRAF2DN diminished the increase in ROS levels similar to the two presented experiments, the third experiment was not included in the graph.

Fig. 3. NF-κB-inducing kinase is not required for TRAF-mediated increase of intracellular ROS levels. Cells were transfected with expression constructs encoding TRAF2 and the indicated proteins: mock, CD28Δtail; CD30, chimeric CD28-CD30 receptor; NIK, NF-κB-inducing kinase; NIKAA/KK, NIK mutant lacking kinase activity (33). A, 24 h after transfection of triplicate samples, changes of intracellular ROS were analyzed as described in Fig. 1C. B, NF-κB activation was analyzed as described previously (9). In brief, 1 million cells were transfected with expression constructs of TRAF2 and the indicated cDNAs as well as luciferase reporter constructs either lacking NF-κB consensus binding sites. Twenty-four hours after transfection, the cells were harvested, washed twice in phosphate-buffered saline, lysed in luciferase lysis buffer (Promega), and analyzed using the Promega luciferase kit according to the manufacturer’s manual. Shown is the average of duplicate transfections.

The adapter protein, which is also critical for other TRAF-mediated signaling events.

Signal Transduction Pathways Regulating ROS Levels and NF-κB Activation Diverge at the Level of TRAF Proteins—The interaction of NIK with TRAF2 has been suggested to result in activation of NIK and, subsequently, NF-κB (33). However, analysis of animals lacking a functional NIK, such as the alymphoplasia (aly) mouse and the NIK-/- mouse, indicates that NIK plays a role in chemokine signaling and immune functions of hematopoietic cells but is not essential for NF-κB activation triggered by TNF-α (34–37). To test if NIK is involved in TRAF-mediated changes of intracellular ROS levels, which could have an indirect effect on NF-κB activation, HEK293 cells were transfected with expression constructs of TRAF2 and either NIK or a mutant of the kinase lacking enzymatic activity (kind gift of Dr. David Wallach). Overexpression of NIK in the presence of exogenous TRAF2 did not result in increased ROS levels (Fig. 3A) but consistent with previously published results was sufficient to induce DNA binding and transcriptional activity of NF-κB in absence of receptor-induced signal transduction (Figs. 3B and 6A) (38, 39). To test further if TRAF-mediated regulation of ROS was independent of NIK, a mutant form of NIK (NIKAA/KK), which lacks kinase activity, was co-expressed with constitutively active CD28-CD30 and TRAF2.
Although NIKAA/KK functioned as a potent dominant negative inhibitor of NF-κB activation (Fig. 3B), it had no detectable effect on increased ROS levels induced by the cytoplasmic domain of CD28-CD30 (Fig. 3A). Overexpression experiments do not always allow conclusive interpretations of signal transduction pathways. However, the lack of effects on the cellular redox status of both a functional NIK and a kinase-dead mutant of this enzyme argue that TRAF-mediated changes of cellular ROS levels are independent of NIK. TRAF-mediated signal transduction pathways regulating NF-κB activation and the cellular redox status must, thus, separate upstream of NIK.

TRAF2-mediated Changes of Intracellular ROS Levels Can Be Regulated by A20.—We have previously shown that co-expression of TRAF2 and distinct TNFR-related molecules including TNFR-II, CD30, Orox40, and 4-1BB results in intracellular redistribution and accumulation of TRAF2 in perinuclear aggregates (27). Increased intracellular ROS levels as result of TRAF2-mediated signaling triggered by TNFR-related proteins suggested an effect of the subcellular localization of TRAF2 on the cellular redox status. However, whereas the distinct receptors caused significant differences in TRAF2 levels in subcellular compartments (27), redistribution of TRAF2 did not result in quantitative differences of ROS levels (Fig. 2), suggesting that other components of TRAF complexes play a critical role in downstream events.

Several cytoplasmic proteins including A20, I-TRAF/TANK (TRAF-interacting protein/TRAF family member-associated NF-κB activator), and TRIP (TRAF-interacting protein) can interact with TRAF2 and, thereby, control TRAF2-mediated signal transduction (40–43). The adapter protein A20 has been suggested to play a regulatory role in a negative feedback mechanism to interfere with TRAF-mediated NF-κB activation (43, 44). To determine whether TRAF2-mediated changes in ROS levels can also be regulated by A20, HEK293 cells were co-transfected with expression constructs encoding A20 (kind gift of Dr. Richard Ulevitch), TRAF2, and constitutively active CD28-CD30. Co-expression of A20, TRAF2, and chimeric CD28-CD30 resulted in intracellular ROS levels, which were indistinguishable from those in control cells (Fig. 4A), and significantly decreased TRAF-mediated NF-κB activation triggered by the recombinant CD28-CD30 molecule (Fig. 4B). A20 is thought to block TRAF-mediated signaling by inhibition of TRAF2 recruitment to multimerized TNFR family members in the plasma membrane (27). To test if recruitment of TRAFs to the plasma membrane is required for changes in the cellular redox potential, a soluble cytoplasmic GST fusion protein containing the intracellular domain of CD30 (GST-CD30) was used. As we have previously shown, both A20 and GST-CD30 interact with TRAF2 and trigger translocation of TRAF2 to an insoluble compartment of the cytosol (27). Like A20, GST-CD30 interacts with TRAF2 and results in depletion of the soluble pool of cytoplasmic TRAF2 and, thereby, interferes with TRAF2 recruitment to the cytoplasmic domain of CD28-CD30 expressed on the cell surface. Co-expression of soluble GST-CD30 abolished the increase of intracellular ROS (Fig. 4A) and NF-κB activity (Fig. 4B) triggered by surface CD28-CD30, whereas co-expression of cytoplasmic GST-CD30 and TRAF2 had no effect on either ROS levels or NF-κB activity. Thus, our findings indicate that formation of TRAF2-containing complexes at the plasma membrane is critical for changes of ROS induced by chimeric CD28-CD30. Taken together, this suggests that additional factors required for TRAF2-mediated ROS production and/or a source for ROS are localized at the plasma membrane.

TRAF2-mediated Changes of Cellular ROS Levels and NF-κB Activation Depend on Electron Transport in Mitochondria.—To gain insight into the mechanism(s) leading to changes of intracellular ROS levels in response to TNFR signaling, cells were treated with specific chemical inhibitors of various known mechanisms of ROS generation as described previously (25). Apocynin has been shown to interfere with the function of NADPH oxidase, an enzyme complex, which generates ROS at the plasma membrane and plays an important role in innate immunity (45). ROS in mitochondria are generated during the transition of electrons from NADPH-CoQ reductase (complex I) to the cytochrome c reductase (complex III). Diphenyleneiodonium chloride (DPI) is an irreversible inhibitor of the mitochondrial complex I as well as endothelial nitric-oxide synthase and inducible nitric-oxide synthase in macrophages. Rotenone, a competitive inhibitor of complex I, blocks effectively ROS generation in mitochondria, which is the major source of ROS in most cell types (46, 47). PDTC (pyrrolidinedithiocarbamate), a chelator of metal ions and scavenger of ROS, inhibits nitric-oxide synthase and served as a negative control (46, 47). Antimycin A was used as positive control because it blocks complex III of the respiratory chain downstream of the site of superoxide (O2•−) generation and, thereby, augments the generation of O2. Subsequently, manganese superoxide dismutase converts O2 to hydrogen peroxide, resulting in increased DCFH-DA oxidation. All pharmacological agents were used at concentrations described in the literature and showed no cytotoxic effects.

![Figure 4](image-url)
on HEK293 cells during the time course (6–24 h) of our experiments (22).

To determine the source of ROS generated in response to TNFR-I signal transduction, HEK293 cells were treated with soluble human TNF-α and the indicated chemical inhibitors or scavengers of intracellular ROS generation. Antimycin A interferes with the respiratory chain in mitochondria by blocking electron transport between complex I and complex III and results in transfer of electrons to free oxygen molecules and, subsequently, generation of H₂O₂. Final concentrations were as follows: 2.5 μg/ml for antimycin A, 100 μM for apocynin (Apo), 10 μM DPI, 25 μM PDTC, and 2.5 μg/ml rotenone (Rot).

**Fig. 5. Inhibitors of the respiratory chain abolish increased ROS levels triggered by TNFR-related proteins.** A, HEK293 cells were incubated with DCFH-DA and treated with 200 units/ml human TNF-α and the indicated chemical inhibitors or scavengers of intracellular ROS generation. Antimycin A interferes with the respiratory chain in mitochondria by blocking electron transport between complex I and complex III and results in transfer of electrons to free oxygen molecules and, subsequently, generation of H₂O₂. Final concentrations were as follows: 2.5 μg/ml for antimycin A, 100 μM for apocynin (Apo), 10 μM DPI, 25 μM PDTC, and 2.5 μg/ml rotenone (Rot). B, DNA binding of NF-κB subunits triggered by TNF-α was analyzed in the presence of compounds, which change the cellular redox potential. Supershift of the specific band in an electromobility shift assay indicates the presence of p65 (Rel A) in the DNA-binding complex. C, changes of cellular ROS levels triggered by the cytoplasmic domain of CD28-CD30 were determined after cotransfection of cells with human TRAF2 and a constitutively active CD28-CD30 (indicated as CD30) fusion protein followed by treatment of the cells with various inhibitors of intracellular ROS generation as described in A. For the last 6 h of a 24-h period, cells transfected with expression constructs encoding the indicated proteins were treated with the indicated pharmacologic agents or left untreated. D, detergent-soluble and -insoluble fractions of the cell lysates were separated, and TRAF2 levels were analyzed by Western blot.

Lack of Mitochondrial Electron Transport Inhibits TRAF-mediated Changes of Intracellular ROS and NF-κB Activation—The concentrations of pharmacological agents used in the above experiments have been previously utilized in studies investigating the cellular redox status and did not cause sig-
significant cytotoxicity in HEK293 cells. However, these agents have putative effects on a variety of different signal transduction events in cells. Thus, to test directly whether mitochondrial electron transport is required for TNF-α/H9251-induced ROS generation, HEK293 cells were generated by prolonged incubation of parental HEK293 cells in low concentrations of ethidium bromide. HEK293/H92670 cells contain mitochondria but lack an intact mitochondrial electron transport chain caused by the loss of mitochondrial DNA, which encodes several components of the respiratory chain (19, 48). Consequently, HEK293/H92670 cells are resistant to apoptosis induced by inhibition of the respiratory chain by rotenone indicating the lack of a functional electron transport chain (data not shown). HEK293/H92670 cells depend upon glycolysis to generate energy but proliferate and survive in culture. Transfection of parental HEK293 cells (containing an intact mitochondrial electron transport chain) with TRAF2 and a constitutively active chimeric CD28-CD30 receptor was sufficient to activate NF-κB, as analyzed in a luciferase reporter assay and caused increased intracellular ROS levels. In contrast, transfection of the HEK293/H92670 cells with expression vectors encoding TRAF2 and CD28-CD30 did not result in DNA binding of NF-κB subunits (Fig. 6A), increased transcriptional activity of NF-κB (Fig. 6B), or changes in cellular ROS levels (Fig. 6C). Thus, our findings are consistent with previously published findings suggesting a role of ROS in NF-κB activation.

Interestingly, although NIK and TRAF2 did not cause increased ROS levels (Fig. 6C), overexpression of NIK and TRAF2 in HEK293 ρ0 cells resulted in increased DNA binding of NF-κB (Fig. 6A) but not in transcriptionally active NF-κB complexes as shown by NF-κB-dependent luciferase reporter assays (Fig. 6B). These results indicate that, in the absence of...
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FIG. 8. TRAF2 as central regulator of pro- and anti-apoptotic signal transduction pathways. Engagement of TNFR-related proteins by their respective ligands can lead to the recruitment of TRAF2 to the plasma membrane. TRAF-interacting proteins, such as A20, interfere with this proximal step of TRAF-mediated signal transduction. Downstream events of TRAF signal transduction include activation of kinases and transcription factors, such as NIK and NF-κB. Recruitment of TRAF2 by TNFR-related proteins can also result in increased levels of intracellular ROS generated in mitochondria, which could result in damage of lipids and proteins by oxidation. Consistent with its anti-apoptotic effects, Bcl-xL efficiently blocks TRAF2-mediated ROS generation in mitochondria without interfering with TRAF2-mediated activation of NF-κB.

mitochondrial electron transport, NIK activity alone is not sufficient to induce transcriptional activity of NF-κB, although the cellular machinery for phosphorylation and degradation of IκB and translocation of NF-κB subunits to the nucleus appear intact. In normal cells, oxidation of NF-κB subunits required for its transcriptional activation in response to NIK could be achieved by low levels of ROS or oxidants, which are not detected by DCFH-DA. The significant increase of ROS levels detected by DCFH-DA in response to TRAF2-mediated signaling triggered by TNFR-related proteins may play a regulatory role in other cellular programs including apoptosis.

Huang and colleagues (49) reported recently that the cellular redox potential plays a critical role in regulating survival of lymphoid cells. Interference in the redox potential by inhibition of cellular superoxide dismutase by estrogen derivatives selectively caused apoptosis in human leukemia cells but not in normal lymphocytes due to the increased ROS production, caused by the higher metabolism of tumor cells. Damage of proteins and lipids in particular in mitochondria could result in disruption of integrity on mitochondria; thus, ROS could sensitize cells to apoptosis or trigger apoptotic pathways. Consistently, CD30-deficient animals have elevated numbers of thymocytes, which are less sensitive to activation-induced cell death triggered by CD3 cross-linking mimicking T cell receptor signaling (50). In contrast, CD30-transgenic thymocytes show increased sensitivity to apoptosis triggered by CD3 cross-linking (51). Overexpression of constitutively active chimeric consisting of the extracellular domain of CD28 and the cytoplasmic tails of CD30, OX40, or 4-1BB is not sufficient to trigger apoptosis. However, although all the tested receptors caused similar levels of NF-κB activation, signaling triggered by these chimeric receptors did not promote survival of HEK293 cells, but rather sensitized the cells to TNF-α-induced apoptosis (27). Incubating cells transfected with human TRAF2 and chimeric CD28-CD30 with TNF-α and PDTC resulted in decreased cell death assessed by microscopic evaluation of the cells (data not shown). Oxidation of proteins or lipids caused by increasing levels of intracellular ROS could be one potential mechanism, which promotes TNF-α-induced apoptosis. Signaling triggered by TNFR-related molecules including CD30, OX40, and 4-1BB, e.g. during the late phase of an inflammatory immune response or during differentiation of thymocytes, could allow to modulate the immune response or thymic development. Although NF-κB activation plays an important role in regulating effector functions, increased ROS levels could sensitize lymphocytes to pro-inflammatory cytokines, such as TNF-α, and result in elimination of excessive effector cells or potentially autoreactive T cells.

Bcl-2 Family Members Abolish Increase of Intracellular ROS Triggered by the Cytoplasmic Domain of CD28-CD30—Proteins, which have been shown to play a crucial regulatory role in apoptosis by maintaining the integrity of mitochondria, include anti-apoptotic members of the Bcl-2 family, such as Bcl-xL. However, the mechanisms by which cells expressing anti-apoptotic members of the Bcl-2 family are protected against apoptosis are not completely understood. Both pro- and anti-apoptotic family members have been shown to translocate to the outer membrane of mitochondria and regulate the cellular redox status. Bcl-xL expression is up-regulated during thymic development of T lymphocytes and in activated lymphocytes in the periphery. It, thus, is an interesting candidate protein, which could inhibit the up-regulation of ROS levels triggered by TNFR-related proteins including CD30. To determine whether Bcl-xL and Bcl-2 have an effect on the changes of ROS triggered by TNFR-related molecules, HEK293 cells were transfected with expression vectors of TRAF2, constitutively active CD28-CD30, and Bcl-xL. Bcl-xL expression had no effect on TRAF2-mediated NF-κB activation triggered by the cytoplasmic tail of CD28-CD30 but interfered with TRAF2-mediated ROS generation (Fig. 7). Expression of Bcl-2 showed similar effects (data not shown). Both anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, abolished the increase of intracellular ROS and kept cells in a redox status similar to control cells without affecting activation of NF-κB. Thus, the ability of TNFR-related proteins to induce NF-κB activation is dependent on changes in mitochondrial function, which can be prevented by inhibition of mitochondrial respiration and fail to occur in mitochondria incapable of oxidative phosphorylation.

We and others have previously shown that recruitment of TRAF2 by surface molecules including members of the TNFR family results in formation of multiprotein complexes and triggers signal transduction events, such as activation of kinases and transcription factors. The results presented in this report indicate that TRAF2-mediated signaling pathways triggered by TNFR-related proteins regulate the cellular redox status by controlling the generation of ROS in mitochondria (Fig. 8). The central role of mitochondria and the cellular redox status in regulating cell survival and apoptosis suggest a novel function of TRAF2 in controlling the fate of cells.

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