

Cytochrome c Release and Apoptosis Induced by Mitochondrial Targeting of Nuclear Orphan Receptor TR3

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TR3, an immediate-early response gene and an orphan member of the steroid-thyroid hormone-retinoid receptor superfamily of transcription factors, regulates apoptosis through an unknown mechanism. In response to apoptotic stimuli, TR3 translocates from the nucleus to mitochondria to induce cytochrome c release and apoptosis. Mitochondrial targeting of TR3, but not its DNA binding and transactivation, is essential for its proapoptotic effect. Our results reveal a mechanism by which a nuclear transcription factor translocates to mitochondria to initiate apoptosis.

The orphan receptor TR3 (also known as nur77 or nerve growth factor-induced clone B NGFI-B) (*1–3*) functions as a nuclear transcription factor in the regulation of target gene expression (*4–7*). TR3 was originally isolated as an immediate-early gene rapidly expressed in response to serum or phorbol ester stimulation of quiescent fibroblasts (*2, 8–10*). Other diverse signals, such as membrane depolarization and nerve growth factor, also increase TR3 expression (*3, 11*). Inactivation of a TR3-related protein results in agenesis of mesencephalic dopamine neurons (*12*). TR3 is also involved in the regulation of apoptosis in different cell types (*13–19*). It is rapidly induced during apoptosis of immature thymocytes and T-cell hybridoma (*13, 14*), lung cancer cells treated with the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (*17*) [also called CD437 (*20*)], and prostate cancer cells treated with different apoptosis inducers (*18, 19*). Inhibition of TR3 activity by overexpression of a dominant-negative TR3 or its antisense RNA inhibits apoptosis (*13, 14, 17, 18*), whereas constitutive expression of TR3 results in massive apoptosis (*15, 16*). How TR3 exerts its proapoptotic effect remains largely unknown.

Requirement of TR3 expression but not its transactivation for apoptosis. We investigated the role of TR3 in apoptosis of LNCaP human prostate cancer cells induced by the AHPN analog 6-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chloro-2-naphthalenecarboxylic acid (MM11453), the retinoid (*Z*)-4-[2-bromo-3-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)propenyl]benzoic acid (MM11384), the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), the calcium ionophore A23187, and the etoposide VP-16. Treatment of LNCaP cells with any of these agents induced extensive apoptosis (Fig. 1A), accompanied by increases in TR3 expression (Fig. 1B). Apoptosis induction by these agents was reduced in an LNCaP clone stably expressing TR3 antisense RNA, in which TR3 expression was lost (Fig. 1, A and C). In contrast, tumor necrosis factor- α (TNF- α)-induced apoptosis of LNCaP cells was not affected by inhibiting TR3 expression (Fig. 1A).

To determine whether induction of TR3 transactivation was involved in initiating apoptosis, a chloramphenicol acetyltransferase (CAT) reporter gene plasmid containing a TR3-binding sequence (NurRE) (*21*) was transiently transfected into LNCaP cells. Basal reporter gene activity was very low (Fig. 1D) because of the lack of endogenous TR3 expression (Fig. 1B). Reporter gene activity was not induced by treatment with apoptosis-inducing agents (Fig. 1D), despite their induction of TR3 expression (Fig. 1B). In contrast, expression of TR3 and reporter gene transcription was increased in cells treated with epidermal growth factor (EGF) (Fig. 1D). These data demonstrate that TR3 induced by growth stimulus from EGF, but not

by apoptotic stimuli, is transcriptionally competent. We also investigated the effect of apoptotic agents on the transcriptional activity of exogenous TR3 protein. In cotransfection experiments, transfected TR3 strongly induced NurRE reporter gene activity, which was strongly inhibited by apoptosis-inducing agents. In contrast, all-*trans*-retinoic acid (RA) and EGF had no effect (Fig. 1E).

Repression of TR3 transactivation function by these apoptosis inducers was unexpected, because TR3 was thought to exert its proapoptotic effect by acting as a transcription factor to regulate gene expression (*13–16*). Repression of TR3 transactivation was not related to direct binding of apoptosis inducers to TR3 protein, because they did not alter TR3 DNA binding in gel-shift assays or TR3 receptor conformation in protease sensitivity assays. Moreover, these agents did not trigger degradation of TR3 protein as could be seen from immunoblotting analysis (*22*).

Translocation of TR3 from the nucleus to mitochondria in response to apoptotic stimuli. We investigated the possibility that repression of TR3 transactivation was due to relocalization of TR3 from the nucleus to the cytoplasm. We used a green fluorescent protein (GFP)-TR3 fusion protein. Pilot experiments confirmed that addition of the GFP tag did not interfere with TR3 transactivation (*22*). The GFP-TR3 fusion protein localized in the nucleus in cells that were not stimulated (Fig. 2A). However, on treatment of cells with TPA for 5 min, GFP-TR3 became diffusely distributed in both the cytoplasm and nucleus. Thereafter, GFP-TR3 was found exclusively in the cytoplasm, displaying a bright punctate pattern (Fig. 2A). Such a relocalization was not observed with GFP alone (*22*).

The cytoplasmic punctate distribution pattern suggested the association of TR3 with one or more intracellular organelles. Because mitochondria play a critical role in many apoptotic pathways (*23*), we examined whether TR3 was associated with mitochondria by immunostaining of the heat shock protein Hsp60, a mitochondria-specific protein (*24*). The distribution patterns for mitochondria and GFP-TR3 in LNCaP cells treated with TPA for 60 min overlapped extensively (Fig. 2A), suggesting that TR3 associates with mitochondria in response to TPA. This association was also observed when LNCaP cells were treated with other apoptosis inducers, including MM11453, MM11384, A23187, and VP-16, but not with nonapoptotic stimuli such as EGF (Fig. 2B). The mitochondrial location of TR3 was further demonstrated by immunoblotting analysis, which showed accumulation of TR3 in the mitochondria-enriched heavy membrane (HM) fraction after treatment with TPA or MM11453 (Fig. 2C). We next studied the location of mitochondria-associated TR3 in mitochondria purified from LNCaP cells that had been treated

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with TPA or MM11453. Incubating isolated mitochondria with trypsin led to proteolysis of both TR3 and Bcl-X_L, a known outer mitochondrial membrane protein, whereas the matrix protein Hsp60 remained intact and associated with mitochondria (Fig. 2D). These data indicate that TR3 resides on the mitochondrial surface.

To determine the domain of TR3 responsible for mitochondrial association, TR3 deletion mutants (25) fused to GFP were analyzed for their ability to target mitochondria. Deletion of 152 amino acids from the NH₂-terminus (TR3/Δ1) completely abolished TPA-induced mitochondrial association. Although deletion of 24 amino acids from the COOH-terminus (TR3/Δ2) had no effect, further deletion of 96 amino acids (TR3/Δ3) caused nuclear localization despite TPA treatment (Fig. 2E). These results demonstrate that COOH-terminal and NH₂-terminal sequences are crucial for mitochondrial targeting of TR3. The TR3 mutant lacking the DNA binding domain (TR3/ΔDBD) exclusively localized in the cytoplasm, even in the absence of treatment with TPA or other apo-

ptosis inducers (Fig. 2E), with more than half of cells displaying mitochondrial association. This finding indicates that the DNA binding domain of TR3 is not required for its mitochondrial targeting, although this domain is necessary for transcriptional activity. Mitochondrial targeting by TR3/ΔDBD was also found in other cell types, including Jurkat T-lymphoblasts and MDA-MB-231 and ZR-75-1 breast cancer cells (22).

Regulation of mitochondrial activities by TR3. To determine whether mitochondrial targeting of TR3 plays a role in regulating the release of cytochrome c (26) from mitochondria into cytosol, the location of cytochrome c was examined. Immunoblotting showed that both TPA and MM11453 caused the release of cytochrome c from the HM fraction into the cytosol (Fig. 3A), but had no discernible effect on the total amount of cytochrome c protein. TR3 appeared to be required for this effect, because no cytochrome c was detected in the cytosolic fraction of LNCaP cells that expressed TR3 antisense RNA (Fig. 3A). In addition, treatment with apoptotic agents led to mitochondrial mem-

brane depolarization in LNCaP cells but not in the antisense-expressing cells (22). We next analyzed whether modulation of cytochrome c release by TR3 correlated with mitochondrial targeting (Fig. 3B). GFP-TR3-transfected and nontransfected LNCaP cells were stained for mitochondria and cytochrome c and analyzed by confocal microscopy. Nontransfected cells exhibited particulate cytochrome c staining, consistent with mitochondrial localization. This pattern was also observed in untreated GFP-TR3-transfected cells, in which GFP-TR3 resided in the nucleus. However, treatment with MM11453 for 30 min caused association of GFP-TR3 with mitochondria, accompanied by the release of cytochrome c from mitochondria.

We investigated modulation of apoptosis by TR3/Δ1, because a TR3 mutant lacking the NH₂-terminal A/B domain acted as a dominant-negative inhibitor of the TR3-induced apoptosis in T-cells (13). Expression of TR3/Δ1 in LNCaP cells prevented the MM11453-induced cytoplasmic localization of TR3 (Fig. 3C) and apoptosis (Fig. 3, D and E), suggesting that TR3/Δ1 inhibits the apo-

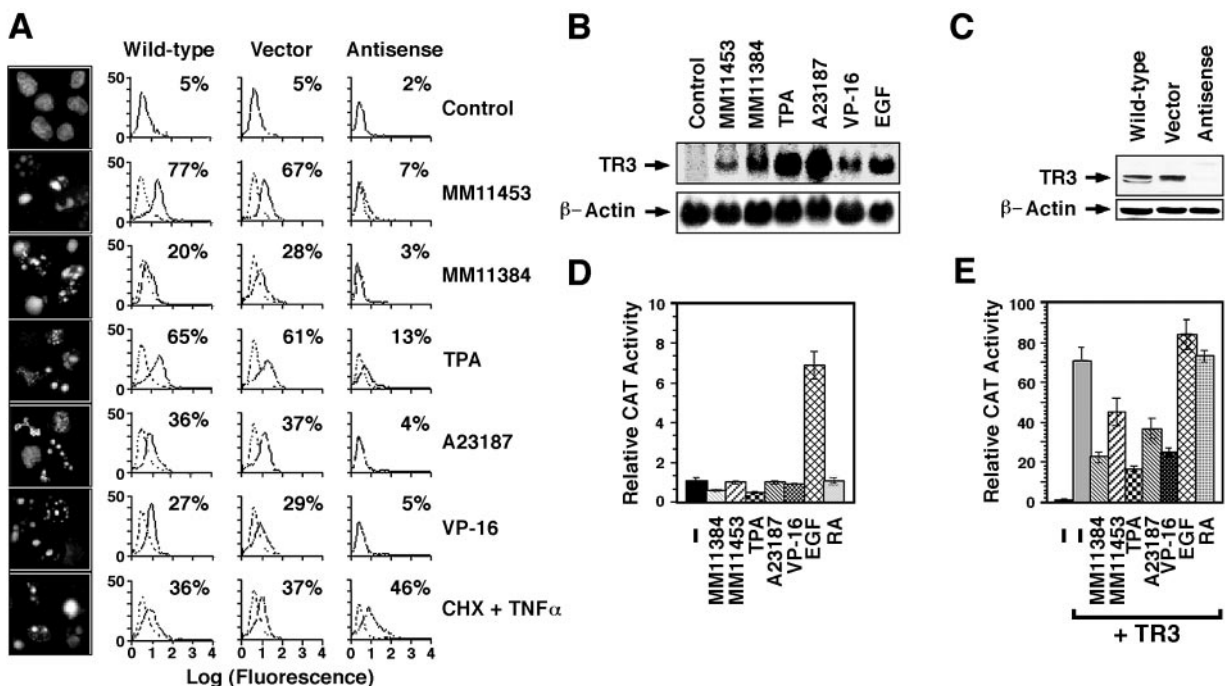


Fig. 1. TR3 is required for induction of apoptosis by retinoids and other apoptosis-inducing agents in LNCaP cells. (A) Induction of apoptosis by MM11453, MM11384, TPA, A23187, VP-16, or TNF- α plus cycloheximide (CHX) in LNCaP cells, LNCaP cells stably transfected with the empty vector (Vector), or LNCaP cells stably expressing TR3 antisense RNA (Antisense). Cells were treated with MM11453 (10^{-6} M), MM11384 (10^{-6} M), TPA (100 ng/ml), A23187 (2×10^{-5} M), VP-16 (5×10^{-4} M), or TNF- α (100 ng/ml) plus cycloheximide (10 μ g/ml) for 2 days. Apoptosis was determined by nuclear staining with DAPI (left panel) and the TdT assay (right panels) (40). (B) Induction of TR3 expression by apoptosis-inducing agents by Northern blotting. LNCaP cells were treated with MM11453, MM11384, TPA, A23187, or VP-16 as above for 3 hours and analyzed for TR3 expression (17). EGF treatment (200 ng/ml) was used for control. β -Actin expression was used to show results for similar

loading of RNA. (C) Inhibition of TR3 expression by overexpression of TR3 antisense RNA. Immunoblot analysis of whole-cell lysates of parental LNCaP, vector-transfected, and TR3-antisense RNA-expressing cells treated with TPA for 3 hours by using anti-TR3 antibody (Santa Cruz Biotechnology) (41). Loading of proteins was controlled by reprobing the blot with anti- β -actin antibody (Sigma). (D) TR3 induced by growth stimulus but not by apoptosis inducers is transcriptionally competent. (E) Apoptosis inducers inhibit the transactivation activity of TR3. (NurRE)₂-tk-CAT (42) (100 ng) and β -galactosidase expression vector (50 ng) were transiently transfected into LNCaP cells without (D) or with (E) 25 ng of TR3 expression vector (43). After transfection, cells were treated as above with MM11453, MM11384, TPA, A23187, VP-16, EGF, or all-*trans*-RA (10^{-7} M) for 24 hours, and CAT activity was determined and normalized relative to β -galactosidase activity.

ptotic effect of TR3 by blocking its mitochondrial targeting.

DNA binding domain of TR3 is dispens-

able for its apoptotic effects. The role of mitochondrial targeting by TR3 in modulating apoptosis was further investigated by us-

ing LNCaP cells transfected with TR3/ Δ DBD (Fig. 2E). Transfected GFP-TR3/ Δ DBD protein was constitutively associated with mito-

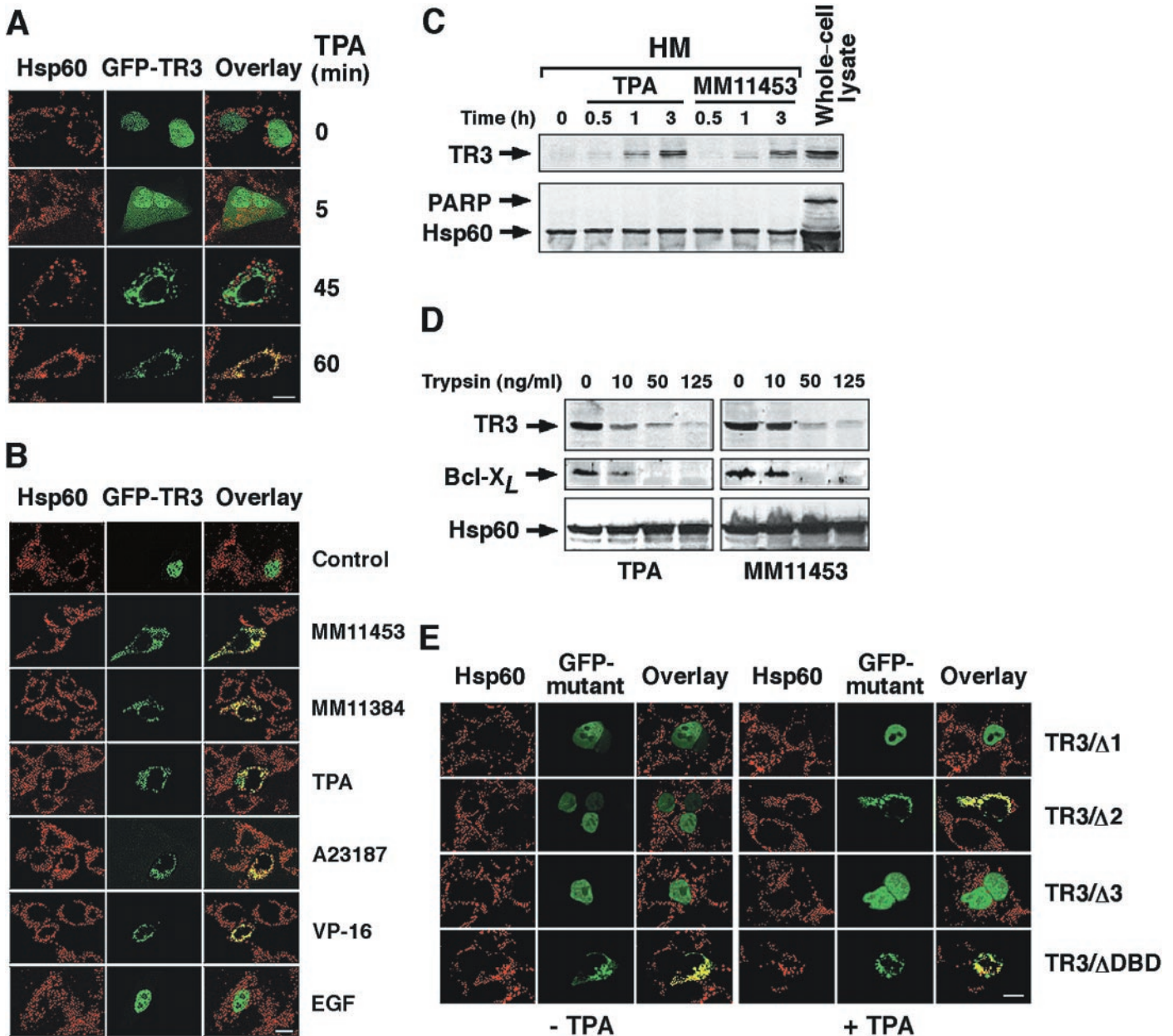
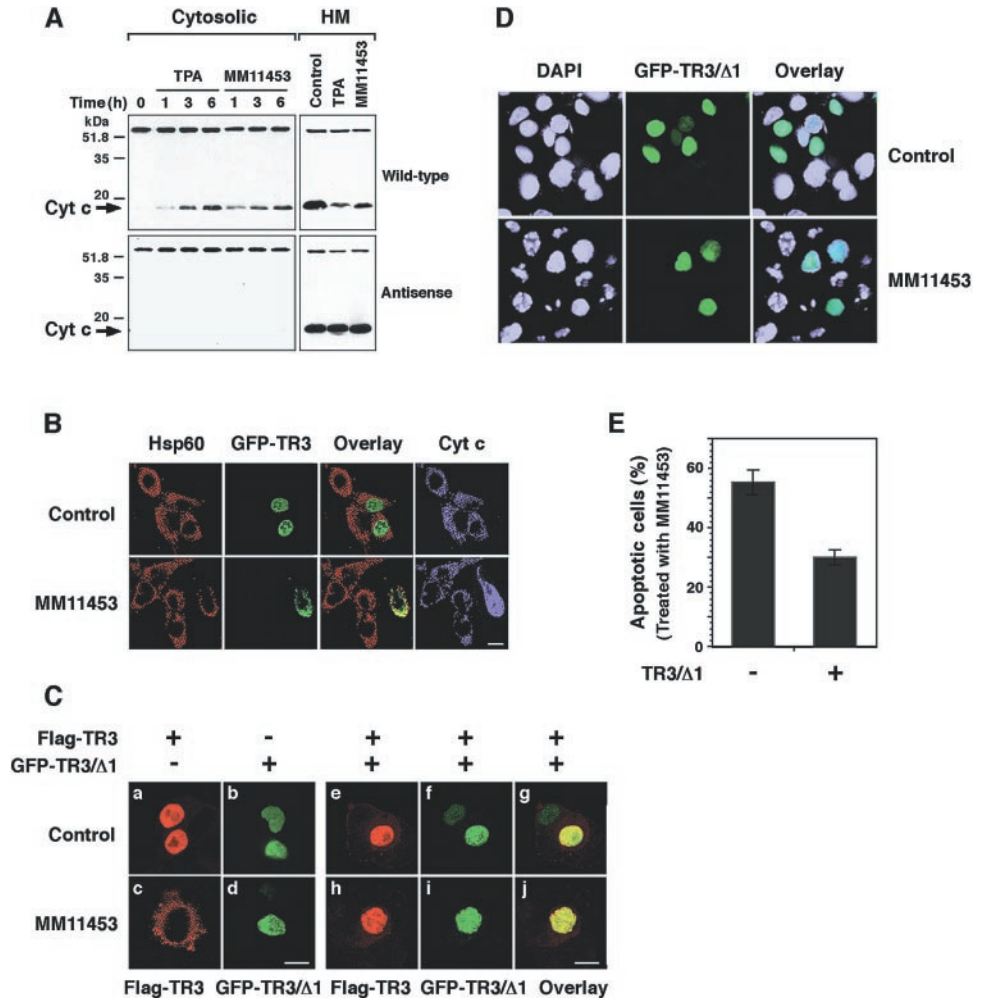


Fig. 2. Apoptosis stimuli induce mitochondrial targeting of TR3. (A) Time course of TPA-induced mitochondrial targeting of TR3. GFP-TR3 expression vector (44) was transiently transfected into LNCaP cells. Cells were treated with TPA (100 ng/ml) for the indicated times, then immunostained with anti-Hsp60 antibody (Santa Cruz Biotechnology) followed by Cy3-conjugated secondary antibody (Sigma) to detect mitochondria. GFP-TR3 and mitochondria (Hsp60) were visualized by using confocal microscopy, and the two images were overlaid (Overlay) (44). About 15% TR3-transfected cells showed the pattern that presented after TPA treatment. GFP-TR3-transfected cells (100%) showed exclusive nuclear localization in the absence of treatment. Scale bar, 10 μ m. (B) TR3 is translocated from the nucleus to mitochondria in response to various apoptosis inducers. GFP-TR3-transfected LNCaP cells were treated with the indicated agents for 1 hour and analyzed using confocal microscopy as described in (A). About 15 to 20% of TR3-transfected cells showed mitochondrial targeting as presented; however, 100% of the GFP-TR3 transfected cells treated with EGF showed nuclear localization. (C) Apoptotic stimuli induce accumulation of TR3 in mitochondria. LNCaP

cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) for the indicated times, and the HM fraction was analyzed for expression of TR3 by Western blotting (45). To demonstrate the purity of the HM fraction, expression of mitochondrial-specific protein Hsp60 and nuclear-specific protein poly(ADP-ribose) polymerase (PARP) is shown. Expression of TR3, PARP, and Hsp60 in whole-cell lysate is shown for comparison. (D) Submitochondrial localization of TR3. Mitochondria purified from LNCaP cells treated with either TPA (100 ng/ml) or MM11453 (10^{-6} M) for 5 hours were incubated at 4°C for 20 min with the indicated concentration of trypsin. The entire contents were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antibodies specific for TR3, Bcl-X_L, and Hsp60. (E) Mitochondrial targeting of TR3 deletion mutants (46) fused with GFP in the presence or absence of TPA treatment (100 ng/ml for 1 hour) as described in (A). In the particular experiment presented, mitochondrial targeting was observed in 65% of TR3/ Δ DBD-transfected cells and 19% of TR3/ Δ 2-transfected cells, while both TR3/ Δ 1 and TR3/ Δ 3 remained exclusively in the nucleus irrespective of the treatment.

Fig. 3. Mitochondrial targeting of TR3 regulates mitochondrial activity. (A) TR3 expression is required for cytochrome c release in response to apoptosis inducers. LNCaP (wild-type) and TR3-antisense RNA expressing (antisense) cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) for the indicated times. Both HM and cytosolic fractions were analyzed for cytochrome c (Cyt c) by immunoblotting (45). HM fractions were from cells treated for 3 hours. A nonspecific band at ~ 70 kD served as a control for equal loading of proteins. (B) Mitochondrial targeting of TR3 is associated with cytochrome c release. GFP-TR3 was transiently transfected into LNCaP cells. Cells were treated with MM11453 (10^{-6} M) for 30 min and immunostained for mitochondria (Hsp60) and cytochrome c (Cyt c). Cytochrome c release was observed in every cell showing TR3 mitochondrial targeting. (C) TR3/ $\Delta 1$ blocks cytoplasmic localization of TR3. Flag-tagged TR3 (Flag-TR3) and GFP-TR3/ $\Delta 1$ were transfected alone (panels a to d) or together (panels e to j) into LNCaP cells. Cells were then treated without (control) or with MM11453 for 1 hour to induce export by Flag-TR3 from the nucleus to the cytoplasm (about 20% of transfected cells showed Flag-TR3 nuclear export, while GFP-TR3/ $\Delta 1$ remained exclusively in the nucleus). Flag-TR3 was visualized by anti-Flag antibody (Kodak, Rochester, New York) followed by Cy3-conjugated secondary antibody using confocal microscopy. Flag-TR3 was translocated from the nucleus to the cytoplasm when cells were treated with MM11453 (panel c), which was blocked when GFP-TR3/ $\Delta 1$ was coexpressed (compare panels c and h) (100% of cells expressing both Flag-TR3 and GFP-TR3/ $\Delta 1$ showed the blocking of Flag-TR3 cytoplasmic localization). (D) TR3/ $\Delta 1$ inhibits MM11453-induced apoptosis. LNCaP cells transfected with GFP-TR3/ $\Delta 1$ were treated with MM11453 (10^{-6} M) for 36 hours and nuclei were stained by DAPI. GFP-TR3/ $\Delta 1$ expression and nuclear morphology were visu-



chondria, and cytochrome c release was observed in these cells in the absence of any apoptotic stimulus (Fig. 4A). In contrast, cytochrome c localized predominantly in mitochondria in nontransfected control cells. GFP-TR3/ Δ DBD also induced extensive apoptosis, as determined by the annexin V-binding assay (27) (Fig. 4B) and by 4,6-diamidino-2-phenylindole (DAPI) staining, which revealed extensive nuclear condensation and fragmentation (Fig. 4C). Similar results were obtained with Jurkat (Fig. 4A), MDA-MB231, and ZR-75-1 breast cancer and other cell lines (22).

Altered mitochondrial targeting abrogates TR3-induced apoptosis. To further explore the relevance of TR3 mitochondrial targeting in cytochrome c release and apoptosis, we studied the effect of leptomyacin B, a blocker of nuclear export (28), on apoptosis induced by TR3/ Δ DBD. Treatment of LNCaP cells with leptomyacin B resulted in

nuclear retention of TR3/ Δ DBD and abrogated its ability to induce cytochrome c release (Fig. 5A). Moreover, TR3/ Δ DBD fused with a nuclear localization sequence (NLS) from the SV40 large T-antigen (29), TR3/ Δ DBD-NLS, was retained exclusively in the nucleus and failed to induce cytochrome c release (Fig. 5B). These results demonstrate that the cytoplasmic localization of TR3/ Δ DBD is essential for its apoptotic effect. We next modified TR3/ Δ DBD by fusing it with heterologous sequences that specifically target either the plasma membrane (30) or the endoplasmic reticulum (ER) (31). When TR3/ Δ DBD was fused with the CAAX box containing the COOH-terminus of K-Ras, TR3/ Δ DBD-CAAX, it resided along the plasma membrane, whereas TR3/ Δ DBD fused with the ER-targeting sequence from the ER-specific isoform of cytochrome b5, TR3/ Δ DBD-cb5, localized to the ER (Fig. 5C). Neither

of these fusion proteins localized to mitochondria (Fig. 5C) or induced cytochrome c release (Fig. 5C) or apoptosis (Fig. 5D). Thus, preventing targeting of TR3 to mitochondria suppresses its ability to induce cytochrome c release and apoptosis.

Release of cytochrome c from isolated mitochondria by TR3. To study whether recombinant TR3 protein or its mutants directly induce cytochrome c release, we incubated them with intact mitochondria isolated from LNCaP cells. Incubating mitochondria with TR3 or TR3/ Δ DBD caused release of cytochrome c. In contrast, TR3/ $\Delta 1$ and TR3/ $\Delta 3$, which were unable to associate with mitochondria (Fig. 2E), did not cause cytochrome c release (Fig. 5E). Because the cytochrome c-releasing activity was antagonized by Bcl-2, a nonspecific effect is excluded. These data further demonstrate that TR3 can act locally on mitochondria to induce cytochrome c release.

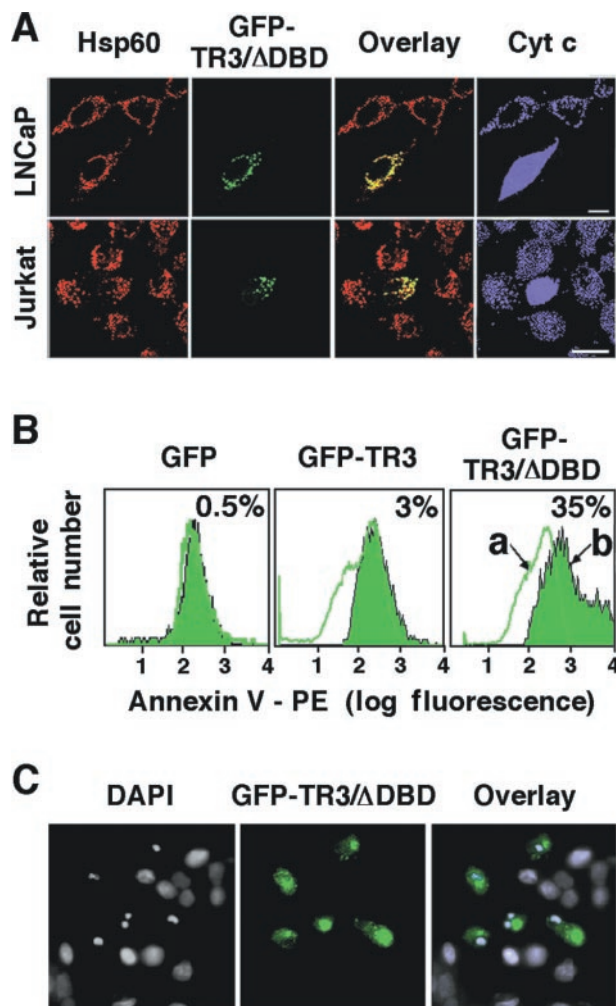


Fig. 4. TR3 lacking the DNA binding domain induces cytochrome c release and apoptosis. (A) Mitochondrial targeting of TR3/ΔDBD is associated with cytochrome c release. GFP-TR3/ΔDBD was transiently transfected into LNCaP and Jurkat cells and analyzed by confocal microscopy as described in Fig. 3B. Cytochrome c release was observed in 100% of cells showing TR3/ΔDBD mitochondrial targeting. (B) Expression of TR3/ΔDBD induces LNCaP cell apoptosis. Wild-type GFP-TR3, GFP-TR3/ΔDBD, or GFP control vector was transfected into LNCaP cells. The GFP-expressing subpopulation of transfected cells (peak b) was identified by their high green fluorescence compared with nontransfected cells (peak a) by using flow cytometry (27, 47). The apoptotic cell population was determined 44 hours after transfection by annexin V staining of the efficiently transfected green-fluorescing cells and the nontransfected (nonfluorescing) cells from the same culture dish. Representative experiment is shown ($n = 4$). (C) Expression of TR3/ΔDBD induces LNCaP cell apoptosis. GFP-TR3/ΔDBD expression vector was transiently transfected into LNCaP cells. Nuclei were

stained by DAPI after 36 hours of transfection. GFP-TR3/ΔDBD expression and nuclear morphology were visualized by fluorescence microscopy, and the two images were overlaid to show the effect of TR3/ΔDBD expression on nuclear condensation and fragmentation.

Discussion and conclusions. The immediate-early response gene TR3 is required for the apoptosis of T-cell hybridomas and cancer cells induced by a variety of stimuli (13–18). Contrary to the current belief that the DNA binding and transactivation of TR3 are required for its apoptotic effect, we demonstrate here that TR3 regulates apoptosis through a mechanism that is independent of transcriptional regulation. In response to apoptotic stimuli, TR3 is translocated from the nucleus to the cytoplasm, where it targets mitochondria to induce cytochrome c release and apoptosis. Our results show that a nuclear transcription factor can function at mitochondria to mediate an important biological function. The observations that TR3 lacking the DNA binding domain localized exclusively in the cytoplasm where it associated with mitochondria and potentially induced apoptosis suggest that target gene regulation by TR3 is not required for its apoptotic effect. TR3 mediates not only apoptosis but also cell proliferation in response to growth factors

(1–3, 8–18). Our present findings and previous observations that TR3 acts as a transcription factor by heterodimerizing with nuclear receptors, such as retinoid X receptor (RXR) (32–34) and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) (35), also suggest that the opposing biological activities of TR3 are regulated by its subcellular localization, i.e., the mitogenic effect of TR3 occurs in the nucleus through target gene regulation, whereas its proapoptotic effect occurs in the cytoplasm through regulation of mitochondrial activity. Abnormal increase of TR3 transactivation may have oncogenic potential because a TR3 fusion protein that is 270 times as active as the native receptor in activating gene expression is produced through chromosomal translocation in extraskelatal myxoid chondrosarcoma (36). Subcellular localization may also regulate activities of other transcription factors, such as c-Myc and c-Jun, which are known to mediate both cell death and proliferation (37–39). Translocation of TR3 between the nucle-

us and the cytoplasm may also represent a new mechanism for cross-talk between different signaling pathways and is likely to play a critical role in regulating the activities of retinoids and apoptosis inducers. As TR3 is often overexpressed in cancer cells because of uncontrolled expression of growth factors (35), our findings suggest that agents, such as MM1453 and MM11384, that induce relocalization of TR3 from the nucleus to mitochondria may have pharmacological value by preferentially inducing the death of cancer cells.

References and Notes

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27. Two-color analysis was performed by flow cytometry (FACS-Star plus, Becton Dickinson, Paramus, NJ) Representative histograms are shown. For annexin V analysis, cells were transiently transfected with GFP expression vectors. Cells were detached from plates with enzyme-free cell dissociation solution (Specialty Media, Phillipsburg, NJ) and were collected in medium containing 10% fetal bovine serum (FBS). Cells were washed with phosphate-buffered saline (PBS), resuspended in 250 μ l buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, and 10 μ l annexin V-PE (PharMingen, San Diego, CA), and analyzed by flow cytometry 30 min later.
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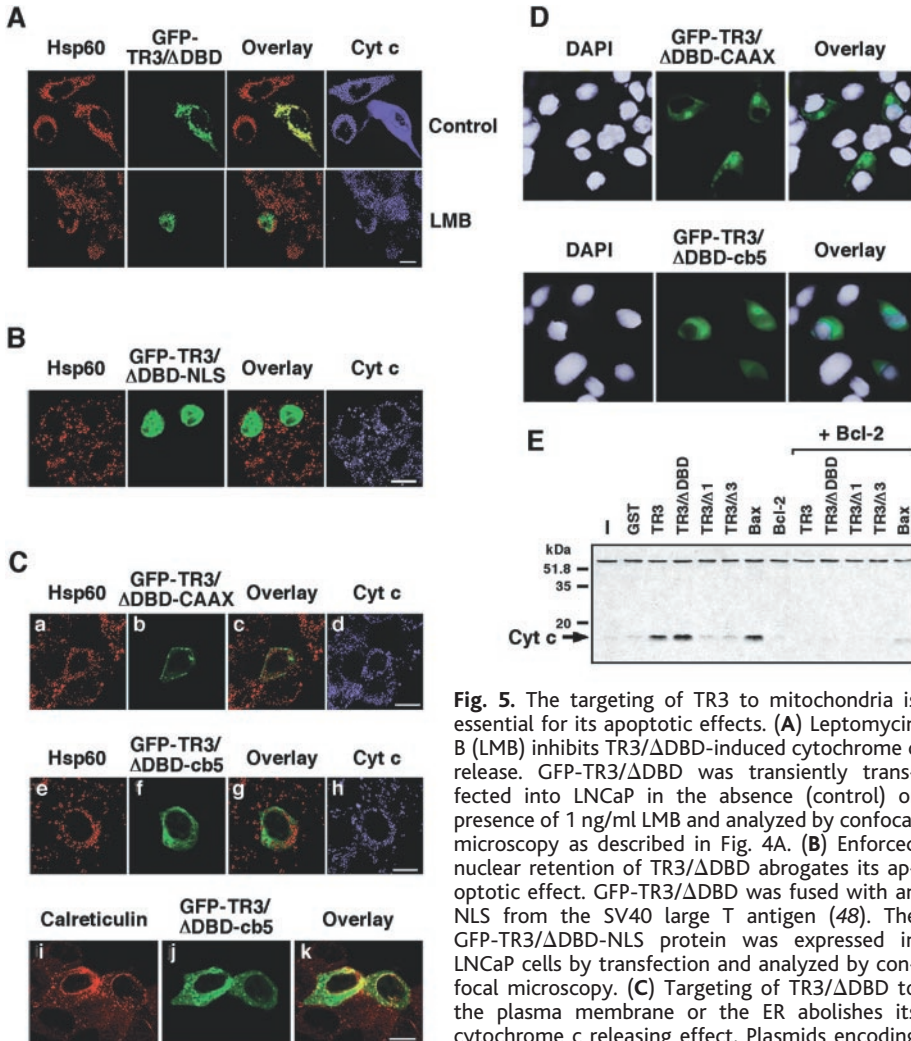


Fig. 5. The targeting of TR3 to mitochondria is essential for its apoptotic effects. **(A)** Leptomycin B (LMB) inhibits TR3/ΔDBD-induced cytochrome c release. GFP-TR3/ΔDBD was transiently transfected into LNCaP in the absence (control) or presence of 1 ng/ml LMB and analyzed by confocal microscopy as described in Fig. 4A. **(B)** Enforced nuclear retention of TR3/ΔDBD abrogates its apoptotic effect. GFP-TR3/ΔDBD was fused with an NLS from the SV40 large T antigen (48). The GFP-TR3/ΔDBD-NLS protein was expressed in LNCaP cells by transfection and analyzed by confocal microscopy. **(C)** Targeting of TR3/ΔDBD to the plasma membrane or the ER abolishes its cytochrome c releasing effect. Plasmids encoding TR3/ΔDBD-CAAX (48) (panels a to d) or TR3/ΔDBD-cb5 (48) (panels e to k) were transfected into LNCaP cells and analyzed by confocal microscopy. To detect ER, cells were immunostained using an antibody for an ER-specific protein calreticulin (37) (Calbiochem, San Diego, California) followed by Cy3-conjugated secondary antibody. **(D)** Targeting of TR3/ΔDBD to the plasma membrane or the ER inhibits its ability to induce apoptosis. TR3/ΔDBD-CAAX or TR3/ΔDBD-cb5 was expressed in LNCaP cells by transfection, and nuclei were stained using DAPI. None of the cells expressing TR3/ΔDBD-CAAX or TR3/ΔDBD-cb5 showed any apoptotic features. **(E)** TR3 induces cytochrome c release from mitochondria in vitro. Mitochondria purified from LNCaP cells were incubated with the indicated recombinant TR3 or its mutant proteins. After a 30-min incubation at 30°C, the samples were centrifuged at 12,000g for 5 min at 4°C. The resulting supernatants were subjected to SDS-PAGE analysis using anti-cytochrome c antibody. A nonspecific band at ~70 kD served as a control for protein loading.

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 40. For analysis of nuclear morphological change, LNCaP cells were trypsinized, washed with PBS, fixed with 3.7% paraformaldehyde and stained with DAPI (0.1 μg/ml) to visualize nuclei by fluorescent microscopy. For the terminal deoxynucleotidyl transferase (TdT) assay, cells were treated with or without the indicated agents for 48 hours, trypsinized, washed with PBS, fixed in 1% formaldehyde and resuspended in 70% ice-cold ethyl alcohol. Cells were then labeled with biotin-16-deoxyuridine 5'-triphosphate by terminal

deoxynucleotidyltransferase and stained with avidin-fluorescein isothiocyanate (Boehringer Mannheim).
 41. The pRC/CMV-TR3 antisense recombinant plasmid was stably transfected into LNCaP cells by using the calcium phosphate precipitation method, and stable clones were screened and selected by using G418 (Gibco-BRL, Grand Island, NY).
 42. The (NurRE)₂-tk-CAT was constructed by ligating two copies of NurRE oligonucleotide (5'-GATCCT-AGTGATATTTACCTCCAAATGCCAGGA-3') into the Bam HI site of the pBLCAT₂ vector.
 43. X-k. Zhang *et al.*, *Nature* **355**, 441 (1992).
 44. pGFP-TR3 fusion construct was generated by cloning TR3 cDNA into pGFP-N2 vector (Clontech, Palo Alto, CA). LNCaP cells were seeded in six-well plates overnight, then transiently transfected with GFP-fusion expression plasmids. After 16 hours, cells were treated with apoptotic agents in medium containing 0.5% FBS, washed with PBS, and fixed in 4% paraformaldehyde. For mitochondrial staining, cells were then

incubated with anti-Hsp60 goat immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-goat IgG conjugated with Cy3 (Sigma). For cytochrome c staining, cells were incubated with monoclonal anti-cytochrome c IgG (PharMingen), followed by anti-mouse IgG conjugated with Cy5 (Amersham International). Fluorescent images were collected and analyzed by using a MRC-1024 MP laser-scanning confocal microscope (Bio-Rad, Hercules, CA).

45. To perform the subcellular fractionation assay, LNCaP cells (1×10^7 cells) suspended in 0.5 ml hypotonic buffer (5 mM tris-HCl, pH 7.4, 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, pH 8.0, and 1 mM dithiothreitol) were homogenized, and cell extracts were centrifuged at 500g for 5 min. Pellet containing nuclei was resuspended in 200 μl 1.6 M sucrose in hypotonic buffer plus protease inhibitors and laid over 1 ml 2.0 M sucrose in the same buffer, then centrifuged at 150,000g for 90 min at 4°C; the supernatant was centrifuged at 10,000g for 30 min at 4°C to obtain the HM fraction. The HM fraction was resuspended in 100 μl of lysis buffer (10 mM tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, pH 8.0) for Western blotting analysis.
46. TR3 deletion mutants were obtained by cloning polymerase chain reaction (PCR) products into the pGFP vector. The following primers 5'-GAAGATCTTCATGTGGGATGGCTCTCGGCCACTTC-3', 5'-CGGAA-TTCCGGCACCAAGCTCCAGCTTGAGGTAG-3', 5'-CGGAATCCCGGTAGCACCAGCCCTGAGCAGAAGATG-3' and appropriate primers (T3 or T7) were used to generate PCR products from pBS-TR3 to produce TR3/Δ1, TR3/Δ2, and TR3/Δ3, respectively. To construct TR3/ΔDBD, a fragment containing the DBD was removed by digestion of pGFP-TR3 plasmid DNA with Stu I.
47. S. K. Kolluri, C. Weiss, A. Koff, M. Goettlicher, *Genes Dev.* **13**, 1742 (1999).
48. To construct pGFP-TR3/ΔDBD-NLS, the DNA sequence corresponding to the nuclear localization sequence from SV40 large T antigen was generated by annealing two complementary DNA sequences (5'-TCGAGCGAAT TGGCCCCCAAGAAAAGAGAAAGGTGG-3' and 5'-AA TTCCACCTTTCTCTTTTCTTGGGGCCCAAT TCGC-3') and was cloned into Xho I- and Eco RI-digested pEGFP-N2 vector to generate pEGFP-N2-NLS. TR3/ΔDBD from pGFP-TR3/ΔDBD was then subcloned into Bgl II-digested pEGFP-N2-NLS vector to generate pGFP-TR3/ΔDBD-NLS. To construct pGFP-TR3/ΔDBD-CAAX, the DNA sequence coding for the COOH-terminal 20 amino acids of human K-Ras, which contains the plasma membrane targeting sequence, was amplified by PCR using forward primer 5'-GATCTCGAGC AAGATGACCAAGATGG-3' and reverse primer 5'-TTCGAATTCATATACACTTTGTC-3' and was cloned into Xho I- and Eco RI-digested pEGFP-N2 vector to generate pEGFP-N2-CAAX. TR3/ΔDBD from pGFP-TR3/ΔDBD was then subcloned into Bgl II-digested pEGFP-N2-CAAX vector to generate pGFP-TR3/ΔDBD-CAAX. To construct pGFP-TR3/ΔDBD-cb5, the DNA sequence coding for the COOH-terminal 34 amino acids of human cytochrome b5, which contains the endoplasmic reticulum targeting sequence was amplified by PCR using forward primer 5'-GATCTCGAGCATCACTACTATTGATTC-3' and reverse primer 5'-GCAGATTTCGCTTCCATGTATAGG-3' and was cloned into Xho I- and Eco RI-digested pEGFP-N2 vector to generate pEGFP-N2-cb5. TR3/ΔDBD from pGFP-TR3/ΔDBD was then subcloned into Bgl II-digested pEGFP-N2-cb5 vector to generate pGFP-TR3/ΔDBD-cb5.
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