Nuclear and cytoplasmic shuttling of TRADD induces apoptosis via different mechanisms

Michael Morgan,1 Jacqueline Thorburn,1 Pier Paolo Pandolfi,2 and Andrew Thorburn1

1Department of Cancer Biology and Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157
2Department of Human Genetics and Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

The adapter protein tumor necrosis factor receptor (TNFR1)-associated death domain (TRADD) plays an essential role in recruiting signaling molecules to the TNFRI receptor complex at the cell membrane. Here we show that TRADD contains a nuclear export and import sequence that allow shuttling between the nucleus and the cytoplasm. In the absence of export, TRADD is found within nuclear structures that are associated with promyelocytic leukemia protein (PML) nuclear bodies. In these structures, the TRADD death domain (TRADD-DD) can activate an apoptosis pathway that is mechanistically distinct from its action at the membrane-bound TNFR1 complex. Apoptosis by nuclear TRADD-DD is promyelocytic leukemia protein dependent, involves p53, and is inhibited by Bcl-xL but not by caspase inhibitors or dominant negative FADD (FADD-DN). Conversely, apoptosis induced by TRADD in the cytoplasm is resistant to Bcl-xL, but sensitive to caspase inhibitors and FADD-DN. These data indicate that nucleocytoplasmic shuttling of TRADD leads to the activation of distinct apoptosis mechanisms that connect the death receptor apparatus to nuclear events.

Introduction

Tumor necrosis factor (TNF)*-α regulates inflammation, survival, proliferation, and apoptosis by activating the membrane-bound receptors, TNF receptors (TNFRs)1 and 2 (for a review of TNF family signaling see Ashkenazi and Dixit, 1998; Locksley et al., 2001). The adapter protein TNFR1-associated death domain (TRADD) is an essential component of the TNFRI complex (Hsu et al., 1995), and functions by recruiting other members of the complex to the receptor. Among these are TRAF-2, which binds the NH2-terminal domain portion of TRADD (Hsu et al., 1996b), and Fas-associated death domain protein (FADD) and receptor-interacting protein (RIP), which binds to its COOH-terminal death domain (DD) (Hsu et al., 1996a, 1996b).

FADD contains a death effector domain (DED) that binds the DED of caspase-8. Recruitment of this caspase through TRADD and FADD results in caspase activation and subsequent apoptosis (Chinnaiyan et al., 1996; Hsu et al., 1996b). RIP is required for activation of NF-κB, which results in the transcription of antiapoptotic genes, whereas TRAF-2 is required for initiation of the JNK signaling pathway (Yeh et al., 1997; Kelliher et al., 1998). In addition, TRAF-2 may recruit inhibitor of apoptosis proteins (IAPs) to the complex, resulting in inhibition of apoptosis (Shu et al., 1996). RIP is also thought to recruit RAIDD, which has a caspase recruitment domain (CARD) that binds caspase-2, and may thus initiate apoptosis though activation of this caspase (Duan and Dixit, 1997). A product generated by caspase-8 cleavage of RIP seems to stabilize the TRADD–FADD interaction, resulting in further caspase-8 activation (Lin et al., 1999).

Thus, a delicate balance is maintained between pro- and antiapoptotic signals that depend on TRADD binding to TNFR1 at the membrane-bound death-inducing signaling complex (DISC). The result of receptor activation (cell survival or death) is dependent on the context of its activation. In some cases, inhibition of caspasess fails to block, and may

---

Supplemental material can be found at: http://doi.org/10.1083/jcb.200204039

© The Rockefeller University Press, 0021-9525/2002/06/975/10 $5.00
The Journal of Cell Biology, Volume 157, Number 6, June 10, 2002 975–984
http://www.jcb.org/cgi/doi/10.1083/200204039
even increase, TNF-induced cell death. However, necrotic rather than apoptotic death occurs in some of these processes (Vercammen et al., 1998; Jones et al., 2000; Luschen et al., 2000; Denecker et al., 2001). In some cells, p53 is required for TNFRI-dependent apoptosis (Cai et al., 1997; Ameyar et al., 1999; Rokhlin et al., 2000), but it is unclear why a nuclear transcription factor should be required for apoptosis when complex formation at the receptor can directly activate caspases. 

The promyelocytic leukemia protein (PML) is a tumor suppressor (Rego et al., 2001) found in discrete bodies in the nucleus known as PML oncogenic domains, or PML nuclear bodies. PML-null cells are resistant to TNF$\alpha$ and the PML–RAR$\alpha$ fusion protein, which is delocalized from nuclear bodies to nonfunctional nuclear microspeckles in acute promyelocytic leukemia patients, and antagonizes Fas ligand– and TNF$\alpha$-induced death (Wang et al., 1998). These data suggest that functional PML (and PML nuclear bodies) is required for death receptor–induced apoptosis. This raises the question of how nuclear PML might be involved in apoptosis that is initiated at the cell membrane by cytoplasmic proteins such as TRADD, FADD, etc. In the case of Fas, this link may involve Daxx, which has been reported to bind Fas in some conditions (Chang et al., 1998; Ko et al., 2001) and be in PML nuclear bodies in other situations (Torii et al., 1999; Zhong et al., 2000b). However, a protein that is at the TNFR1 DISC and can mediate apoptosis from PML nuclear bodies has not been identified.

Here we report that TRADD contains both nuclear export and import sequences, allowing it to shuttle through the nucleus. Upon inhibition of nuclear export with leptomycin B (LMB), TRADD accumulates in nuclear structures that are associated with PML nuclear bodies. A fragment of TRADD-DD that is localized exclusively to these structures induces death through a mechanism that involves p53 and PML. These data suggest that shuttling of TRADD between the cytoplasm and the nucleus activates different apoptotic mechanisms and provides a link between components of the TNF receptor signaling machinery and the nucleus.

Results

TRADD translocation to the nucleus

To study the subcellular localization of TRADD in living cells, we transfected HeLa cells with a plasmid encoding a green fluorescent protein (GFP)–TRADD fusion protein. GFP–TRADD was excluded from the nucleus and had a perinuclear or diffuse cytoplasmic distribution (Fig. 1 A, top left) similar to what has been described previously in fixed cells (Jones et al., 1999). At higher expression levels, GFP–TRADD accumulated in what appeared to be the previously observed death effector filaments (Siegel et al., 1998; Guiet and Vito, 2000; Cottin et al., 2001). Expression of a GFP–TRADD construct with an exogenous SV40-nuclear localization signal (NLS) fused to the NH$_2$ terminus of the GFP (NLS–GFP–TRADD) failed to change the cytoplasmic distribution, suggesting that GFP–TRADD might be actively exported from the nucleus. LMB binds to and inhibits the nuclear export receptor chromosomal region maintenance protein (CRM)1, and has been widely used to test whether proteins are actively exported from the nucleus (Henderson and Eleftheriou, 2000). Cells expressing either GFP–TRADD or triple yellow fluorescent protein (YFP$_3$) proteins were followed by time-lapse video microscopy before and after LMB treatment. Although both proteins were initially cytoplasmic, LMB treatment led to rapid accumulation of GFP–TRADD localized in dots after LMB treatment. These data suggest that GFP–TRADD moves between the cytoplasm and the nucleus.
dispersed to a diffuse cytosolic pattern upon treatment with Brefeldin A (unpublished data). LMB treatment resulted in the accumulation of endogenous TRADD in the nucleus, where it formed dot-like structures in many cells (Fig. 1 B).

We investigated whether endogenous TRADD would colocalize with PML, which is found in subnuclear domains known as PML oncogenic domains or PML-nuclear bodies (for review see Maul et al., 2000; Zhong et al., 2000a). Upon expression of GFP-PML in LMB-treated cells, a clear correlation could be detected between the nuclear TRADD structures and GFP-PML. Where present in the same nuclei, the two proteins appeared to be partially colocalized or in structures juxtaposed to each other (Fig. 1 C, top).

A second protein that localizes to nuclear dots is HIPK2, which has been reported to bind to TRADD (Li et al., 2000). A related protein, HIPK3, binds both FAS and FADD and colocalizes with PML (Rochat-Steiner et al., 2000). HIPK2 colocalizes with PML and activates p53-dependent apoptosis in nuclear bodies (D’Orazi et al., 2002). Like GFP-PML, nuclear dots containing HIPK2-GFP colocalized with, or were adjacent to, nuclear dots containing endogenous TRADD in LMB-treated cells (Fig. 1 C, bottom).

TRADD contains a nuclear export sequence
Rapid nuclear accumulation of endogenous TRADD and GFP–TRADD in LMB-treated cells suggests that TRADD contains a nuclear export sequence (NES) that is dependent on CRM1, or binds to another protein that contains a CRM1-dependent NES. To map this region, combinations of NH₂- and COOH-terminal deletions of TRADD were created as COOH-terminal GFP fusion proteins (left). The table indicates whether fusion proteins were primarily nuclear or cytoplasmic in the presence or absence of an exogenous SV40-NLS fused to the NH₁ terminus of GFP. (Bottom) Overlapping 20-aa segments of TRADD. The ability of these sequences to export GFP or NLS-GFP is indicated to the right. The TRADD NES sequence is shown in alignment with other known NES sequences (right).
the cytoplasm. Conversely, constructs that lacked this region were primarily localized to the nucleus when an NLS was present. To more precisely map the NES, we expressed overlapping 20-aa segments of this region as COOH-terminal YFP fusion proteins (Fig. 2 B, bottom). Proteins containing aa 144–163 or 154–173 were excluded from the nucleus, indicating that the overlapping aa 154–163 could function as an NES. YFP fusion proteins of these two fragments were absent from the nucleus even when an SV40-NLS was fused to the NH$_2$ terminus of the YFP (Fig. 2 A), and relocated to the nucleus in LMB-treated cells (unpublished data). The solved solution structure of TRADD indicates that these 10 aa are part of an exposed alpha helix (Tsao et al., 2000). This sequence displays homology to other NESs (Henderson and Eleftheriou, 2000), with three of four leucines being conserved (Fig. 2 B, right). The combination of L155A and L162A mutations in the wild-type GFP–TRADD abolished nuclear export, and resulted in nuclear accumulation (Fig. 2 A). These data indicated that aa 154–163 function as a CRM1-dependent NES.

TRADD contains a nuclear import sequence
The accumulation of nuclear GFP–TRADD in LMB-treated cells and in the L155A/L162A mutant suggests that TRADD has a nuclear import signal. Consistent with this hypothesis, we found that a YFP fusion protein containing the core helices of the DD (aa 222–289) of TRADD (YFP–TRADD-DD) was exclusively nuclear and localized in dots (Fig. 3 A). This localization pattern in nuclear dots appears to be specific for the TRADD-DD, as expression of the DDs of FADD and death-associated protein kinase (DAPK) in similar YFP fusion proteins resulted in diffuse localization patterns similar to YFP alone.

To further define the sequence important for nuclear localization, we made NH$_2$- and COOH-terminal truncations of the YFP–TRADD-DD construct. A construct containing TRADD aa 222–245 was sufficient to retain YFP in the nucleus in a pattern similar to YFP fused with an SV40-NLS (Fig. 3 A). This region contains a stretch of several basic aa, and loosely matches the consensus for a bipartite NLS. To verify that the putative NLS region was responsible for nuclear import, we fused the TRADD-DD to a YFP$_3$ construct. YFP$_3$ is normally excluded from the nucleus because it is too large to diffuse through nuclear pores (Fig. 3 B). The resulting construct (YFP$_3$–TRADD-DD) was imported into the nucleus where it accumulated in sub-nuclear dots (Fig. 3 B). Individual mutations in four basic residues in this region (K229M, R231L, K232M, and R242A) increased the cytoplasmic distribution of the protein (unpublished data), and a triple point mutant (K229M/R231L/K232M) resulted in a protein that was completely excluded from the nucleus (Fig. 3 B), indicating that this region is a functional NLS.

The previously used anti-TRADD antibody failed to recognize YFP- or GFP–TRADD-DD. Therefore, we used this antibody to determine whether the nuclear DD colocalized with endogenous TRADD in LMB-treated cells. A strong colocalization was observed between GFP–TRADD-DD and endogenous TRADD in treated cells (Fig. 3 C), suggesting that the nuclear structures are identical.

Figure 3. Mapping of a nuclear localization sequence in TRADD. (A and B) Fluorescence microscopy of cells expressing YFP (A) or YFP$_3$ (B) fusion proteins (left) as labeled. (Middle) Cell nuclei stained with Hoechst dye; (Right) The respective merged images. (C) The merged image (right) shows colocalization of endogenous TRADD (left, red) and GFP–TRADD-DD (middle, green) after 6 h LMB treatment. Bars, 10 mM.

A COOH-terminal element of TRADD regulates the kinetics of nuclear import
A recent report suggested that the COOH-terminal portion of the TRADD-DD (aa 245–312) may associate with acidic keratins 14 and 18, localizing TRADD to cytoplasmic filaments and decreasing sensitivity to TNF (Inada et al., 2001). We noticed that some TRADD constructs lacking the NES accumulated in the nucleus in some cells, and in cytoplasmic filaments in other cells. It is presently unclear whether these cytoplasmic filaments occur under physiological conditions, as we have observed them only at higher expression levels. Nuclear accumulation of TRADD occurred at slower rates for constructs where these filaments were present, and appeared to be dependent on the COOH-terminal 23 aa. Therefore, we compared the cellular localization of TRADD-DD constructs that differ only in the COOH terminus. As shown in Fig. 4, YFP constructs con-
Src. Relocation of YFP–TRADD-DD to the cell membrane plasma membrane using the myristoylation sequence from hypothesis, we mislocalized the tagged minimal DD to the nucleus was important for its ability to kill cells. To test this, we used the minimal YFP-tagged DD construct (YFP–TRADD-DD) consisting of aa 222–289, which localizes exclusively to the nucleus in PML-associated structures.

Nuclear YFP–TRADD-DD induces apoptosis

Time-lapse microscopy indicated that YFP–TRADD-DD–expressing cells died displaying the typical hallmarks of apoptosis, such as membrane blebbing and condensed nuclei (Fig. 5 A; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200204039/DC1). Quantitative cell survival analysis of HeLa cells expressing YFP–TRADD-DD indicated that <25% of these cells remained alive 24 h after microinjection (Fig. 5 B). As controls, we injected plasmids that expressed either YFP alone, YFP fused to an SV40-NLS, or the YFP-tagged DDs of FADD or DAPK. None of these molecules contained aa 196–289 or 222–289 that were exclusively nuclear, whereas constructs containing aa 196–312 or 222–312 had significant cytoplasmic distribution at 24 h after transfection. These data suggest that the COOH-terminal aa contain an element that regulates the kinetics of nuclear accumulation of TRADD, perhaps by inhibiting the NLS, functioning as another NES, or binding to cytoplasmic proteins such as cytoskeletal proteins. Accordingly, for experiments to address the function of TRADD-DD in the nucleus, we used the minimal YFP-tagged DD construct (YFP–TRADD-DD) consisting of aa 222–289, which localizes exclusively to the nucleus in PML-associated structures.

Expression of p53 in p53-null cells sensitizes them to YFP–TRADD-DD death

p53 is essential for TNF-dependent apoptosis in some cell types (Cai et al., 1997; Ameyar et al., 1999; Rokhlin et al., 2000). p53 has also been shown to localize in PML nuclear bodies, where PML acts as a transcriptional coactivator of p53 and regulates its activity (Fogal et al., 2000; Guo et al., 2000). In addition, HIPK2, which binds to TRADD (Li et al., 2000) and colocalizes in nuclear dots (Fig. 1), can activate p53 (D’Orazi et al., 2002). To test whether p53 was involved in death induced by nuclear TRADD-DD, we performed microinjection experiments in HT7 cells, which are a derivative of the p53-null cell line H1299. These cells contain a p53 transgene regulated by a tetracycline-inducible promoter, and express p53 in the presence, but not in the absence, of doxycycline (Koumenis et al., 2001) (Fig. 5 E, bottom). As shown in Fig. 5 E, H7 cells failed to undergo TRADD-DD–induced apoptosis in the absence of doxycycline. However, doxycycline-induced expression of p53 sensitized these cells to death by the TRADD-DD, suggesting that p53 plays an important role in apoptosis induced by nuclear TRADD-DD.

YFP–TRADD-DD requires PML to induce death

We investigated whether PML was required for YFP–TRADD-DD–induced death in mouse embryo fibroblast (MEF) cells. As shown in Fig. 5 D (left), about half of the wild-type MEF cells died in response to YFP–TRADD-DD at 24 h, when compared with YFP-injected control cells. However, MEF cells derived from PML knockout mice were completely resistant to YFP–TRADD-DD when compared with control cells, indicating that PML is required for the death signaling by nuclear YFP–TRADD-DD (Fig. 5 D, left). As a control, an expression plasmid for wild-type FADD was injected into PML-deficient and wild-type MEFs. There was no difference in the sensitivity of PML knockout cells to FADD compared with wild-type cells. This suggests that FADD-dependent caspase activation pathways are intact in PML −/− MEFs, and that the PML-dependent signal generated by TRADD-DD is likely independent of FADD recruitment. To verify the requirement for PML, we coexpressed PML with TRADD-DD or YFP in PML −/− MEFs. Injection of TRADD-DD or PML alone in conjunction with YFP or control vector failed to kill cells at this time point (Fig. 5 D, right). However, ~1/2 of the cells injected with both TRADD-DD and PML died after 24 h. These data suggest that PML plays an essential role in a nuclear apoptotic pathway induced by TRADD-DD.
TRADD induces apoptosis by different mechanisms in the nucleus and cytoplasm

The previous data suggest that nuclear TRADD-DD can induce apoptosis via a mechanism that is different from the current model, by which TRADD induces apoptosis at the TNFR1. Therefore, we compared the effects of various inhibitors on death induction by the exclusively nuclear YFP–TRADD-DD and the nuclear-excluded wild-type...
TRADD (unpublished data). A FADD-DN molecule lacking the DED has been widely used to inhibit apoptosis induced by death receptors, including TNFR1. Consistent with the previously established role for TRADD (Hsu et al., 1996b), coexpression of a dsRED version of the FADD-DN molecule prevented death induced by GFP–TRADD (Fig. 6B). However, this molecule failed to inhibit death induced by the nuclear DD construct YFP–TRADD-DD (Fig. 6B). Conversely, microinjection of Bcl-xL with YFP–TRADD-DD resulted in an almost complete inhibition of YFP–TRADD-DD–induced death, but failed to block apoptosis in GFP–TRADD–expressing cells (Fig. 6C). These results indicate that when in the nucleus, the TRADD-DD activates an apoptosis pathway that is mechanistically distinct from that activated by TRADD in the cytoplasm. In addition, lack of inhibition by dsRED–FADD-DN on YFP–TRADD-DD implies that the isolated DD does not cause death by upregulating machinery in death receptor pathways upstream of FADD (i.e., FAS, FASL, TNFR1, TNFα, etc.).

Discussion

In summary, we have shown that TRADD contains both NES and NLS elements capable of nuclear export and import, and causing the protein to shuttle between the cytoplasm and the nucleus. The export activity is sensitive to LMB, implicating a CRM1-dependent export, and treatment with LMB causes nuclear accumulation of endogenous TRADD in dot-like structures that are associated with PML nuclear bodies. The core portion of the TRADD-DD localizes exclusively to such structures even in the absence of LMB, where it can induce apoptosis through a mechanism that is different from TRADD’s previously defined role at the cell membrane. This mechanism requires PML and p53, and may involve the mitochondria because it is inhibited by Bcl-xL.

Detailed structure–function analyses have been performed previously with the TRADD-DD (Hsu et al., 1995; Park and Baichwal, 1996). However, these studies utilized a larger construct (aa 196–312) than our minimal DD construct (aa 222–289), which lacks the inhibitory COOH-terminal aas that delay nuclear localization and death. Based on these previous studies, our YFP–TRADD-DD protein should be unable to self associate, bind to TNF-R1, or activate NF-κB, consistent with our hypothesis that TRADD acts by a different mechanism in the nucleus than at the TNFR1 DISC.

Previous studies suggest that TRADD can use alternate mechanisms to induce apoptosis in addition to the well-known pathway involving FADD and caspase-8 recruitment. For example, a significant percentage of FADD-deficient cells die upon TRADD overexpression (Yeh et al., 1998). This incomplete rescue implies that TRADD is able to kill cells independent of FADD. Moreover, several studies show that caspase inhibition often fails to prevent cell death associated with TNFα treatment (Vercammen et al., 1998; Jones et al., 2000; Luschen et al., 2000; Denecker et al., 2001), suggesting a possible role for TRADD in caspase-independent death.

Previous studies also link TNF to apoptotic signaling in the nucleus involving PML and p53. Consistent with our results, nuclear PML structures are essential for TNFα-medi-
ated death (Wang et al., 1998), and PML has been linked to both caspase-dependent and -independent death pathways (Wang et al., 1998; Quignon et al., 1998). As YFP–TRADD-DD is localized in PML-associated structures where it can induce caspase-independent death, we hypothesize that nuclear shuttling of TRADD may provide a link between nuclear PML and TNFα signaling. p53 is also localized in PML-associated nuclear dots in some instances, and is required for TNF signaling in some contexts (Cai et al., 1997; Ameyar et al., 1999; Roklin et al., 2000). Therefore, nuclear shuttling of TRADD may link the TNF receptor with p53 through HIPK2 and/or PML, both of which regulate p53-dependent transcription and apoptosis (Fogal et al., 2000; Guo et al., 2000; Wang et al., 2001; D’Orazi et al., 2002). We have not been able to demonstrate nuclear translocation of TRADD upon TNFα treatment. This may be due in part to technical difficulties in studying such responses in cells that are rapidly undergoing apoptosis. However, we cannot exclude the possibility that nuclear TRADD functions in another pathway outside of TNF signaling.

Death induced by nuclear TRADD-DD is inhibited by Bcl-xL, suggesting that mitochondrial dysfunction occurs in response to nuclear TRADD. Anti-apoptotic members of the Bcl-2 family can block some caspase-independent, as well as caspase-dependent, death (Monney et al., 1998; Okuno et al., 1998), consistent with the ability of Bcl-xL to block the caspase-independent death seen here. Bax and other p53 target genes lead to mitochondrial dysfunction (Miyashita and Reed, 1995; Green and Reed, 1998; Oda et al., 2000a, 2000b; Nakano and Vouven, 2001), and activation of the mitochondrial pathway is required for the p53-dependent TNF toxicity seen in MCF-7 cells (Ameyar-Zazoua et al., 2002).

In conclusion, our data suggest that rather than functioning solely in the cytoplasm, TRADD can shuttle through the nucleus where it can activate a different apoptotic pathway involving PML and p53. Therefore, the subcellular distribution of TRADD may modulate its activity and behavior, adding an additional level of complexity to TRADD signaling.

Materials and methods

Plasmid construction
The YFP–FADD-DD and YFP–DAPK-DD kinase expression plasmids have been previously described (Morgan et al., 2001). The dS-RED–FADD-DD expression construct was made by removing the YFP coding sequence at the NH2 terminus of YFP–FADD-DD and replacing it with dS-RED (CLONTECH Laboratories, Inc.). Expression vectors for p35 and BCL-xL were obtained from previously described (Morgan et al., 2001). The dS-RED–FADD-DN constructs were by transfection or microinjection. In mapping experiments, nuclei of living cells were stained with Hoechst 33258 at a final concentration of 10 μg/mL. To quantitate cell death, healthy living fluorescent cells were counted 1–2 h after microinjection, and the percent survival was determined after overnight incubation. Quantitative data shown in the histograms represent the mean percent survival (± SEM) from 3 or 15 separate experiments, with typically 50–200 cells per sample.

Cell culture and transfection
All cells were maintained in DMEM in the presence of 10% fetal bovine serum. Additionally, HT7 cells were supplemented with 100 μg/mL Genetin to maintain the p53 transgene. Where indicated, cells were incubated in the presence of the caspase inhibitor z-VAD-fmk (150 μM; Calbiochem) or the corresponding volume of DMSO as a control. Expression of all constructs was by transfection or microinjection. In mapping experiments, nuclei of living cells were stained with Hoechst 33258 at a final concentration of 10 μg/mL. Where used, LMB (Sigma-Aldrich) was added to the media at a final concentration of 10 μg/mL. Transfections were performed with FuGENE-6 reagent (Roche) according to the manufacturers’ protocols, at a concentration of 2 μg plasmid DNA/3.5-cm dish.

Immunofluorescence
For immunofluorescence experiments, cells were grown on glass coverslips, and where indicated, transfected or treated with LMB before permeabilizing-fixing in a 1:1 mixture of methanol/acetone at –20°C for 10 min. Cells were then air dried and blocked in 1% fetal bovine serum in PBS for 10 min. Cells were then stained with a monoclonal anti-TRADD antibody (BD Transduction Laboratories) for 45 min and rinsed three times with PBS for 5 min. For detection, cells were incubated with a rhodamine-conjugated goat anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch Laboratories) for 30 min followed by rinsing three times with PBS for 5 min. Images were obtained with a Hamamatsu digital camera using the Openlab 3.0 software (Improvision).

Online supplemental material
2 h after microinjection of HeLa cells with YFP–TRADD-DD, time-lapse video microscopy was used to create Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200204039/DC1), which represents apoptosis by the nuclear TRADD-DD and shows apoptotic death of HeLa cells expressing YFP–TRADD-DD (green). Images were performed 2 h before the start of the video. Images were captured at 30-min intervals for 24 h. Cells were maintained in an environmental chamber at 37°C and with 5% CO2. Fluorescence images and phase contrast images were obtained at half-hour intervals over 24 h with a Hamamatsu digital camera using the Openlab 3.0 software (Improvision). Images were merged using Openlab 3.0 and exported in Quicktime format.

We thank Constantinos Koumenis (Wake Forest University School of Medicine, Winston-Salem, NC) for the HT7 cells. This work was supported by grants from the North Carolina Biotechnology Center, American Heart Association, Mid-Atlantic Affiliate, and the National Institutes of Health (NS42662). Initial experiments were supported by the Huntsman Cancer Foundation at the University of Utah during the tenure of a predoctoral fellowship from the American Heart Association, Western States Affiliate (M.J. Morgan).

Submitted: 8 April 2002
Accepted: 19 April 2002
References


