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Regulation of Tumor Necrosis Factor- and Fas-Mediated Apoptotic Cell Death by a Novel cDNA, $TR2_L$

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A novel cDNA, $TR2_L$, isolated from murine NIH 3T3 fibroblasts, was found to modulate tumor necrosis factor (TNF)-mediated apoptosis in murine L929 fibrosarcoma cells. The full-length cDNA (853 bp) encodes a predicted coding region of 56 amino acids (6.3 kD), with 53.6% identity to the C-terminus of rat transcriptional activator FE65. When expressed stably in L929 cells, $TR2_L$ protein inhibited TNF cytotoxic response. In contrast, $TR2_L$ enhanced anti-Fas antibodies/ actinomycin D (ActD)-mediated L929 apoptosis. Alteration of $TR2_L$ function occurred by tagging this protein with a 6xHis fragment to the *N*-terminus (designated 6xH-$TR2_L$). L929 cells which stably expressed 6xH-$TR2_L$ acquired a significantly enhanced TNF apoptotic response and increased genomic DNA fragmentation compared to control cells. Enhanced cell death also occurred in these 6xH-$TR2_L$-expressing cells under serum starvation conditions. In contrast, the anti-Fas/ActD-mediated apoptosis was blocked by the 6xH-$TR2_L$ protein. Functional role of $TR2_L$ protein in regulation of cancer cell susceptibility to TNF- and Fas ligand-mediated apoptosis is suggested.

Not all cancer cells are sensitive to tumor necrosis factor (TNF)-mediated apoptotic cell death. The likely mechanism is that overexpression of certain cellular protein(s) protects the cells from TNF-mediated death. For example, overexpression of oncoprotein HER-2/*neu* (p185) enables ovarian cancer cells to resist lysis by TNF-α and lymphokine-activated killer cells (1). As structurally related to HER-2/*neu*, epidermal growth factor (EGF) receptor alters TNF cytotoxic signal transduction and controls TNF responsiveness in ME-180 squamous carcinoma cells (2). Other types of oncogene products such as *E1b* and *met* also confer TNF resistance in tumor cells (3,4). How these oncogene products contribute to tumor cell resistance to TNF is unknown. Additionally, overexpression of several intracellular proteins restricts TNF cytotoxicity. These proteins are mitochondrial manganous superoxide dismutase (MnSOD) (5), A20 (6), heat-shock protein hsp70 (7), metallothionein (8), Bcl-2 and Bcl-x (9).

Recently we demonstrated that transforming growth factor-β (TGF-β) stimulates murine L929 fibrosarcoma cells to resist TNF-mediated cell death or cytotoxicity (10). TGF-β is shown to induce a rapid tyrosine phosphorylation of cellular protein substrates in L929 cells, which may alter the TNF killing pathway (10). In addition, TGF-β-treated L929 cells secrete a novel extracellular matrix TNF-resistance triggering (TRT) protein (10), as well as express a novel TR1 (TNF resistance) gene (Chang et al, manuscript in preparation). In an effort to further elucidate TNF-resistance in cancer cells, we have isolated a cDNA from a murine NIH-3T3 cDNA library, designated $TR2_L$, which was shown to partially inhibit TNF-mediated apoptosis of L929 cells. In contrast, $TR2_L$ enhanced anti-Fas antibody-induced L929 cell death

* H.C. contributed solely to the technical aspect of cDNA library screening, sequencing, and establishing stable cell transfectants.

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in the presence of actinomycin D (ActD). Functional alteration of $TR2_L$ was found when this protein was tagged with a 6 histidine-containing fragment (6xHis) to its *N*-terminus.

MATERIALS AND METHODS

Cell lines. TNF-sensitive murine L929 fibrosarcoma cells were cultured as previously described (10).

Expression cloning of $TR2_L$ cDNA. A cDNA library was constructed from phorbol 12-myristate 13-acetate (PMA)-differentiated monocytic U937 cells (72-hr treatment) using a CMV-based eukaryotic expression phagemid, λpCEV27, a kind gift of Toru and Aaronson (11). PMA induced U937 cell differentiation and increased TNF-resistance in these cells (12). Expression of the entire cDNA library in L929 cells was performed as described (13). Briefly, purified DNA of cDNA-vector constructs or vector alone was prepared by our CsCl-column method, and L929 cells were electroporated with the purified DNA (40 μg DNA/ 1×10^7 cells) (13). After 24 hr, G418 (200 μg/ml), an analogue of neomycin (neo), was added to the cell culture to select neo-resistant cells. Following 7-day in culture, the cells were treated with recombinant human TNF-α (5000 units/ml; Genzyme, Boston, MA) for 24 hr. The surviving cells were again treated with TNF-α for 24 hr. The resulting TNF-resistant cells were allowed to grow for 1-2 month in the presence of G418, and again the TNF treatment was repeated at least three times during this culture period. TNF-resistant cell colonies were obtained, and the cDNA inserts were amplified by PCR from the chromosomal DNA of these cells. Primers used in PCR amplification were the sequences of T7 and Sp6 promoters. One of the amplified cDNA, designated TR2, was used to screen cDNA libraries from NIH 3T3 fibroblasts (Clontech, Palo Alto, CA) and the PMA-treated monocytic U937 cells. Two related cDNA clones, designated $TR2_L$ (long) and $TR2_S$ (short), were isolated from NIH 3T3 fibroblasts and U937 cells, respectively. Sequence and functional analyses of $TR2_L$ cDNA are described in this manuscript. The $TR2_L$ cDNA insert of 853 bp was subcloned into a CMV-based mammalian expression vector, pCR3 (Invitrogen, San Diego, CA), for stable expression and functional analysis in the TNF-sensitive L929 cells.

DNA sequencing and sequence analysis. Alkaline denaturation of plasmid DNA (14) and standard dideoxy chain terminating sequencing reaction (15), using T7 DNA polymerase (16) (Sequenase version 2.0 from United States Biochemicals, Cleveland, OH), were performed. The MacDNASIS software program (Hitachi software, San Bruno, CA) was used for sequence analysis. Homologous searching against the universal database was performed using the NCBI BLAST program (17).

TNF cytotoxicity assays. L929 cells were electroporated with $TR2_L$-pCR3 DNA or vector DNA alone (40 μg DNA/ 1×10^7 cells), followed by selection of stable transfectants for 1-2 months as described above. Analysis of the cDNA function was performed by challenging the stable L929 transfectants (3×10^5 cells/ml precultured in 96-well plates for 48 hr) with TNF-α (62.5-2000 units/ml) for 24 hr. The extent of cell death was determined by staining cells with crystal violet (10). Where indicated, the transfected cells were treated with monoclonal anti-Fas antibodies (Pharmingen, San Diego, CA) in the presence of actinomycin D (ActD; 1 μg/ml) for 24 hr. Unless otherwise indicated, each experiment, containing 8 replicates, was repeated 4-5 times, and only one set of representative data is presented.

DNA fragmentation. Internucleosomal DNA fragmentation was measured in agarose gel electrophoresis as described (18). Briefly, L929 cells stably transfected with $TR2_L$-cDNA or vector were plated onto petri dishes (3×10^5 cells/ml), cultured overnight and treated with TNF (2000 units/ml) for 6 hr. The cells were lysed by 500 μl of a 10 mM Tris buffer, pH 7.4, containing 0.5% NP-40 and 0.1% SDS, extracted with 500 μl of phenol/chloroform (1:1) three times and chloroform twice, and precipitated with an equal volume of isopropanol. One-third of each resuspended DNA sample (30 μl) was treated with 1 μl of ribonuclease A (1 mg/ml; Sigma) for 5 min at room temperature, loaded and electrophoresed in a 2% agarose gel. The gel was stained with ethidium bromide.

Tagging of $TR2_L$ protein with a 6xHis sequence to the N-terminus. Expression of a *N*-terminally 6xHis-tagged $TR2_L$ protein in L929 cells was performed (19). A 5′ end PCR primer, encoding the 6xHis sequence (MRGSHHHHHH) and the first 6 amino acid residues of $TR2_L$ coding region, was synthesized: 5′-ATGCGGGGTTCTCATCATCATCAT-CATCATATGTTGCGGTATCAGAAGTG. The 3′ end primer was: 5′-AAAAAAAAAAAAAACGAACT. PCR amplification of $TR2_L$ cDNA was then performed with a predicted product of 868 bp. The PCR product was cloned into the CMV-based pCR3 vector by T-A cloning, and subjected to sequence verification and expression in L929 cells. L929 cells which stably expressed the 6xHis-tagged $TR2_L$ protein (6xH-$TR2_L$) were subjected to functional testing by challenge with TNF as described above.

Northern hybridization. Standard Northern hybridization was performed using RNA from L929 cells. Total cellular RNA from L929 cells was isolated (20), and 10 μg of RNA was electrophoresed in duplicate in a 1.2% agarose gel containing formaldehyde. The separated RNA was blotted onto a nitrocellulose membrane and probed with [^{32}P] labeled $TR2_L$ cDNA. The membrane was washed twice with 2×SSC containing 0.1% SDS at room temperature for 10 min and once with 0.2×SSC containing 0.1% SDS at 70°C for 20 min, followed by autoradiography.

RESULTS

Figure 1 shows the full length cDNA of $TR2_L$ (854 bp), which encodes a predicted amino acid sequence of 56 amino acids (6.3 kD). Homology searching in the database shows that

A. $TR2_L$ Predicted Coding Region (56 aa)

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    gcg gcc gcg tcg acc ATG TTG CGG TAT CAG AAG TGC TTG GTT GCC AGG CCA CCT   54
 1                      M   L   R   Y   Q   K   C   L   V   A   R   P   P

    TCG CAG AAA GTC CGG CCC CCG CCC CCG CCA GCA GAT TCA GTG ACC CGA AGA GTC   108
14   S   Q   K   V   R   P   P   P   P   P   A   D   S   V   T   R   R   V

    ACG ACC AAT GTG AAA CGA GGG GTC TTA TCC CTC ATT GAC ACT TTG AAA CAG AAG   162
32   T   T   N   V   K   R   G   V   L   S   L   I   D   T   L   K   Q   K

    CGG CCG GTC ACA GAG ACT CCC TAG CAG CCC AGG TCT GGA GGA GTC TGA CAC CAT   216
50   R   P   V   T   E   T   P   *

    tac ctg agg atc aag tag cta cac taa aga agc cga tac ctg gcc ttc cac tca   270
    gtt gcc gat gct ttg tct cca gag att tta tct tta acc aga cag tgt tag aca   324
    agc atg tga cct cgt ctt gcc gcc acc atg tga tat aaa gag gag cat gat ttt   378
    ttc ttt cct gta agt tac atc atg agc agc aga agg tcg tct tct tgt aaa tat   432
    tgt gaa cag gac ttc cga gca cac aca cag aag aat gcg gcc aca ccc ttc cta   486
    gcc aac taa cac tgc atc ctt gga cga acg gtg ccc aag ttc att ttg ctg tcc   540
    tct tga ctg gat ctg tca caa gcc aac ttg ggg tcc ctc agc act ttg ccc tga   594
    ttt cgg atc ctg agt aac aga tac cac tga att gct gta aca atg ggg tga cac   648
    ctt tgg gtt tta gtt ttt cat aac act gag cct gtt ggt tga caa aac ttg gct   702
    gtt ggc gtc ggt aca gac acc agc cac cga gct ctc tga cac atg gac act ggt   756
    tca cat cta cag cta ttt gag gtg ccg ggc aaa gga gaa ctt taa aat tga tag   810
    aaa act aaa ttt gag gaa tta aaa tca agc aaa aaa aaa aaa aa                 854
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B. Sequence aglignment between $TR2_L$ and rat transcriptional activator FE65

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TR2L      1   MLRYQKCLVARPPSQKVRPPPPPADSVTRRVTTNVKRGVLSLIDTLKQKRPVTETP 56
              MLRYQKCL AR  +     P PPA SV RRV   V+RGV SL  +LK KR  ++TP
FE65    444   MLRYQKCLDARSQTSTSCLPAPPAESVARRVGWIVRRGVQSLWGSLKPKRLGSQTP 499
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FIG. 1. Nucleotide sequence of the full-length $TR2_L$ cDNA and the predicted amino acid sequence (56 aa; 6.3 kD). (A). The open reading frame is marked by capital letters. The start codon and the stop codon are underlined. The ''aattaaa'' sequence (underlined) is a possible polyadenylation signal. The deduced amino acid residues are given under the nucleotide sequence and the positions are indicated at the left (italics). Prosite searching shows that there are several conserved phosphorylation sites: Thr-32 for cAMP- and cGMP-dependent protein kinase; Ser-14, Thr-28, and Thr-45 for protein kinase C; Ser-41 for caesin kinase II. (B). The bottom panel demonstrates the alignment between the $TR2_L$ amino acid sequence and the C-terminus of rat transcriptional factor FE65 (21), using the NCBI BLAST program (17). The consensus residues are shown between these two sequences.

$TR2_L$ is 53.6% homologous to the *C*-terminal non-DNA binding region (#444 - 499) of rat transcriptional activator FE65, a polypeptide of 499 amino acids (21) (Fig. 1). Prosite searching (22) shows that a conserved cAMP- and cGMP-dependent protein kinase phosphorylation site is located at Thr-32 (consensus pattern: R/K-X-S/T), 3 conserved protein kinase C phosphorylation sites at Ser-14, Thr-28 and Thr-45 (consensus pattern: S/T-X-R/K), and a conserved casein kinase II phosphorylation site at Ser-41 (consensus pattern: S/T-X-X-E/D). $TR2_L$ is not homologous to any protein known to regulate TNF- and Fas ligand-mediated apoptotic cell death. $TR2_L$ possesses no death domain (23).

To analyze $TR2_L$ function, L929 cells were electroporated with DNA of $TR2_L$ cDNA-vector construct or vector only, followed by selecting stable neo-resistant cell colonies using G418 for 1-2 months. The resulting $TR2_L$-expressing cells acquired a restricted TNF cytotoxic response. That is, by transfection with $TR2_L$ cDNA (40 μg per 10^7 cells), the cells partially resisted cell death mediated by TNF (approximately 30% resistance), compared to control cells transfected with vector only (Fig. 2A). In contrast, cross-linking of the $TR2_L$-expressing

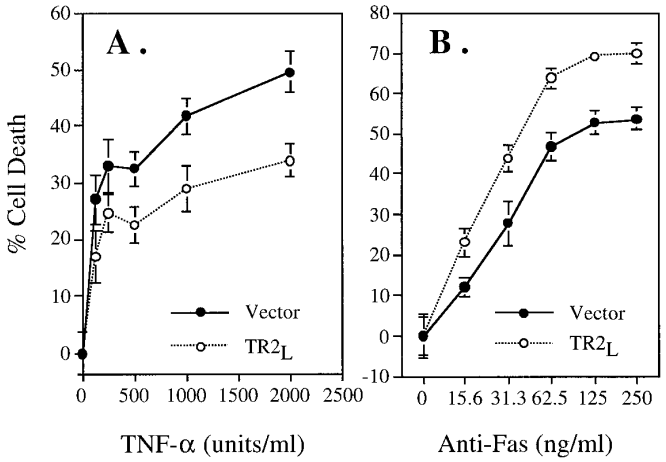

FIG. 2. Modulation of TNF- and anti-Fas/ActD-mediated L929 cell death by $TR2_L$ cDNA. Stable, neo-resistant L929 cell colonies, which were transfected with DNA of the $TR2_L$ cDNA-vector construct or vector (pCR3) only, were established as described in the Materials and Methods. These cells were seeded onto 96-well plates and cultured for 48 hr, followed by treating with various concentrations of TNF-α (A) or monoclonal anti-Fas antibodies (B) in the presence of ActD (1 μg/ml) for 24 hr. The extent of cell death or cytolysis was quantified as described in the Materials and Methods. Compared to controls, TNF-mediated cell death was partially inhibited by $TR2_L$ (approximately 30%). In contrast, $TR2_L$ enhanced anti-Fas/ActD-dependent cell death.

L929 cells with anti-Fas monoclonal antibodies in the presence ActD resulted in enhancement of cell death as compared to the vector control cells (Fig. 2B). Without ActD, the antibodies alone failed to induce L929 cell death (data not shown).

The putative $TR2_L$ protein is somewhat hydrophobic (average hydrophobicity, -0.62; analyzed according to the method of Kyte-Doolittle) (24), and a hydrophobic region is located at the C-terminus starting from amino acid residue #34 to 42. Tagging of the protein with the 6xHis sequence to the *N*-terminus, referred to as 6xH-$TR2_L$, reduces the hydrophobicity (average hydrophobicity, -0.82). The purpose of this experiment was originally intended to scale-up purification of $TR2_L$ protein from L929 cells by affinity chromatography. Notably, the functional properties of the 6xH-tagged $TR2_L$ protein were altered. For example, expression of the 6xH-$TR2_L$ cDNA in L929 cells dramatically enhanced TNF apoptosis compared to control cells as determined in a time-course study (Fig. 3A). More chromosomal DNA fragmentation was found in the 6xH-$TR2_L$-transfected cells than in control cells after 6-hr treatment with TNF-α (Fig. 3B). When serum was deprived from the culture, the 6xH-$TR2_L$-expressing cells had an increased cell death compared to the vector controls (Fig. 3C). In stark contrast, these cell resisted anti-Fas antibodies/ActD-mediated cytotoxicity (Fig. 3D).

Northern hybridization showed that L929 cells expressed 3 major mRNA transcripts of approximately 4.3, 1.9 and 1.2 kb, respectively, as identified by the ^{32}P-labeled $TR2_L$ cDNA probe (Fig. 4). In addition, L929 cells expressed a very low level of a 0.8 kb transcript, probably corresponding to our isolated $TR2_L$ cDNA from the NIH 3T3 cDNA library.

DISCUSSION

In this study we report the isolation of a novel cDNA, $TR2_L$, which modulates both TNF- and Fas/ActD-mediated apoptotic cell death in L929 cells. L929 cells when transfected with $TR2_L$ cDNA acquired a partially increased TNF resistance. The resistance could be further increased by transfecting L929 cells with higher amounts of $TR2_L$ cDNA (data not shown). In contrast, expression of $TR2_L$ protein enhanced monoclonal anti-Fas antibodies/ActD-medi-

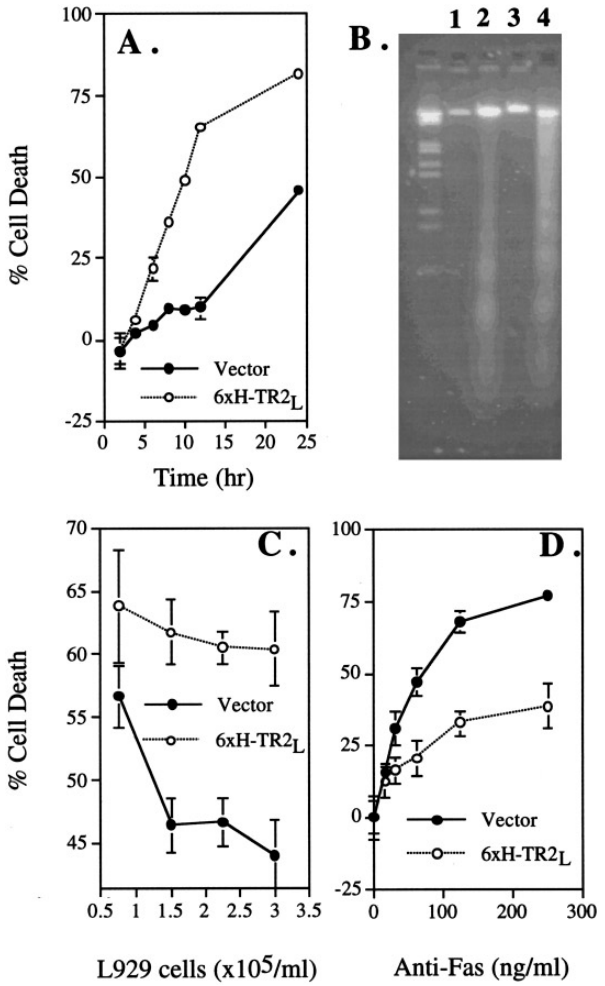

FIG. 3. Enhancement of TNF-mediated cell death by tagging of $TR2_L$ protein with a 6xHis containing peptide (MRGSHHHHHH) to the *N*-terminus. Stable neo-resistant L929 cell colonies, transfected with either 6xH-$TR2_L$-vector DNA or vector (pCR3) DNA, were established as described in the Materials and Methods. These cells were seeded onto 96-well plates and cultured for 48 hr, followed by treating with TNF-α (2000 units/ml) for 2-24 hr (A), or starving the cells by serum deprivation for 48 hr (using various cell concentrations) (C), or treating with anti-Fas antibodies in the presence of ActD (1 μg/ml) (D) for 24 hr. Enhanced cell death, respectively, by TNF-α and serum deprivation were observed in 6xH-$TR2_L$ cDNA-transfected cells. In contrast, these cells resisted cell death by anti-Fas/ActD. Increased chromosomal DNA fragmentation in 6xH-$TR2_L$ cDNA-transfected cells (6-hr TNF treatment) is shown in (B) as follows: 1. vector-transfected cells; 2. vector-transfected cells treated with TNF-α; 3. 6xH-$TR2_L$-expressing cells; 4. 6xH-$TR2_L$-expressing cells treated with TNF-α. The first lane is a DNA molecular size standard.

ated L929 cell death. These observations indicate that there is a fundamental difference in the regulation of TNF- and Fas-mediated cell death by $TR2_L$.

Mechanisms by which $TR2_L$ modulates apoptotic cell death are unknown. Whether $TR2_L$ interacts with intracellular signal proteins, such as TRAIL-1 and FADD-1 (23), during TNF- or Fas-mediated signaling transduction remains to be elucidated. Although there is a striking similarity between $TR2_L$ protein sequence and that of the *C*-terminal non-DNA binding domain of rat transcriptional activator FE65, $TR2_L$ is probably not involved in activation of transcription due to its lack of DNA binding domain. However, the presence of several conserved serine

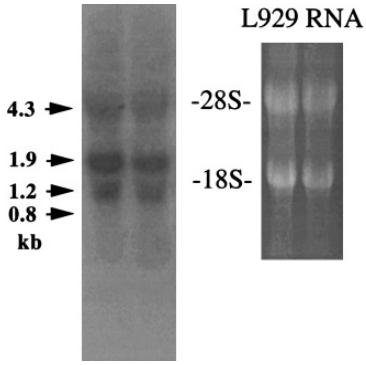

FIG. 4. Northern hybridization of L929 RNA. Total cellular RNA from L929 cells were isolated and 10 μg of RNA were electrophoresed in duplicate in 1.2% agarose gels containing formaldehyde. The separated RNA were blotted onto nitrocellulose membranes and probed with [^{32}P] labeled $TR2_L$ cDNA. The membranes were washed twice with 2×SSC containing 1% SDS at room temperature for 10 min and once with 0.2×SSC containing 1% SDS at 70°C for 20 min. The positions of 28S and 18S tRNA on the gel (left panel) were aligned corresponding to their exact positions on the membranes (right panel). Three major positive transcripts are approximately 4.3, 1.9 and 1.2 kb (see top three arrows). L929 cells expressed a very low level of 0.8 kb transcript (see the bottom arrow), which probably corresponds to our isolated $TR2_L$ cDNA from the NIH 3T3 cDNA library.

and/or threonine phosphorylation sites in $TR2_L$ suggests that phosphorylation of these amino acid residues may alter TNF killing function. Previously we reported that TGF-β induces a rapid tyrosine phosphorylation of cellular protein substrates in L929 cells, which apparently interrupts the TNF killing pathway (10). Thus, we do not exclude the possibility that phosphorylation of $TR2_L$ protein occurs in the $TR2_L$-expressing L929 cells upon treatment with TNF.

We have performed block analysis (25) to examine the potential intrinsic properties of $TR2_L$, which is based on the presence of consensus protein patterns related to other proteins. $TR2_L$ has a conserved structural motif especially related to phosphotyrosine interaction domain (PID; approximately 160 amino acids) proteins such as FE65 (21) and Shc transforming protein (26). The presence of serine/threonine phosphorylation sites and part of PID in $TR2_L$ suggests that this protein plays a possible role in protein-protein interaction during signal transduction.

We have also subcloned the $TR2_L$ cDNA in frame to the 3′ end of green fluorescent protein (GFP) in a CMV-based expression vector (Clontech). No apparent functional alteration in this fusion protein was observed. That is, the GFP-tagged $TR2_L$ protein still inhibited TNF-mediated cell death in L929 cells. The expressed GFP-$TR2_L$ is distributed evenly in the cytosol, as determined by fluorescent microscopy. Whether $TR2_L$ is also located in the nucleus remains to be established.

Northern analysis shows that there are several mRNA transcripts in L929 cells as identified by using the $TR2_L$ cDNA as a probe. This observation suggests that our isolated $TR2_L$ cDNA of 0.8 kb from NIH-3T3 fibroblasts is an alternatively spliced product. As shown in the Northern analysis, L929 cells expressed little or no mRNA transcript corresponding to the 0.8 kb $TR2_L$ cDNA. However, we have indeed isolated a 1.1 kb cDNA from L929 cells, which codes for a larger size of TR2 protein. The functional property of this protein is still being characterized in our laboratory. An additional $TR2_L$-related cDNA which we have isolated from the monocytic U937 library is $TR2_S$. The $TR2_S$ cDNA is truncated product of 0.5 kb, coding for a different protein from that of the $TR2_L$ cDNA (Cao et al, unpublished). Notably, the $TR2_S$ protein of only 31 amino acid residues is a potent inhibitor of TNF-mediated death of L929 cells.

Normally, tagging of proteins with an *N*- or *C*-terminal 6xHis sequence fails to alter their

functions. However, tagging of $TR2_L$ protein with the 6xHis sequence to the *N*-terminus altered its functional properties. This is probably caused by the relatively small size of $TR2_L$. The 6xH-$TR2_L$ protein became a potent enhancer of cell death caused by TNF and by serum deprivation. In contrast, enhancement of anti-Fas antibodies/ActD-mediated cell death by $TR2_L$ turned into inhibition by 6xH-$TR2_L$. Again, the molecular mechanism by which 6xH-$TR2_L$ differentially regulated TNF- and Anti-Fas/ActD-mediated cell death is unknown. Conceivably, the 6xHis sequence provides a structural motif which interacts with proteins associated with apoptotic cell death. Expression of 6xH-$TR2_L$ protein in TNF-resistant cancer cells would potentially increase their TNF susceptibility.

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