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Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-κB

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Background: Two receptors that contain the so-called 'death domain' have been described to date: tumor necrosis factor receptor 1 (TNFR1) and Fas/Apo-1 (CD95); both belong to the TNFR gene family. The death domain of TNFR1 mediates the activation of programmed cell death (apoptosis) and of the transcription factor NF-κB, whereas the death domain of CD95 only appears to activate apoptosis.

Results: We have identified an additional member of the TNFR family, which we have named Apo-3. Apo-3 is a transmembrane protein of approximately 47 kDa that has similarity to members of the TNFR family in its extracellular, cysteine-rich domains. In addition, Apo-3 resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain. The Apo-3 gene mapped to human chromosome 1p36.3, and Apo-3 mRNA was detected in several human tissues, including spleen, thymus, peripheral blood lymphocytes, small intestine and colon. Ectopic expression of Apo-3 in HEK293 or HeLa cells induced marked apoptosis. CrmA, a poxvirus inhibitor of Ced-3-like proteases which blocks death signaling by TNFR1 and CD95, inhibited Apo-3-induced apoptosis. Ectopic expression of Apo-3 also induced the activation of NF-κB. Apo-3 did not specifically bind to the Apo-2 ligand, suggesting the existence of a distinct ligand for Apo-3.

Conclusions: These results identify Apo-3 as a third member of the TNFR family that activates apoptosis, and suggest that Apo-3, TNFR1 and CD95 engage a common apoptotic cell-death machinery. Apo-3 resembles TNFR1 because it can stimulate NF-κB activity and regulate apoptosis. Apo-3 mRNA is expressed in various tissues, consistent with the possibility that this receptor may regulate multiple signaling functions.

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Background

The mammalian tumor necrosis factor receptor (TNFR) gene family encodes ten different cell-surface proteins that interact with a corresponding TNF-related ligand family [1,2]. The receptors share homology in their extracellular domain (ECD), which contains 3–6 cysteine-rich pseudo-repeats, but are generally not related in their cytoplasmic regions. Two TNFR family members, TNFR1 and Fas/Apo-1 (CD95), can activate apoptotic cell death [3]. These two receptors have additional homology in their intracellular domain (ICD), in an oligomerization interface known as the death domain [4,5]. Death domains are also found in several other metazoan proteins that regulate apoptosis — namely, the *Drosophila* protein Reaper [6,7] and the mammalian proteins FADD/MORT1 [8,9], TRADD [10] and RIP [11]. Upon ligand binding and receptor clustering, TNFR1 and CD95 recruit the FADD/MORT1 protein into a death-inducing signaling complex; CD95 binds to FADD/MORT1 directly [8,9], whereas TNFR1 binds indirectly to FADD/MORT1 *via* TRADD [12,13]. FADD/MORT1 in turn recruits the thiol protease MACHα/FLICE into the death signaling complex [14,15]. MACHα/FLICE appears to be the first component in a cascade of apoptotic proteases related to the product of the *Caenorhabditis elegans* gene *Ced-3* and to mammalian interleukin-1β-converting enzyme (ICE). Upon activation by proteolytic cleavage, these proteases execute essential aspects of the cell-death program [3]. In addition to initiating apoptosis, activation of TRADD by TNFR1 can lead to stimulation of the transcription factor NF-κB [4] through the interaction between TRADD and another cytoplasmic protein called TRAF2 [12,16]. NF-κB resides in the cytoplasm in a latent form, and upon activation by various stimuli, it migrates to the nucleus and induces the transcription of immune-response genes [17].

To search for new members of the TNFR family that may regulate apoptosis, we screened the DNA databases for

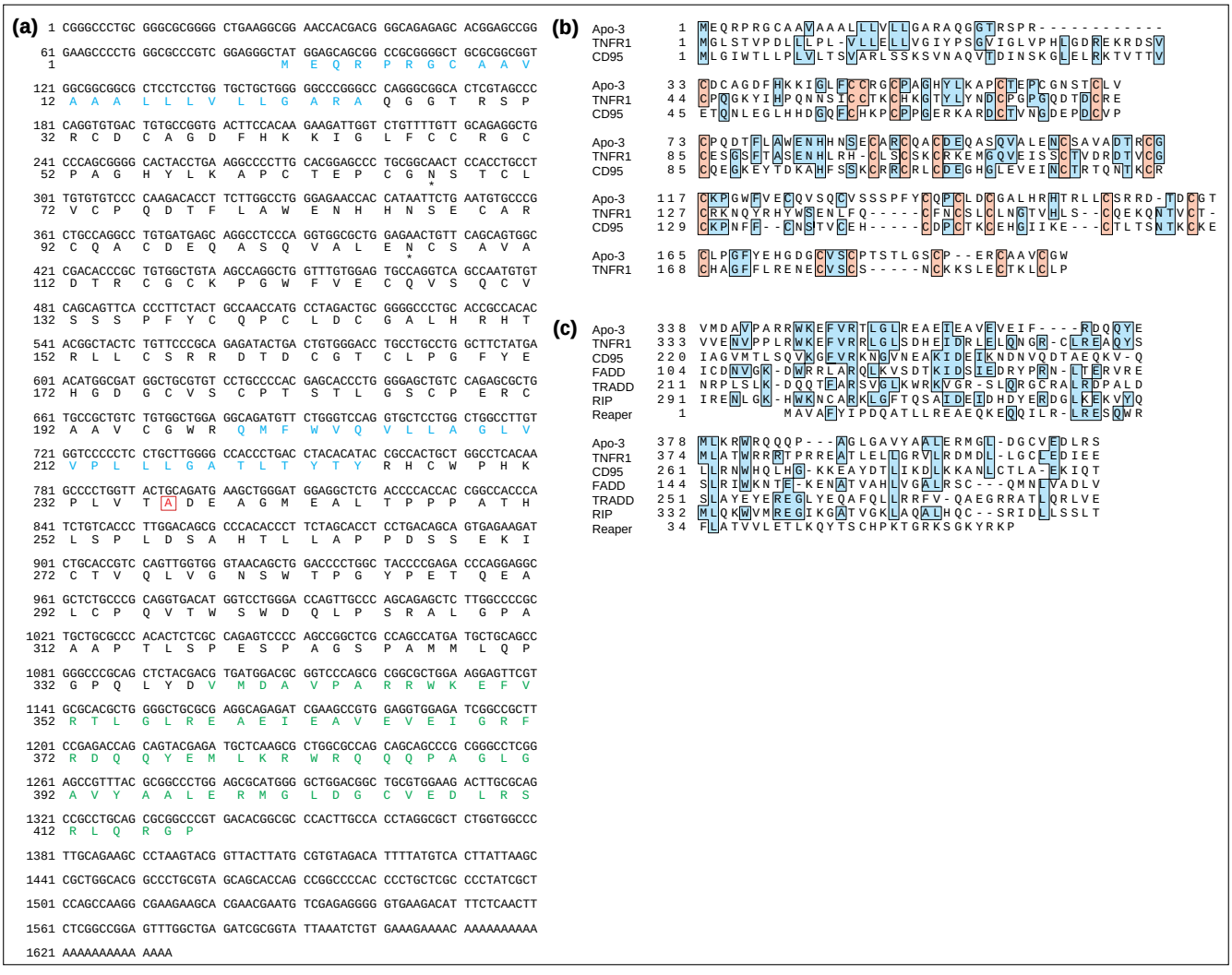
expressed sequence tags (ESTs) that had sequence similarity to TNFR1 and CD95. We identified two sets of ESTs; one set had some sequence similarity to the ICDs of TNFR1 and CD95, and the other set had some similarity to the ECDs of these proteins. Based on the ICD-related ESTs, we isolated cDNAs from human fetal heart and fetal lung libraries that encoded a transmembrane protein that resembles members of the TNFR family. We named this protein Apo-3. The ECD-related ESTs corresponded to the amino-terminal region of Apo-3. In this paper, we report the primary structure of Apo-3 and provide evidence that this receptor plays a role in the regulation of apoptosis and of NF-κB.

Results

Primary structure of Apo-3

We screened human fetal heart and lung cDNA libraries using synthetic oligonucleotide probes based on ESTs that showed homology to the ICDs of TNFR1 and CD95 (see Materials and methods). We isolated a cDNA clone from fetal heart which contains a single open reading frame with an apparent translational initiation site [18] surrounding a methionine encoded by nucleotides 89–91, and which ends with a stop codon at nucleotide positions 1339–1341 (Fig. 1a); a polyadenylation sequence is present at the 3′ end. The predicted polypeptide precursor is 417 amino-acids long and has a calculated molecular

Figure 1



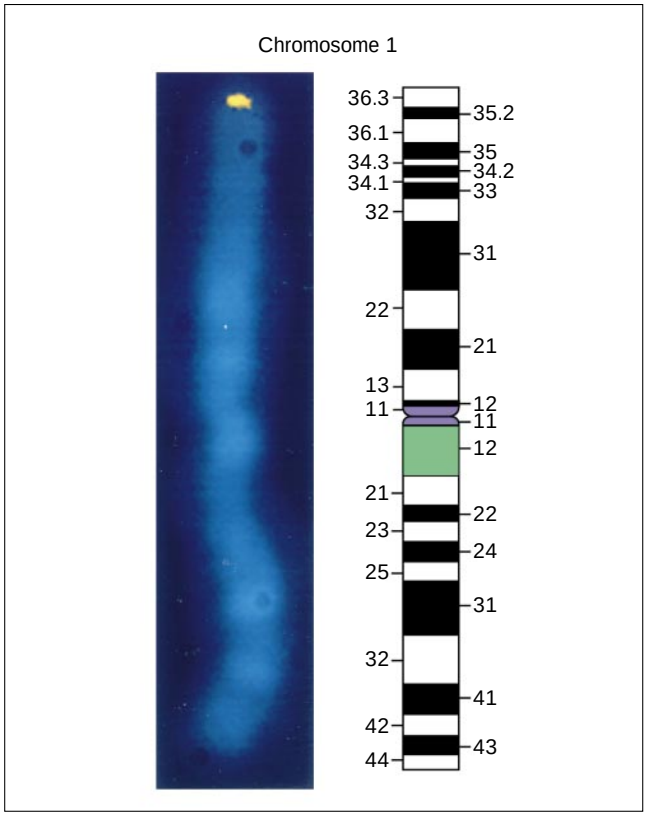
(a) Nucleotide and predicted amino-acid sequence of human Apo-3. The putative signal peptide and transmembrane domain are shown in blue, the death-domain sequence is shown in green, and the potential N-linked glycosylation sites are marked by asterisks. Also indicated is the alanine residue (red) which was present in the fetal lung but not the fetal heart cDNA clone. (b) Comparison of the ECD sequences of human Apo-3, TNFR1 and CD95. Amino acids conserved in at least two sequences are boxed in blue. Cysteines that form the framework of each cysteine-rich domain in the crystal structure of TNFR1 [37] are boxed in red. (c) Comparison of the death-domain sequences of human Apo-3, TNFR1, CD95, FADD/MORT1, TRADD, RIP and *Drosophila* Reaper. Amino acids conserved in at least three of the sequences are boxed in blue.

weight of approximately 45 kDa and a PI of 6.4. Hydropathy analysis (data not shown) suggested the presence of a signal sequence (amino acids 1–24), followed by an extracellular region (amino acids 25–198), a transmembrane domain (amino acids 199–224) and an intracellular region (amino acids 225–417) (Fig. 1a).

The cDNA sequence suggests that there are two potential N-linked glycosylation sites at amino-acid positions 67 and 106. The ECD contains four cysteine-rich pseudorepeats which resemble the corresponding regions of human TNFR1 (four repeats) and CD95 (three repeats) (Fig. 1b), and of the other known TNFR family members (data not shown). The ICD contains a sequence which resembles the death domains found in the ICDs of TNFR1 and CD95 and in other death signaling proteins, such as human FADD/MORT1, TRADD, RIP and *Drosophila* Reaper (Fig. 1c). Notably, four out of the six amino-acid residues in the death domain of TNFR1 that are essential for signaling (F345, R347, L351 and W378 [4]) are identical in Apo-3 (F350, R352, L356 and W382), whereas the remaining two TNFR1 residues (E369 and I408 [4]) are semiconserved in Apo-3 (D371 and L409) (Fig. 1c). Both globally and in individual regions, Apo-3 is related more closely to TNFR1 than to CD95; the respective percent identities are 29 % and 23 % overall, 28 % and 25 % in the ECD, 32 % and 18 % in the ICD, and 48 % and 20 % in the death domain.

We isolated a related cDNA clone from fetal lung (data not shown); this clone was identical to the fetal heart clone, except that it was 172 bp shorter at the 5′ end, suggesting that it was not full-length. In addition, the fetal lung cDNA clone lacked the alanine residue at position 236, possibly due to differential mRNA splicing *via* two consecutive splice-acceptor consensus sites (Fig. 1c).

Figure 2



Chromosomal localization of the Apo-3 gene. A human genomic clone of Apo-3 was identified by screening a PAC library, and the PAC clone was mapped by FISH to normal human lymphocyte chromosomes. The chromosome 1 hybridization image, shown alongside a map of human chromosome 1, is representative of 20 metaphase spreads. Positive hybridization signals at 1p36.3 were noted in more than 95 % of the cells. Signals were seen in both chromosome 1 homologues in more than 90 % of the positive spreads.

Chromosomal localization of the Apo-3 gene

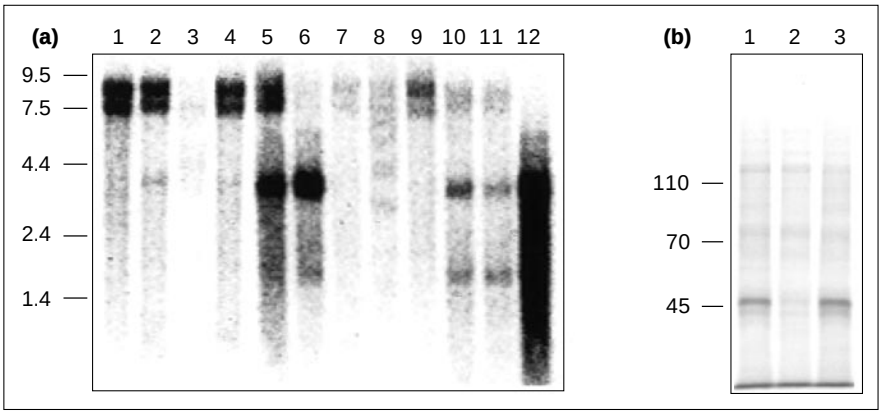
We investigated the chromosomal location of Apo-3 by fluorescence *in situ* hybridization (FISH) to normal human lymphocyte chromosomes. Direct hybridization using the Apo-3 cDNA as a probe gave a poor signal-to-background ratio (data not shown); however, the results suggested that the gene was located on chromosome 1p36. To confirm this finding, we screened a human genomic P1-derived artificial chromosome (PAC) library using the Apo-3 probe. We identified a positive PAC clone, which mapped by FISH to the short arm of chromosome 1, at position 1p36.3 (Fig. 2). A second Apo-3-positive PAC clone mapped to the same position (data not shown).

Expression of Apo-3

We investigated Apo-3 mRNA expression in human tissues by northern-blot hybridization to a 206 bp DNA probe based on the 3′ untranslated region of the Apo-3 cDNA (Fig. 3a). A predominant mRNA transcript of approximately 4 kb was detected in adult spleen, thymus and peripheral blood lymphocytes; the Apo-3 mRNA was less abundant in small intestine, colon, fetal lung and fetal kidney. Additional transcripts of approximately 7 kb and 9 kb were seen mainly in fetal brain, lung and kidney, and in adult spleen and ovary.

We expressed the Apo-3 protein by transiently transfecting HEK293 cells with pRK5–Apo-3 (a pRK5-based plasmid expressing Apo-3), and immunoprecipitated lysates of metabolically labeled cells with a mouse antiserum raised against an Apo-3–IgG fusion protein (see Materials and methods). We observed a predominant radioactive band with a relative molecular mass of approximately 47 kDa in cells transfected with pRK5–Apo-3, but not in control cells transfected with pRK5 (Fig. 3b, lanes 1,2); this band was not present in control immunoprecipitates in which preimmune mouse serum was used (data not shown). Given the potential glycosylation sites of Apo-3, the observed size

Figure 3



(a) Expression of Apo-3 mRNA in human tissues. Apo-3 mRNA expression was analyzed by northern-blot hybridization to a 206 bp DNA probe based on the 3′ untranslated region of the Apo-3 cDNA. Lane 1, fetal brain; lane 2, lung; lane 3, liver; lane 4, kidney; lane 5, adult spleen; lane 6, thymus; lane 7, prostate; lane 8, testis; lane 9, ovary; lane 10, small intestine; lane 11, colon; lane 12, peripheral blood lymphocytes. The sizes of molecular weight standards (in kb) are shown on the left. (b) Ectopic expression of Apo-3 in HEK293 cells. Cells were transfected with 10 μg pRK5–Apo-3 plus 10 μg pRK5 (lane 1), 20 μg pRK5 (lane 2), or 10 μg pRK5–Apo-3 plus 10 μg pRK5–CrmA (lane 3). After 24 h, cells were metabolically labelled and extracts were immunoprecipitated using a mouse antiserum raised against an Apo-3–IgG fusion protein; samples were subjected to SDS–PAGE and phosphorimager analysis. The sizes of molecular weight markers (in kDa) are shown on the left.

is consistent with the size of approximately 45 kDa predicted for the Apo-3 polypeptide precursor.

Ectopic expression of Apo-3 triggers apoptosis

Microscopic examination of Apo-3-transfected HEK293 cells 36 hours after transfection revealed that, compared with pRK5-transfected controls, there was a substantial loss of cell viability; membrane blebbing and loss of cell volume were seen in many Apo-3-transfected cells (Fig. 4a,b), suggesting cell death by apoptosis [19]. To assess this possibility directly, we analyzed the integrity of cellular DNA. Apo-3-transfected cells showed a marked increase in DNA fragmentation as compared with controls (Fig. 4j, lanes 1,2); the fragmented DNA migrated on agarose gels as a series of bands with incrementally increasing size, indicating internucleosomal DNA cleavage, a hallmark of programmed cell death [19]. As an additional assay for apoptosis, we measured a combination of two parameters by fluorescence-activated cell sorting (FACS) analysis — staining by the DNA-binding dye propidium iodide (PI), which indicates loss of plasma membrane integrity, and binding of Annexin-V, which interacts with phosphatidylserine on the surface of apoptotic cells [20,21]. Double-negative cells are viable; Annexin-V-positive/PI-negative cells are in the early stages of apoptosis and still maintain plasma membrane integrity; double-positive cells are in the later stages of apoptosis, or are post-apoptotic and have lost plasma membrane integrity; Annexin V-negative/PI-positive cells are necrotic [20,21]. The transient transfection efficiency of HEK293 cells was 60–70 % (data not shown); in order to limit our analysis to those cells that had taken up plasmid DNA, we co-transfected the cells with a pRK5–CD4 expression vector; these cells were then gated by staining for the CD4 marker. Transfection with pRK5–Apo-3 resulted in a marked increase in the level of double-positive cells, as compared with pRK5-transfected controls (Fig. 4e,f). We also used FACS analysis to determine the effect of plasmid dose on apoptosis. Transfection with plasmids encoding either Apo-3 or TNFR1 was associated with a dose-dependent increase in apoptosis; notably, the effect of Apo-3 was more pronounced than that of TNFR1 (Fig. 4i). Taken together, these results indicate that ectopic expression of Apo-3 in HEK293 cells triggers apoptosis. Similar results were seen with HeLa cells transfected with Apo-3 (data not shown), suggesting that the apoptotic response to Apo-3 transfection is not unique to the HEK293 cell line.

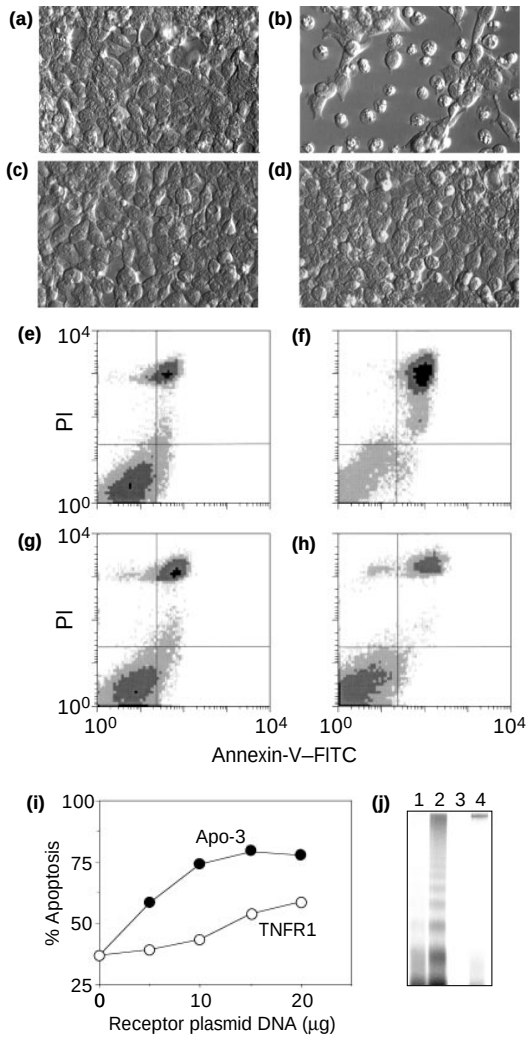
CrmA inhibits induction of apoptosis by Apo-3

The apoptotic activity of the proteases ICE and Yama/CPP32 can be inhibited effectively by the product of the cowpox virus gene *crmA* [22,23]. CrmA inhibits TNFR1- and CD95-induced cell death [24,25]. Co-transfection of HEK293 cells with pRK5–CrmA and pRK5–Apo-3 did not affect the expression levels of Apo-3 (Fig. 3b, lane 3); however, CrmA blocked Apo-3-associated apoptosis as assessed morphologically (Fig. 4c,d), by FACS (Fig. 4g,h) and by DNA fragmentation (Fig. 4j lanes 3,4). CrmA also inhibited apoptosis associated with Apo-3-transfection in HeLa cells (data not shown). These results suggest that the activity of proteases such as ICE and Yama/CPP32 is required for Apo-3-induced apoptosis.

Apo-3 activates NF-κB

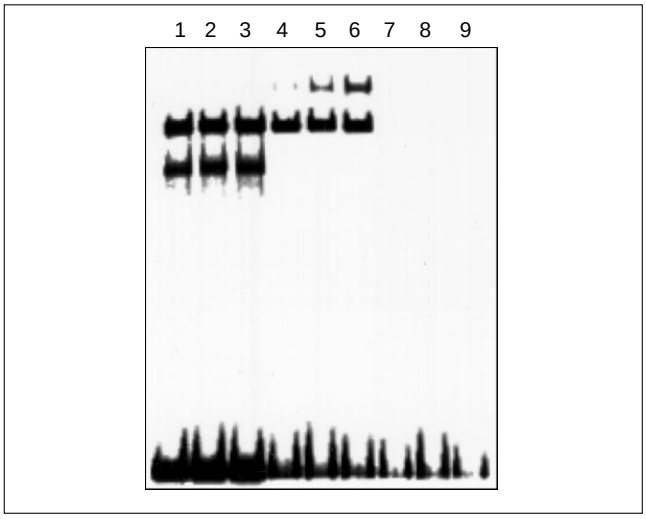
Given the similarity between the death-domain sequences of Apo-3 and TNFR1, we investigated whether Apo-3 activates NF-κB. Cells transfected with pRK5–-Apo-3 showed a significant increase in NF-κB-specific DNA-binding activity relative to pRK5-transfected controls (Fig. 5); NF-κB was also activated in cells transfected with pRK5–TNFR1. The levels of NF-κB activity appeared higher in TNFR1-transfected cells than in

Figure 4



Induction of apoptosis by ectopic expression of Apo-3 in HEK293 cells. Apoptosis was assessed 36 h after transfection by morphological analysis (**a–d**), by FACS (**e–i**), and by analysis of DNA fragmentation (**j**). For morphological analysis, cells were transfected with 10 μg pRK5 (a), 5 μg pRK5 plus 5 μg pRK5–Apo-3 (b), 5 μg pRK5 plus 5 μg pRK5–CrmA (c), or 5 μg pRK5–Apo-3 plus 5 μg pRK5–CrmA (d), and photographed at 400× magnification by Hoffmann-based light microscopy. For FACS analysis, cells were transfected with 3 μg pRK–CD4 as a marker for plasmid uptake, together with 10 μg pRK5 (e), 5 μg pRK5 plus 5 μg pRK5–Apo-3 (f), 5 μg pRK5 plus 5 μg pRK5–CrmA (g), or 5 μg pRK5–Apo-3 plus 5 μg pRK5–CrmA (h). The cells were gated by staining with a phycoerythrin-conjugated anti-CD4 antibody before FACS analysis of staining by Annexin-V–FITC and PI. The percent apoptosis in (e–h), respectively, was 37 %, 66 %, 36 % and 26 %. In (i), cells were transfected with the indicated amount of pRK5–Apo-3 or pRK5–TNFR1, together with pRK–CD4 and the appropriate amount of pRK5 to adjust the total amount of plasmid to 20 μg, and analyzed by FACS as above. For the DNA fragmentation assay (j), cells were transfected with 10 μg pRK5 (lane 1), 5 μg pRK5 plus 5 μg pRK5-Apo-3 (lane 2), 5 μg pRK5 plus 5 μg pRK5–CrmA (lane 3), or 5 μg pRK5–Apo-3 plus 5 μg pRK5–CrmA (lane 4). The DNA was extracted, radiolabeled by ^{32}P in a terminal transferase reaction, and subjected to agarose gel electrophoresis and phosphorimager analysis.

Figure 5



Activation of NF-κB by ectopic expression of Apo-3 in HEK293 cells. Cells were transfected with 10 μg pRK5 (lanes 1,4,7), 10 μg pRK5–Apo-3 (lanes 2,5,8), or 10 μg pRK5–TNFR1 (lanes 3,6,9). Nuclear extracts were prepared 36 h later and reacted with a non-specific ^{32}P-labeled oligonucleotide probe (lanes 1–3), or with a ^{32}P-labeled, NF-κB-specific probe, alone (lanes 4–6), or together with a 50-fold excess of unlabelled oligonucleotide of the same sequence (lanes 7–9). The reactions were subjected to electrophoretic mobility-shift assay and the gel was analyzed by phosphorimager analysis. The band at the bottom of the gel in all lanes is the free labeled probe. The two other bands seen in lanes 1–3 represent non-specific interactions, as does the band common to lanes 1–3 and 4–6. The top band in lanes 4–6 represents the labelled NF-κB-specific probe, whose migration is delayed by specific interaction with activated NF-κB protein in the nuclear extracts.

Apo-3-transfected cells (Fig. 5, compare lanes 3 and 4). These results suggest that Apo-3 is linked to an NF-κB activation pathway.

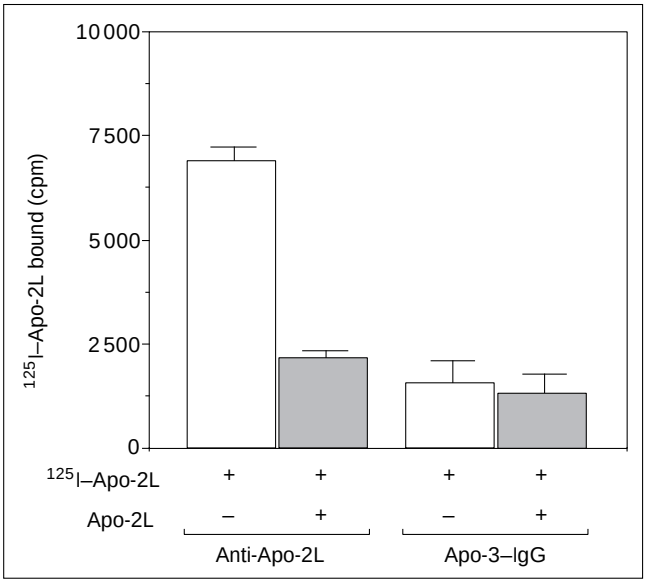
Apo-2 ligand does not bind to Apo-3

Recent studies have identified a new member of the TNF cytokine family — called Apo-2L (for Apo-2 ligand [26,27]), or TRAIL (for TNF-related apoptosis-inducing ligand [28]) — which resembles CD95 ligand and TNF in its apoptosis-inducing activity. Because the receptor for Apo-2L is unknown, we investigated whether Apo-2L interacts with Apo-3 by testing the ability of ^{125}I-labeled Apo-2L to bind specifically to an Apo-3–IgG fusion protein (Fig. 6). The ^{125}I-labeled Apo-2L did not bind specifically to Apo-3–IgG, but bound specifically to a monoclonal anti-Apo-2L antibody. The ^{125}I-labeled Apo-2L also failed to bind selectively to the surface of HEK293 cells transfected with pRK5–Apo-3 or with pRK5–Apo-3 plus pRK5–CrmA (data not shown). These results suggest that Apo-3 is not a receptor for Apo-2L.

Discussion

We have identified Apo-3 as an additional member of the TNFR family. Apo-3 has four cysteine-rich domains in its

Figure 6



Lack of specific interaction between Apo-3–IgG and Apo-2 ligand. The binding of 1 nM ^{125}I-labeled Apo-2L (^{125}I–Apo-2L) to a monoclonal anti-Apo-2L antibody or to an Apo-3–IgG fusion protein was analyzed in the absence (white bars) or presence (grey bars) of 100 nM unlabeled Apo-2L. The data are means ± standard errors (n = 4).

extracellular region. In addition, Apo-3 has a cytoplasmic death domain that is related to the death domains of TNFR1 and CD95, as well as to the death domains of intracellular regulators of apoptosis such as FADD/MORT1, TRADD, RIP and Reaper. Apo-3 mRNA is expressed both in lymphoid and in non-lymphoid human tissues, suggesting that it may regulate signaling functions in multiple types of cells. Apo-3 does not appear to interact with Apo-2L, suggesting the existence of a separate Apo-3 ligand and a distinct Apo-2 receptor.

The Apo-3 gene mapped to position 1p36.3 on human chromosome 1. Recent work shows that a genomic region which is deleted in certain human neuroblastomas maps between 1p36.2–1p36.3, suggesting the presence of a tumor suppressor gene at this locus [29]. Four additional TNFR gene family members — TNFR2, CD30, 4-1BB and OX40 — reside in 1p36 [2], but are outside the deleted region [29].

Ectopic expression of Apo-3 resulted in apoptosis, as judged by morphological and biochemical criteria. Hence, one biological role of Apo-3 may be to trigger a cell-death program upon binding of its cognate ligand. CrmA inhibited Apo-3-induced apoptosis, suggesting that proteases such as ICE and/or Yama/CPP32 may be involved in executing the cell-death signal emanating from Apo-3. Thus, Apo-3, TNFR1 and CD95 appear to activate a common apoptotic cell-death machinery. Transfection with a plasmid encoding Apo-3 also resulted in the activation of NF-κB, indicating that, like TNFR1, Apo-3 is capable of regulating not only cell death, but also transcription of inflammatory response genes. Therefore, the eventual outcome of Apo-3 activation may be determined by the cellular context in which it is triggered. This possibility is consistent with the expression of Apo-3 mRNA in a variety of tissues.

Our conclusion that Apo-3 regulates apoptosis and NF-κB is based on the premise that overexpression of this receptor mimics ligand-dependent activation. This notion is supported by the previous observation that ectopic expression of TNFR1 leads to spontaneous self-association of the receptor and to activation of apoptosis and NF-κB-dependent gene expression [30]. In addition, ligand-independent dimerization of CD95, induced intracellularly by a chemical dimerizing agent, is sufficient to trigger apoptosis [31]. Self-association appears also to be an activation mechanism of other death-domain-containing proteins, such as TRADD, FADD, RIP and Reaper, whose overexpression leads to activation of apoptosis [8–11,32].

Conclusions

Apo-3 is a novel member of the TNFR family. The Apo-3 gene is encoded on human chromosome 1, and its mRNA is expressed in multiple tissues. Like TNFR1 and CD95, Apo-3 contains a cytoplasmic death domain, and appears to trigger a similar apoptotic cell-death pathway which is inhibited by CrmA. In addition, Apo-3 appears to be capable of activating the transcription factor NF-κB, suggesting that, like TNFR1, this receptor may regulate distinct signaling pathways in different cellular contexts. Future studies should aim at isolating a specific ligand for Apo-3 to help elucidate the physiological role of this receptor.

Materials and methods

Isolation of Apo-3 cDNAs

Human fetal heart and human fetal lung λgt10 bacteriophage cDNA libraries (Clontech) were screened by hybridization with synthetic oligonucleotide probes based on a set of ESTs represented by GenBank site W71984, which showed some degree of homology to the ICDs of human TNFR1 and CD95. The oligonucleotide probes were: 5′–GGCGCTCTGGTGGCCCTTGCAGAAGCC; and 5′–TTCGGCCGAGAAGTTGAGAAATGTC. Another EST set (for example, GenBank site H41522), which showed some degree of homology to the ECDs of human TNFR1 and CD95, corresponds to the ECD of Apo-3. One positive clone each from the fetal heart (FH20A.57) and the fetal lung (FL8A.53) libraries was isolated and sequenced in its entirety.

Accession number

The GenBank accession number for the Apo-3 cDNA is U74611.

Northern-blot analysis

Expression of Apo-3 mRNA in human tissues was examined by northern-blot analysis. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were hybridized to a 206 bp ^{32}P-labeled

DNA probe based on the 3′ untranslated region of Apo-3. The probe was generated by PCR using the oligonucleotides that were used to screen the fetal heart and lung libraries.

FISH mapping

Mapping was performed by FISH [33] to normal human lymphocyte chromosomes counterstained with PI and DAPI. Biotinylated Apo-3 probes were detected with avidin–FITC. The Apo-3 cDNA probe was also used to identify clones in a genomic PAC library that contained the Apo-3 gene, and the PACs were used as confirmatory probes in FISH. The regional assignment of the genomic probe was determined by analyzing 20 metaphase spreads.

Expression of Apo-3

pRK5-based mammalian expression plasmids carrying clone FH20A.57 (pRK5–Apo-3), TNFR1 (pRK5–TNFR1), or CD4 (pRK5–CD4) were constructed and transiently transfected into HEK293 cells by calcium phosphate precipitation; HeLa cells were transfected by electroporation. The total amount of plasmid DNA was kept constant for each set of transfections.

Analysis of apoptosis

Cells were analyzed for apoptosis 36 h after transfection. Apoptosis was assessed morphologically, or quantitated by FACS analysis of cells stained with FITC-conjugated Annexin V (Brand Applications), which binds to exposed phosphatidylserine on the surface of apoptotic cells, and by staining with PI, which penetrates cells that have lost membrane integrity [20,21]. DNA fragmentation was analyzed by extracting DNA from cells, and ^{32}P-labelling the 3′ ends with terminal transferase using an Apoptotic DNA Laddering kit (Trevigen); samples were separated by 1.5 % agarose gel electrophoresis, as described [26].

Radioimmunoprecipitation

Transfected cells (5×10^5 per lane) were metabolically labeled by adding 50 μCi ^{35}S-labelled methionine and ^{35}S-labelled cysteine to the growth media 24 h after transfection. After a 6 h incubation, the cells were washed several times, lyzed, and subjected to immunoprecipitation using anti-Apo-3 antiserum as described [34]. The anti-Apo-3 antiserum was raised in mice against a fusion protein comprised of the Apo-3 ECD linked to the human IgG1 hinge and Fc, which was constructed and purified essentially as described [35]. Pre-immune serum was derived from the same mice before immunization.

NF-κB activation

Cells were harvested 36 h after transfection, nuclear extracts were prepared, and 1 μg of nuclear protein was incubated with a duplex, ^{32}P-labeled NF-κB-specific synthetic oligonucleotide probe (top strand sequence: 5′–ATCAGGGACTTTCCGCTGGGGACTTTCCG), alone or together with a 50-fold excess of unlabelled probe, or with a non-specific duplex ^{32}P-labelled synthetic oligonucleotide (top strand sequence: 5′–AGGATGGGAAGTGTGTGATATATCCTTGAT). DNA binding was analyzed by electrophoretic mobility-shift assay, as described [12,34,36].

Apo-2L binding assay

Soluble human Apo-2L [26] was radio-iodinated using Enzymobeads (BioRad) to a specific activity of 67 μCi μg^{-1}. The biological activity of the iodinated protein was equivalent to that of non-iodinated Apo-2L, as measured by apoptosis induction in the human 9D cell line [26]. Microtiter wells were coated with goat anti-mouse Fc or anti-human Fc antibody and blocked with bovine serum albumin. A neutralizing monoclonal mouse anti-Apo-2L antibody, or Apo-3–IgG fusion protein (5 μg ml^{-1}) were bound to the anti-mouse or anti-human Fc antibodies, respectively; incubations were carried out using ^{125}I-labelled Apo-2L (1 nM) for 2 h at 24 °C. Non-specific binding was determined in the presence of 100 nM unlabeled Apo-2L.

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