Contrasting Effects of IG20 and Its Splice Isoforms, MADD and DENN-SV, on Tumor Necrosis Factor α-induced Apoptosis and Activation of Caspase-8 and -3*

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We identified a novel cDNA (IG20) that is homologous to cDNAs encoding a protein differentially expressed in normal and neoplastic cells (DENN-SV) and human MADD (MAPK-activating death domain-containing protein). Furthermore, we show that the above variants most likely result from alternative splicing of a single gene. Functional analyses of these variants in permanently transfected HEK 293 cells revealed that IG20 and DENN-SV render them more susceptible or resistant to tumor necrosis factor α (TNF-α)-induced apoptosis, respectively. All variants tested could interact with TNF receptor 1 and activate ERK and nuclear factor κB. However, relative to control cells, only cells expressing IG20 showed enhanced TNF-α-induced activation of caspase-8 and -3, whereas cells expressing DENN-SV showed either reduced or no caspase activation. Transfection of these cells with a cDNA encoding CrmA maximally inhibited apoptosis in HEK-IG20 cells. Our results show that IG20 can promote TNF-α-induced apoptosis and activation of caspase-8 and -3 and suggest that it may play a novel role in the regulation of the pleiotropic effects of TNF-α through alternative splicing.

Apoptosis is utilized by multicellular organisms for regulating development, growth, and homeostasis and is controlled by a group of signaling molecules (1, 2). One such molecule is tumor necrosis factor α (TNF-α), which plays a role in both apoptosis and cell proliferation (3). TNF-α acts on target cells by interacting with either TNF receptor 1 (TNFR1) or 2. Upon TNF-α binding, these transmembrane proteins mediate intracellular signal transduction by a diverse array of cytoplasmic adaptor proteins. Some of these adaptor proteins contain a death domain through which they interact with the cytoplasmic death domain of TNFR1 (4–6).

Previously, we and others identified some cDNAs that are differentially expressed in insulinomas (7, 8). One of the clones, IG20 (insulinoma-glucagonoma clone 20), hybridized strongly with a 5-kilobase transcript from insulinoma and weakly with that from brain. Northern blot analyses showed that IG20 is undetectable in normal tissues, but is expressed at higher levels in human tumors and transformed cell lines, with substantially higher levels in neuroendocrine tumors (8). The cDNA sequence of IG20 shows a high homology to that of MADD (MAPK-activating death domain-containing protein) (8–10). MADD associates with TNFR1 through a death domain-death domain interaction and activates MAPK. Overexpression of MADD or its death domain activates both ERK and Jun and leads to phosphorylation of cytoplasmic phospholipase A2 (9) and induction of TNF-α expression (11). Additionally, MADD can mediate TNF-α-induced proliferation of Kaposi’s sarcoma cells, which can be inhibited by blocking MADD transcription (12).

Another cDNA, DENN (differentially expressed in normal and neoplastic cells), was independently cloned and is identical to MADD (10). MADD/DENN undergoes alternative splicing, creating a shorter form, designated DENN-SV (where SV is splice variant), the function of which is unknown. Other cDNAs with considerable sequence homology to IG20 are human KIAA0358 with unknown function (13) and rat GTP/GDP exchange protein (14). The ace-3 gene of Caenorhabditis elegans shows very modest homology to IG20 (15).

In this report, we demonstrate that KIAA0358, MADD/DENN, and DENN-SV are different splice variants of the same gene, which we have designated as IG20. HEK cells stably transfected with IG20 showed enhanced susceptibility to TNF-α-induced apoptosis, whereas cells transfected with DENN-SV showed resistance. Upon treatment with TNF-α, cells transfected with different IG20 isoforms showed enhanced ERK and NF-κB activation. However, only cells expressing exogenous IG20 showed increased activation of caspases upon treatment with TNF-α, whereas DENN-SV-transfected cells showed little or no caspase activation. Consistent with a previous report (9), MADD/DENN did not alter the susceptibility to TNF-α-induced apoptosis. Transfection of cells with cDNA encoding CrmA rendered HeLa-IG20 cells more resistant to TNF-α-induced apoptosis. Together, our results show that splice variants of IG20 differentially affect TNF-α-induced, caspase-mediated apoptosis.
**EXPERIMENTAL PROCEDURES**

**RT-PCR Using RNA from Human Cell Lines**—The U937 cell line was obtained from American Type Culture Collection. HeLa cells were a gift from Dr. David Ucker. All other cell lines, including HU446, were a gift from Dr. William Beck (University of Illinois, Chicago, IL). Tissue samples were provided by the Cooperative Human Tissue Network. Total RNAs were extracted from various cell lines and tissues using the RNeasy kit (QIAGEN Inc., Chatsworth, CA). 0.1 μg of RNA from each cell line or tissue sample was used in the SuperScript-One-Step RT-PCR system (Life Technologies, Inc.) according to the manufacturer’s protocol. A first incubation at 50 °C for 30 min was followed by another incubation at 94 °C for 2 min. Subsequent 30 cycles of PCR were carried out at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by one incubation at 72 °C for 7 min. Two primer sets were separately used at 10 μM each to amplify the desired fragment of IG20: the first was F-1 and B-1 primer pair (5'-CTT GCT CAG GAC-3' and 5'-ACT GAT CTC CAC ATC CG-3'), respectively, and the second was the F-2 and B-2 primer pair (5'-GGG ACT CTG ACT CCG AAC CTA CTG-3' and 5'-CCG ACT GTG CTC CAG CGG CGG CTG-3'), respectively. These primers flanked the region of IG20 that contains the deletions and unique restriction sites (Fig. 1A). Sequences of RT-PCR products—10 μl of cDNA from each of the RT-PCR products were done on a 5% polyacrylamide gel until the desired fragments were clearly separated. Four bands representing different RT-PCR products were run on a 5% polyacrylamide gel until the desired fragments were clearly separated. Four bands representing different RT-PCR products were run on a 5% polyacrylamide gel until the desired fragments were clearly separated. Four bands representing different RT-PCR products were run on a 5% polyacrylamide gel until the desired fragments were clearly separated. Four bands representing different RT-PCR products were run on a 5% polyacrylamide gel until the desired fragments were clearly separated.

**Cloning of Desired cDNAs into Mammalian Expression Vectors**—IG20 was cloned into the multiple cloning site of pBKRSV (Stratagene, La Jolla, CA) and used to transform E. coli XL-1 cells. pBKRSV containing IG20 in the correct orientation was identified. The plasmid was digested with AatII, which produced two fragments of ~8.3 and 1 kilobases. The 1-kilobase fragment was further digested with AfeI to allow cloning of different cDNA fragments obtained by PCR. cDNAs corresponding to fragments of IG20, MADD/DENN, IG20-SV2, and DENN-SV were released from pGEM-Teasy vector (Promega, Madison, WI) and used to transform E. coli DH5α cells. Clones containing the desired fragments were identified by restriction analysis with NotI and sequenced using the F-1 and B-1 primers described above.

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**Comparison of IG20 cDNA and Genomic Sequences**—IG20 cDNA sequence was used in the FASTA program of the NCBI Protein Database to search for homologous sequences in the human genome. Different portions of IG20 cDNA were aligned with homologous sequences on multiple clones of the human genome. This was carried out using the ClustalW multiple sequence alignment program. The existence of exons and the intron-exon splice junctions were determined by the presence of the invariant GU and AG at the 5'- and 3'-splice sites of the introns, respectively, and the adenosine residue in the intron that is necessary for the formation of the lariat structure.

**Transfection of HeLa Cells with cDNAs Encoding Different Isoforms of IG20**—IG20, MADD/DENN, or DENN-SV, cloned into pBKRSV, pcDNA3.1-His, or empty vectors, was used to transfect HeLa cells. Cells were plated in 6-well plates at 1.5 × 105 cells/well in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 2 mM (29.2 mg/ml)-l-glutamine. 24 h later, cells were separately transfected with each of the above constructs using Super-Fect transfection reagent (QIAGEN Inc.) according to the manufacturer’s protocol. To select for permanently transfected cells, growth medium was replaced at 48 h post-transfection and the medium was replaced every 3 days with fresh medium containing 400 μg/ml G418. Expression of transfected cDNAs was confirmed by immunoblotting as described below.

**Antibodies**—Anti-IG20 peptide polyclonal antibodies were raised in rabbits by immunization with keyhole limpet hemocyanin-coupled synthetic peptides. The antibodies were used to select from three different regions on IG20 and had the following sequences: peptide N, PESTEELKHLKQALASM (amino acids 419–435); peptide M, SSEEDLRTPPRPVSS (amino acids 1594–1608). Antibody titers and specificity for IG20 were determined by enzyme-linked immunosorbent assay.

**Immunoprecipitation and Interaction with TNFR1**—Permanently transfected cells were plated in 100-mm dishes at 1.2 × 107 cells/dish and grown overnight. The cells were then washed with PBS, grown in methionine-free medium for 1 h, washed again with PBS, incubated for 1 h in medium containing 100 μCi/ml [35S]methionine, and harvested by trypsinization. Further processing of cells and cell lysates was carried out at 4 °C. Cells were washed once with ice-cold PBS and incubated in lysis buffer (20 mM Tris-Cl [pH 7.5], 1% Triton X-100, 157 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM Na3VO4, 2 mM sodium pyrophosphate, 10% (v/v) glycerol, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride) for 1 h. Lysates were spun at 13,000 rpm for 30 min; supernatants were transferred to other tubes; and protein concentration was determined by incorporation of radioactivity (i.e. [35S]methionine) and spectrophotometry. Equal amounts of total protein from each sample were used in further analyses. Samples were precleared by incubation with 5 μl of normal rabbit sera/sample for 30 min.
min, followed by the addition of 25 μl of protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and further incubation for 30 min. Samples were centrifuged at 13,000 rpm for 30 s, and 4.1 × 10^6 cpm/sample was used with 5 μl of a mixture of anti-IG20 peptide N, M, and C antibodies, incubated overnight, and subjected to immunoprecipitation.

Co-immunoprecipitation of IG20 and its splice isoforms with TNFR1 was performed according to previously described protocols (9). Briefly, cells were plated and grown as described above and treated with 10 ng/ml TNF-α and 10 μg/ml cycloheximide (Sigma) for 15 min. Cells were then lysed, used to obtain equal amounts of total proteins, and pre-cleared as described above. IG20 protein isoforms were co-immunoprecipitated using 5 μl of anti-TNFR1 polyclonal antibodies/sample (CLONTECH, Palo Alto, CA). To detect specific IG20 isoforms, the above immunoprecipitated samples were separated and then transferred to nitrocellulose membrane and detected using anti-His monoclonal antibodies (CLONTECH).

**FIG. 2. Expression of IG20 and its splice isoforms in HeLa cells.** IG20 isoforms are seen at ~215 kDa, as expected. A, expression of the transfected IG20 isoforms in HeLa cells. The data do not discriminate between different IG20 isoforms or between endogenous (weak band under HeLa) or exogenous IG20. B, expression of His-tagged IG20 cDNA constructs in HeLa cells. The tagged proteins were seen at the expected size (~215 kDa), indicating that the proteins were encoded by the transfected cDNAs. Abs, antibodies.
FIG. 3. **TNF-α-induced apoptosis in transfected HeLa cells.** A and B, TNF-α-induced mitochondrial depolarization of cells. A, FACS histograms of a representative experiment showing mitochondrial depolarization due to TNF-α treatment. Shown are percentages of cells with depolarized mitochondria (TMRE negative) over total cell count within the same sample. B, percentages of cells with depolarized mitochondria due to TNF-α treatment. These values were obtained by subtracting percentage of cells with depolarized mitochondria without TNF-α treatment from percentage of cells with depolarized mitochondria after TNF-α treatment. Values shown represent averages of three independent experiments. Error bars indicate means ± S.D. of three samples. C, trypan blue staining of TNF-α-treated cells. Each bar represents the average of trypan blue-stained cells due to TNF-α-cycloheximide treatment only. This was calculated by subtracting percent trypan blue-positive cells transfected with a given construct from percent trypan blue-positive cells transfected with the same construct but treated with TNF-α. Error bars indicate mean ± S.D. of three samples. At least 300 cells were counted from each sample. D, chromatin condensation due to TNF-α treatment only. Values were calculated by subtracting percentage of cells with condensed chromatin without TNF-α treatment from percentage of cells with TNF-α treatment. Four groups of fields, each containing at least 200 cells, were counted for each sample. Error bars represent mean ± S.D. E, chromatin staining of HeLa cells transfected with different IG20 isoforms untreated or treated with TNF-α. Cells with bright Hoechst staining represent apoptotic cells with condensed chromatin.
Contrasting Effects of IG20 Splice Isoforms on Apoptosis

RT-PCR Reveals Putative Splice Variants of IG20—To determine whether different isoforms can be identified, initially we carried out RT-PCR using RNA from U937 and H446 cell lines and F1-B1 and F2-B2 primer pairs. Separation of PCR products on a polyacrylamide gel revealed the existence of up to four distinct PCR products. These cDNA fragments ranged in size from 935 to 1124 base pairs (Fig. 1A) and were designated IG20, IG20-SV1, IG20-SV2, and IG20-SV3, from the largest to the smallest. Similarly, up to four bands, but in different combinations, were obtained when we used RNA as template from U937 and H446 cell lines (Fig. 1B). Sequencing of IG20 cDNAs revealed deletions in the putative splice sites.

RESULTS

RT-PCR Reveals Putative Splice Variants of IG20—To determine whether different isoforms can be identified, initially we carried out RT-PCR using RNA from U937 and H446 cell lines and F1-B1 and F2-B2 primer pairs. Separation of PCR products on a polyacrylamide gel revealed the existence of up to four distinct PCR products. These cDNA fragments ranged in size from 935 to 1124 base pairs (Fig. 1A) and were designated IG20, IG20-SV1, IG20-SV2, and IG20-SV3, from the largest to the smallest. Similarly, up to four bands, but in different combinations, were obtained when we used RNA as template from 10 other human cell lines and 18 normal and neoplastic human tissues (data not shown). These results indicate that expression of multiple splice variants of IG20 is a common physiological phenomenon.

Sequencing of IG20 cDNAs Reveals Deletions in the Putative Splice Variants—The cDNAs from each of the four bands in
were cloned and sequenced. Sequence analyses revealed that IG20-SV1 and IG20-SV3 are identical to corresponding regions on MADD/DENN and DENN-SV cDNAs, respectively. Fig. 1B shows that differences between splice isoforms of IG20 are due to alternative splicing of exons 13 and 16. IG20 is the longest, and its sequence includes an additional 60 base pairs (positions 2819–2878) that are not present in MADD/DENN (IG20-SV1) and DENN-SV (IG20-SV3) sequences (9, 10). IG20-SV2 sequence is unique and is 129 and 69 base pairs shorter than IG20 and MADD/DENN, respectively. DENN-SV (IG20-SV3) is 189 and 129 base pairs shorter than IG20 and MADD/DENN, respectively; and its sequence is identical to that reported earlier (10). cDNAs from several cell lines and tissues were used to confirm that bands of the same size have identical sequences.

The data show that IG20 cDNA were on chromosome 11p11. The ATG start codon that is used for all IG20 splice isoforms is located in exon 2 at position 164 of IG20 cDNA. Exon 1 (shown in Table I) contains a 5′-untranslated region that is specific for IG20. Generation of IG20 splice variants results from alternative splicing of exons 13L, 16, 21, 26, and 34 since sequence differences between various isoforms are limited to splicing of these exons. Exon 13 (referred to as exon 13L) may be internally spliced, deleting 129 nucleotides (positions 2448–2576) and generating a shorter form (exon 13S). Splicing of exons 21 and 26 generates IG20. Additional splicing of exons 16, 13L, and 13L and 16 generates MADD/DENN (IG20-SV1), IG20-SV2, and DENN-SV (IG20-SV3), respectively. Splicing of exon 34 generates KIAA0358. With the exception of the above deletions, all five isoforms have identical sequences.

Table I shows all 36 exons of IG20, ranging in size from 47 to 991 nucleotides. To confirm that isoforms of IG20 can be generated by RNA splicing, we searched for intron-exon splice junctions and found that our results (Table I) are consistent with mRNA splicing rules (16). These rules include the invariant GU and AG at the 5′- and 3′-splice junctions of the intron, respectively, and the adenosine residue necessary for the formation of the lariat required for mRNA splicing.
Splicing of the IG20 Gene Generates Functional IG20, MADD/DENN, and DENN-SV Protein Isoforms—

Full-length cDNAs representing various splice isoforms were constructed and cloned into mammalian expression vectors and used to transfect HeLa cells. To demonstrate that HeLa cells stably transfected with IG20, MADD/DENN, and DENN-SV do indeed express the corresponding proteins, equal amounts of total cell lysates from methionine-labeled cells were immunoprecipitated using anti-IG20 antibodies, separated by SDS-PAGE, and autoradiographed. Fig. 2A shows overexpression of IG20 at the expected size (~215 kDa) in HeLa cells transfected with different variants of IG20 compared with minimal levels of endogenous IG20 in HeLa-vector cells.

To confirm that the overexpression of IG20 proteins shown in Fig. 2A was specifically due to expression of transfected cDNA and not due to overexpression of endogenous counterparts, we...
immunoprecipitated cell lysates from HeLa cells stably transfected with His-tagged IG20 constructs with anti-IG20 antibodies and used anti-His monoclonal antibodies for detection. Fig. 2B shows the expression of corresponding variants in HeLa cells transfected with different isoforms, but not in HeLa-vector cells.

**Different Isoforms of IG20 Differentially Affect TNF-α-induced Apoptosis**—To assess the in vivo functional effects of IG20, MADD/DENN, and DENN-SV on TNF-α-induced apoptosis, we used HeLa cells stably transfected with these variants. Cells were subjected to TNF-α and cycloheximide treatment to induce programmed cell death. We quantitated apoptosis by staining cells with TMRE for mitochondrial depolarization (Fig. 3, A and B), with trypan blue for cell viability (Fig. 3C), and with Hoechst stain for chromatin condensation (Fig. 3, D and E). Consistently, the results showed that, relative to vector-transfected controls, HeLa-IG20 cells were most susceptible and HeLa-DENN-SV cells were most resistant to TNF-α-induced apoptosis. Susceptibility of HeLa-IG20 cells to TNF-α-induced apoptosis was even more profound compared with HeLa-DENN-SV cells. Collectively, our studies demonstrate that HeLa-IG20 and HeLa-DENN-SV cells can show contrasting responses to TNF-α-induced apoptosis. Results obtained with HeLa-MADD/DENN cells were comparable to those obtained with HeLa-vector cells and were consistent with an earlier report that MADD does not affect TNF-α-induced apoptosis (9).

**IG20, MADD/DENN, and DENN-SV Interact with TNFR1 and Activate ERK1/2 and NF-κB**—An earlier report had shown that MADD associates with TNFR1 through a death domain-death domain interaction and can activate ERK (9). Therefore, we investigated whether the differential effects of IG20 and DENN-SV on TNF-α-induced apoptosis are due to either their ability/ inability to interact with TNFR1 or to activate ERK. Upon treatment with TNF-α, all three variants were co-immunoprecipitated with the receptor, indicating that they can interact with TNFR1 (Fig. 4A). Similarly, ERK activation was seen in cells transfected with different IG20 splice isoforms (Fig. 4B).

We next investigated the ability of these isoforms to affect TNF-α-induced activation of NF-κB. As shown in Fig. 5, upon TNF-α treatment, IG20, MADD/DENN, and DENN-SV mediated activation of NF-κB to comparable levels. Our results show that there were no significant differences between cells transfected with different IG20 isoforms regarding the ability of NF-κB to bind its consensus DNA sequence (Fig. 5A) or levels of NF-κB-dependent protein expression (Fig. 5B). Thus, differences noted in HeLa cells transfected with the different IG20 isoforms in response to TNF-α-induced apoptosis could not be explained by differences in their ability to activate ERK or NF-κB.

**Enhanced Activation of Caspases, Particularly Caspase-8 and -3, Is Mediated by IG20, but Not by MADD/DENN or DENN-SV**—We investigated potential differences in downstream signaling in the TNFR1-mediated apoptotic pathway by measuring overall caspase activation. Fig. 6A shows differences in the activation of caspases among HeLa-vector, HeLa-IG20, HeLa-MADD/DENN, and HeLa-DENN-SV cells. These differences were consistently seen at different time points tested with continuing increases in a time-dependent manner (data not shown).

To test involvement of specific caspases in TNF-α-induced, IG20-mediated apoptosis, we measured two different key caspases. Caspase-8 is an early caspase that is activated through TNFR1. As shown in Fig. 6B, upon treatment with TNF-α, maximal induction of caspase-8 activity was seen in HeLa-IG20 cells. As expected, HeLa-vector and HeLa-MADD/DENN cells showed a moderate activation of caspase-8. However, surprisingly, HeLa-DENN-SV cells did not show any induction of caspase-8 activity upon TNF-α treatment. Consistent with the above data regarding activation of caspase-8, we saw higher and lower levels of cleaved active caspase-8 in HeLa-IG20 and HeLa-DENN-SV cells, respectively, compared with control cells (Fig. 6C).

We next measured TNF-α-induced activation of caspase-3, which has been implicated as the execution phase of apoptosis. As shown in Fig. 6D, HeLa-IG20 cells showed maximal induction of caspase-3 activity upon TNF-α treatment. HeLa-vector cells showed caspase-3 activation, but it was less than that seen in HeLa-IG20 cells. Both HeLa-MADD/DENN and HeLa-DENN-SV cells showed only minimal activation.

Similarly, we saw higher and lower levels of cleaved caspase-3 in HeLa-IG20 and HeLa-DENN-SV cells, respectively, compared with control cells (Fig. 6E). Additionally, FACS analyses using anti-active caspase-3 monoclonal antibody showed that a higher percentage of HeLa-IG20 cells and a lower percentage of HeLa-DENN-SV cells have active caspase-3 compared with control cells (Fig. 6F).

**Inhibitory Effects of CrmA on the Pro-apoptotic Activities of IG20**—To further confirm our results pertaining to IG20 mediated-inhibition of TNF-α-induced apoptosis and activation of caspases, we used CrmA, an inhibitor of caspase-8. Cells were transiently cotransfected with cDNAs encoding CrmA and GFP and then treated with TNF-α. Transfection with CrmA showed significant inhibition of TNF-α-induced apoptosis in HeLa-IG20 cells compared with minimal inhibition in all other cell types tested (Fig. 7). The CrmA experiment was repeated several times, and the results consistently showed that maximal effects of CrmA on TNF-α-induced, IG20-mediated apoptosis were specifically seen in HeLa-IG20 cells.

**DISCUSSION**

Identification, cloning, and genomic organization of IG20 clearly show that the gene is located on human chromosome 11p11 and consists of 36 exons. Our results further demonstrate that IG20, KIAA0358, MADD/DENN, IG20-SV2, and DENN-SV are different isoforms that could arise from alterna-
tive splicing of exons 13L, 16, 21, 26, and 34. Our RT-PCR results revealed that multiple IG20 isoforms are expressed in different patterns and levels in all human cell lines and tissues tested. However, the functional relevance of expression of different IG20 isoforms and their relative levels in a given cell or tissue is not clear. Further studies are necessary to fully understand their mutual regulation, if any, and their role in cell survival and/or death.

In an attempt to understand the functional differences between IG20 isoforms, we generated HeLa cell lines permanently transfected with cDNAs encoding IG20, MADD/DENN, and DENN-SV. These isoforms were selected because MADD/DENN and DENN-SV were previously characterized, and IG20 is a novel isoform that could be readily detected by RT-PCR in several tissues and cell lines using exon-specific primers. In contrast, we were unable to detect KIAA0358 in any of the human tissues tested so far; therefore, it was not included in our studies. Exon 13L was spliced from both SV2 and DENN-SV; therefore, SV2 was also not further investigated in this study.

Earlier studies had shown that MADD interacts with TNFR1 through its death domain (8). This domain was present in all study. SV; therefore, SV2 was also not further investigated in this study. Exon 13L was spliced from both SV2 and DENN-SV; therefore, SV2 was also not further investigated in this study.

To understand why cells transfected with different IG20 isoforms responded differently to TNF-α treatment, we searched for potential functional motifs in the spliced regions. An extensive search failed to reveal any apparent functional domains in the spliced regions. This would suggest that the splicing of exon 13L seen in MADD/DENN or exons 13L and 16 seen in DENN-SV most likely results in conformational changes that affect their cellular localization or interactions with other proteins involved in TNF-α-induced signaling. Our findings that IG20 has both exons 13L and 16 and is pro-apoptotic raise the possibility that these regions might directly or indirectly modulate one of the functions of IG20. However, in DENN-SV, one of the two deletions is identical to that found in MADD/DENN, and yet its effects are different in that these cells are more resistant to TNF-α-induced apoptosis and show little or no caspase activation. At this time, it is not clear whether both deleted regions of DENN-SV are important or only the exon 13L deletion (as in IG20-SV2) is sufficient. Further systematic studies, which are underway, might help us resolve the relevance of various deletions to the function of this protein.

To begin to understand the potential mechanism of action of IG20 and its splice variants in the context of TNFR1 signaling, we carried out additional studies. We tested for protein expression in transfected cells and found that splice isoforms corresponding to the transfected cDNAs were expressed. Furthermore, we showed that, like MADD/DENN, IG20 and DENN-SV contain the death domain and are capable of interacting with TNFR1. TNF-α exerts its effects via multiple pathways, leading to diverse biological effects, including activation of the cell cycle and apoptosis (3, 17–20). ERK activation is linked to various physiological functions in mammalian cells, including cell proliferation, mediation of cell survival, control of gene expression, and induction of apoptosis (reviewed in Ref. 21). An earlier study showed that MADD mediates activation of ERK by TNFR1 (9). In this study, we showed that IG20, MADD/DENN, and DENN-SV can mediate TNF-α-induced ERK activation. Similarly, within 15 min of addition, TNF-α induced significant activation of NF-κB, which reached comparable levels in all cells, although the basal levels were somewhat different in different cells. Together, these data show that all variants are produced and that, upon TNF-α treatment, they can be recruited to the TNFR1 complex, leading to activation of ERK and NF-κB. Therefore, the differences in response to TNF-α treatment by cells transfected with different IG20 isoforms are most likely not due to differences in these properties.

TNF-α-induced apoptosis involves recruitment of FADD (Fas-associated death domain-containing protein) to TNFR1 through TRADD (22). Subsequently, FADD activates caspase-8, which in turn activates effector caspases (e.g., caspase-3). Activation of caspase-3 is an irreversible step in the execution phase that commits the cell to undergo apoptosis. Therefore, we tested the effects of IG20 and its isoforms on activation of total caspases induced by TNF-α. Relative to vector controls, upon TNF-α treatment, HeLa-IG20 cells showed maximal caspase activity, whereas HeLa-DENN-SV cells showed the lowest levels of caspase activity. These findings were further confirmed and extended by showing maximal activation of caspase-8 and -3 in HeLa-IG20 cells, with little or no activation of these caspases in HeLa-DENN-SV cells. Collectively, these data show that differential activation of caspases might be responsible for the differential susceptibility of these cells to TNF-α-induced apoptosis.

To further confirm that activation of caspases is critical, we tested the ability of IG20 and its splice isoforms to induce apoptosis in the presence of a caspase inhibitor, CrmA. CrmA is a cytokine response-modifying protein of cowpox viruses that has been shown to inhibit apoptosis in cowpox virus-infected cells (23). It is a homolog of mammalian serpins (serine proteinase inhibitors) that has been shown to inhibit apoptosis induced by a number of agents (including TNF-α and FasL) by inhibiting different caspases, preferentially caspase-1 and -8 (24–27). Upon transient transfection of CrmA, maximal inhibition of apoptosis was noted in HeLa-IG20 cells relative to other cells. HeLa-IG20/CrmA cells were even more resistant to TNF-α-induced apoptosis than HeLa-DENN-SV/CrmA cells. These studies provide further confirmation that enhanced apoptosis of HeLa-IG20 cells is most likely a consequence of enhanced caspase-8 activation. Moreover, our results strongly indicate that IG20 is acting primarily upstream of caspase-8 and that its interaction with TNFR1 modulates TNF-α-induced caspase-8 activation. Inhibition of this caspase activity can block activation of downstream caspases, including caspase-3, and thus prevent TNF-α-induced, IG20-mediated apoptosis. Further studies are needed to fully delineate different signaling pathways selectively activated by different IG20 isoforms and to determine whether and how they may mutually regulate susceptibility to TNF-α-induced apoptosis.

In summary, our results demonstrate a novel regulatory function for IG20 splice variants in TNFR1 signaling. In this study, we focused our efforts on understanding the role of IG20 and its splice isoforms in TNF-α-induced apoptosis. However, preliminary studies strongly indicate that the effects noted are not limited to TNF-α-induced apoptosis since similar differential effects of splice variants were also evident in response to other death-inducing agents, including TRAIL (data not shown). Relative to other species, there is a higher degree of alternative splicing in human genes that could result in multiple proteins with different functions from the same gene. It is
apparent that IG20 is one such gene that can undergo splicing resulting in different isoforms, which might play an important regulatory role in physiological cell death.

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