Identification of a Novel Tumor Necrosis Factor-α-inducible Gene, SCC-S2, Containing the Consensus Sequence of a Death Effector Domain of Fas-associated Death Domain-like Interleukin-1β-converting Enzyme-inhibitory Protein*

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We report here the isolation and characterization of a novel tumor necrosis factor-α (TNF-α)-inducible gene, SCC-S2. Based on the nucleotide sequence, the SCC-S2 open reading frame contains a sequence in the amino terminus that shows a significant homology to death effector domain II of cell death regulatory protein, Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (FLIP). Unlike FLIP, the SCC-S2 open reading frame contains only one death effector domain and lacks the carboxyl-terminal caspase-like homology domain, raising the possibility that SCC-S2 may be a novel member of the FLIP family. SCC-S2 mRNA expression is found in most normal tissues and malignant cells. The steady state level of SCC-S2 mRNA is significantly induced by TNF-α in different tumor cells (TNF-α at 20 ng/ml for 3 h: A549, -2–9-fold; SKOV-3, -3-fold; PCI-04A, -3–6-fold). TNF-α treatment (100 ng/ml, 4 h) of HeLa cells transiently transfected with FLAG epitope-tagged SCC-S2 cDNA or expression vector alone led to an increase in the number of apoptotic cells as compared with the untreated counterpart. Interestingly, however, SCC-S2 transfectants revealed a significant decrease in the number of apoptotic cells as compared with the vector transfectants (p < 0.001). These data implicate a role of SCC-S2 as a negative mediator of apoptosis in certain cell types.

Increasing evidence suggests that apoptosis requires activation of members of the interleukin-1β-converting enzyme-like family of cysteine proteases, also known as caspases. The caspase activation appears to be triggered by some members of the TNFR1 superfamily, including TNFR1 (p55/CD120a) and TNFR2 (p75/CD120b), and Fas/Apo-1 (CD95). TNF binds to TNFR1, and FasL binds to Fas. TNFR1 and Fas, also known as death receptors, are characterized by the presence of a cytoplasmic sequence motif called the death domain, which interacts with the death domain of the adaptor molecules FADD and TNF-associated death domain, recruiting them to the membrane. TNF-associated death domain interacts with FADD, and FADD, in turn, associates with an apical caspase, FLICE (caspase 8/MACH/Mch5), through death effector domains (DEDs) present at the carboxyl terminus of FADD and the amino terminus of FLICE, resulting in the assembly of a receptor-associated death-inducing signaling complex. Death-inducing signaling complex-associated FLICE signals proteolytic activation of downstream caspases, ultimately leading to apoptosis (reviewed in Ref. 1). FADD mutant containing only the death domain or FLICE containing two DEDs can act as a dominant negative inhibitor of apoptosis (2–4). Because ligand activation of a death receptor does not lead to apoptosis in all cell types, it has been suggested that natural cell death inhibitory molecules may exist in certain cells. Indeed, FLICE-inhibitory proteins (FLIP, CASH, I-FLICE, and FLAME-1) containing two sequence motifs with significant homology to DEDs have been identified (5–9). FLIPs contain two DEDs in the amino terminus and are represented by two splice variants: FLIP(L), the long form, and FLIP(S), the short form. Carboxyl-terminal extension of the longer variant shows homology to the caspase-like protease homology domain but lacks active site cysteine, suggesting that it is devoid of proteolytic activity. These proteins bind to FLICE through DEDs, blocking the binding and proteolytic activation of effector caspases. Consistent with these findings, a viral homologue of cellular FLIP (v-FLIP) identified in herpes and Molluscum contagiosum viruses exhibits antiapoptotic activity, and overexpression of cellular FLIP suppresses FasL and TNF-α-induced apoptosis (5, 10–12).

Several reports indicate that negative regulators of apoptosis, including the FLIP family of proteins, may also trigger tumorigenesis in appropriate cells (8, 13). For example, increased expression of FLIP has been found in Fas ligand-resistant melanoma cell lines and in metastatic cutaneous mel...
anoma lesions from patients, whereas no expression was detected in melanocytes surrounding the hair follicle of the skin (8). Second, activation of the Ras/Raf-1/mitogen-activated protein kinase kinase (MKK)/mitogen-activated protein kinase (MAPK) pathway is known to play major roles in tumorigenesis and protection against cytotoxic agents (reviewed in Refs. 14 and 15), and activation of MKK1 has been shown to abrogate Fas-initiated apoptosis through the induction of FLIP expression (16).

During the course of a search for genes differentially expressed in human tumor cell lines established from primary and matched (from the same patient) metastatic head and neck squamous cell carcinoma (HNSCC), we identified a 2.0-kb transcript, corresponding to a partial cDNA clone SCC-S2, amplified in a metastatic and radioresistant HNSCC-derived cell line (PCI-06B) as compared with its matched primary tumor-derived cell line (PCI-06A) (17). PCI-06B cells are also resistant to TNF-α-induced cytotoxicity (18). The present studies were undertaken to isolate the full-length SCC-S2 cDNA, determine the effect of TNF-α on SCC-S2 mRNA level in cancer cells, and examine the possible antiapoptotic function of SCC-S2. Our data suggest that SCC-S2 cDNA encodes a novel protein. The putative open reading frame (ORF) of SCC-S2 revealed significant homology with DED II of mouse and human FLIP proteins. SCC-S2 mRNA was expressed in most human normal tissues and cancer cell lines. We have confirmed the previous observation of a relatively higher steady state level of SCC-S2 mRNA in PCI-06B cells compared with PCI-06A cells and demonstrated a significant TNF-α-inducible expression of SCC-S2 mRNA in different tumor cell types. In addition, transient expression of FLAG epitope-tagged SCC-S2 protein in HeLa cells led to a decrease in the number of cells undergoing apoptosis in the presence or absence of TNF-α as compared with the vector transfectants.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HNSCC cell lines PCI-06A, PCI-06B, and PCI-04A (19) were grown in minimal essential medium supplemented with 15% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 1 mM non-essential amino acids, 2 mM L-glutamine, 25 μg/ml gentamicin (all from Life Technologies, Inc.), and 0.4 mg/ml hydrocortisone (Sigma). The other human tumor cell lines were grown in improved minimal essential medium (Cellgro) containing 10% heat-inactivated fetal bovine serum. The cells were grown in 75-cm² tissue culture flasks in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

**cDNA Cloning**—A human heart cDNA library in λZapII vector (Stratagene) was screened using a 32P-labeled SCC-S2 partial cDNA probe (large box) and an overlapping EST clone (nucleotides 1–396) are shown. Nucleotide positions are indicated by numbers on the right. The predicted longest ORF (188 amino acids) is shown. Amino acid positions are numbered on the left. The poly(A)⁺ signal sequence is shown in boldface in a small box. The proposed main structural features of the SCC-S2 protein are the putative DED (shaded) and the protein kinase C and casein kinase II phosphorylation sites (boldface and underlined, respectively). The nucleotide sequence is deposited in the GenBank™ DNA data base (accession numbers AA406630 (nucleotides 1–396), AF098933 (nucleotides 397–911), U68132 (nucleotides 912–1170), and AF098934 (nucleotides 1171–1915)).
screened. The filters were hybridized at 42 °C in buffer containing 50% formamide, 5x SSC, 1x Denhardt's solution, 20 mM sodium phosphate buffer (pH 6.8), and 200 μg/ml sheared salmon sperm DNA, followed by washings at 55 °C, three times in 2x SSC and 0.1% SDS and three times in 0.2x SSC and 0.1% SDS. The filters were rinsed twice in 2x SSC, damp dried, and autoradiographed. The positive clones were isolated after five cycles of amplification and screening. The cDNA insert (1519 bp) from a positive clone (ID DK721) was subcloned into pBlue-script (1) vector by in vivo excision according to the manufacturer's instructions (Stratagene).

Sequence Analysis and Data Base Search—Both strands of the SCC-S2 cDNA (1519 bp) were sequenced by automated sequencing using Applied Biosystems Prism 377 DNA sequencer and an Applied Biosystems Prism Dye terminator cycle reaction kit. Raw data files from the ABI 377 sequencer were imported into the Auto Assembler program (ABI). Contigs were generated by comparing all fragments in one project with the parameters of at least 50 bp of overlap and at least a 75% level of homology. The assembled sequence was used to find a matching IMAGE consortium EST clone AA 406630 from the human EST data base (20). The Integrated Molecular Analysis of Genomes and their Expression EST clone AA 406630 was purchased from Genome Systems and sequenced as above. The sequences were assembled using the Auto Assembler program, and the complete sequence was then subjected to data base search. Sequence data base search and ORF prediction were done using the National Center for Biotechnological Information BLAST and ORF finder programs on the World Wide Web (21). Multiple sequence alignment was performed using the MultiAlign program (22). A search for the presence of different motifs and signature sequences was conducted. The prediction of the possible nature of putative protein based on structural characteristics was done by Reinhart's method (23).

TNF-α Treatment, Northern Blotting, and Hybridization—Logarithmically growing cells were switched to serum-free medium for 2 h prior to the addition of the indicated amounts of TNF-α (R & D Systems), followed by incubations for various times as described before (24, 25). The cells were washed with cold phosphate-buffered saline, and total RNA was isolated with Trizol reagent according to the manufacturer's specifications (Life Technologies, Inc.). For Northern analysis, total RNA was electrophoresed on 1% agarose-formaldehyde gel, transferred

During the preparation of this report, a GenBank™ data base search revealed that the SCC-S2 sequence reported in this study matched with GG2–1 mRNA (accession number AF070671; Ref. 43) and MDC-3.13 isoform 1 mRNA (accession number AF099936). In addition, expressed sequence tags representing potential mouse and Drosophila homologues of human SCC-S2 cDNA were identified (accession numbers AA116718 and AA817954).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or described before (17, 26). Membranes were reprobed with radiolabeled room temperature and 0.1 m

Fig. 4. Expression of SCC-S2 transcript in human cancer cell lines. Left panels, a cancer cell line blot (CLONTECH) was probed with a radiolabeled ~1.5-kb SCC-S2 cDNA fragment and reprobed with β-actin cDNA. Middle and right panels, blots were sequentially hybridized to ~1.5-kb SCC-S2 cDNA and GAPDH cDNA probes. Autoradiographs were computer-scanned, and SCC-S2 mRNA expression was normalized to β-actin or GAPDH. G361, melanoma; A549, lung carcinoma; SW480, colorectal adenocarcinoma; MOLT-4, lymphoblastic leukemia; K562, chronic myelogenous leukemia; HL60, promyelocytic leukemia; U273MG, glioblastoma; MDA-MB231, breast carcinoma; RCC-RR, renal cell carcinoma; SW900, lung carcinoma; SKOV-3, ovarian carcinoma; PC-3, prostate carcinoma; PCI-06A and PCI-06B, head and neck squamous cell carcinoma.

overnight to nylon membrane (Qiagen), and fixed by UV cross-linking, and the membrane was baked at 80 °C for 2 h. The multitissue blots H, H2, H3, F, and C, containing poly(A)+ RNA from adult and fetal tissues and various cancer cell lines, were purchased from CLONTECH. 10⁶ cpm/ml ³²P-labeled SCC-S2 cDNA (~1.5 kb, ID DK721) was used as probe, and hybridizations were performed at 68 °C using ExpressHyb (CLONTECH), followed by washings with 2× SSC and 0.1% SDS at room temperature and 0.1× SSC containing 0.1% SDS at 68 °C as described before (17, 26). Membranes were reprobed with radiolabeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin probe as an internal control. The autoradiographs were scanned and bands quantified using ImageQuant software, version 3.3 (Molecular Dynamics personal densitometer).

Polymerase Chain Reaction Amplification and Cloning of FLAG Epitope-tagged SCC-S2 cDNA in Mammalian Expression Vector—cDNA fragment encoding the open reading frame of SCC-S2 (nucleotides 1–697, Fig. 1) was amplified by polymerase chain reaction using human placental cDNA (CLONTECH). The 5′- and 3′-primers used for amplification were 5′-CCTAGGCTTCTCGCCGGCCTTCACCC-3′ and 5′-CCAGGATTCCTCCTTATGTTCTCTGTCGATGATCCAC-3′, respectively. The sequence underlined in the 3′-primer corresponds to the FLAG octapeptide (Sigma). The amplified product (734 bp) was verified by automated sequence analysis of both strands and cloned into the mammalian expression vector PCR 3.1 according to the instruction manual (Invitrogen).

Transient Transfection and Immunoblotting—HeLa cells were seeded in six-well plates (1–2 × 10⁵ cells/well) and transfected with the expression vector PCR 3.1 or recombinant vector containing FLAG-tagged SCC-S2 cDNA (2 μg/well) using the LipofectAMINE method (Life Technologies, Inc.). 36 h after transfection, cells were harvested and lysed at 4 °C for 30 min in lysis buffer (100 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin), followed by microcentrifugation for 5 min at 4 °C. Protein concentration was determined using Coomasie G250 protein assay reagent (Pierce). Cell lysates (25–50 μg) were resolved by 15% SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Millipore), and immunoblotted with 1 μg/ml mouse monoclonal FLAG-M2 antibody (Sigma). Enhanced chemiluminescence method (Luminol, NEN Life Science Products) was used to detect the signal. The blot was reprobed with human polyclonal anti-GAPDH antibody (Trevigen).

Apoptosis Assay—HeLa cells were transiently transfected with vector or FLAG epitope-tagged SCC-S2 cDNA as described above. 30 h after transfection, cells were switched to medium containing 1% fetal bovine serum for 1 h and then treated with TNF-α (100 ng/ml) for an additional 4 h. After treatment, floating cells were pooled with the adherent cells collected by trypsinization and fixed in 2 ml of 75% ethanol for at least 30 min at 4 °C. For the FACS analysis of sub-G₁ cells, the fixed cells were pelleted and resuspended in 1 ml of phosphate-buffered saline solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (Sigma). The stained cells were analyzed using a FACSort (Becton Dickinson), and Repproman computer software. The percentage of cells containing sub-G₁ DNA content was used as an index of apoptosis as described (27, 28).
RESULTS AND DISCUSSION

The screening of a human heart cDNA library with a partial SCC-S2 cDNA probe (259 bp; Ref. 17) led to the identification of a clone containing 1519-bp cDNA insert (ID DK721). A BLAST search of the EST data base with this sequence resulted in the identification of a 5′-overlapping EST clone (AA 406630). The assembled nucleotide sequence was 1915 bp, with a predicted ORF of 188 amino acids and an in-frame stop codon 5′ to the first ATG (Fig. 1). The sequence contained 133 bp of the 5′-untranslated region and 1215 bp of the 3′-untranslated region. The polyadenylation signal sequence could be located in the 3′-untranslated region. SCC-S2 cDNA encoded a putative cytosolic protein with predicted relative molecular mass of 21 kDa. A search for the known motifs and protein family signature sequences revealed three putative casein kinase II phosphorylation sites and one protein kinase C phosphorylation site (Fig. 1).

A BLAST search of the ORF suggested that SCC-S2 is a novel protein. The sequence contained a putative DED that showed significant homology with DED II of the FLIP family of cell death regulatory proteins. The putative DED domain in SCC-S2 showed identities (similarities) as follows: mouse CASH α/β, 35% (58%); human CASH α/β, 27% (50%); mouse FLIP(L), 32% (53%); and human FLIP(L), 27% (58%) (Figs. 1 and 2A). Identity higher than 25% is considered significant (29). The death domains and/or DEDs are important protein-protein interaction domains in death receptors, including TNFR1, and adaptor molecules such as TNFR-associated death domain, FADD, FLICE, and receptor-interacting protein. Based on the known structure-function relationships of FLIP proteins, the presence of a putative DED domain in the amino terminus and the absence of a caspase catalytic domain in the carboxyl terminus suggest that SCC-S2 may serve as a dominant negative inhibitor of the DED containing molecules such as FLICE. Interestingly, the SCC-S2 DED shared only 9 and 11% identity with DED in mouse FLICE and human FLICE (32 and 38% similarity), respectively (Fig. 2). It is not known as yet whether SCC-S2 interacts with and/or inhibits FLICE.

Viral genomes are known to code for apoptosis inhibitory proteins, allowing increased viral replication to combat the host’s apoptotic defense mechanism (5, 30–36). These inhibitors interact with Fas, TNF-receptor-related apoptosis-mediated protein, TNF-related apoptosis-inducing ligand receptor, and TNFR1 and block apoptotic signaling events. The poxvirus-encoded serpin CrmA and baculovirus gene product p35 exert inhibitory effects by binding directly to FLICE (36). The putative SCC-S2 DED showed significant homology to the corresponding domains present in some viral proteins, sharing 30 and 46% identity (58 and 66% similarity) to human poliovirus coat proteins and canine adenoavirus DNA polymerase, respectively (Fig. 2B). Relatively weak identity (21%) and similarity (54%) of the SCC-S2 DED to vaccinia virus DNA polymerase were observed (Fig. 2). Other features of the SCC-S2 ORF included the signature sequence for vinculin family talin binding region proteins (Fig. 2C). This sequence indicated 20% identity (44% similarity) to human α1(E)- and α2(E)-catenins, a class of proteins known to play a role in epithelial cell-cell contacts (37).

SCC-S2 transcript (∼2.0 kb) was detectable in most human normal tissues, with relatively higher levels in spleen, lymph node, thymus, thyroid, bone marrow, and placenta and lower levels in spinal cord, ovary, lung, adrenal glands, heart, brain, testis, and skeletal muscle (Fig. 3). Among the fetal tissues examined, a prominent signal was seen in liver, lung, and kidney, whereas expression could not be detected in brain (Fig. 3). SCC-S2 mRNA was expressed in all cancer cell lines tested, with relatively higher levels in K562 chronic myelogenous leukemia cells, MOLT 4 lymphoblastic leukemia cells, and A549 lung carcinoma cells and lower levels in SW480 colorectal adenocarcinoma cells (Fig. 4). Consistent with our original findings (17), a 2.0-kb transcript was detected in PCI-06B cells, and SCC-S2 mRNA expression was reproducibly higher in PCI-06B cells than in PCI-06A cells (∼2-fold) (Fig. 4).

Engagement of TNFR1 by its cognate ligand leads to increased expression of a number of pro- and antiapoptotic genes. We asked whether TNF-α treatment of cells results in the induction of SCC-S2 mRNA. Data shown in Fig. 5 indicate a significant increase in the steady state level of SCC-S2 mRNA in A549 lung carcinoma cells, SKOV-3 ovarian carcinoma cells, and PCI-04A HNSCC cells (TNF-α at 20 ng/ml for 3 h: A549, ∼2–9-fold; SKOV-3, ∼3–fold; PCI-04A, ∼3–6-fold). It should be noted that A549 cells and SKOV-3 cells are resistant to TNF-α (38, 39). TNF-α-induced SCC-S2 mRNA was also noted in U373MG cells and human hepatoma HepG2 cells (data not shown). TNF-α-inducible gene expression has been associated with the presence of binding motifs of transcription factors NF-κB and AP-1 in the promoter region of several genes. Whether SCC-S2 promoter contains a TNF-α-responsive element(s) remains to be determined.

To address the possibility of an antiapoptotic function of SCC-S2, HeLa cells were transiently transfected with FLAG epitope-tagged SCC-S2 cDNA expression vector (Fig. 6, left panel). The efficiency of transient transfection was initially determined by co-transfection with pCMV β-galactosidase expression vector (CLONTECH), and the percentage of blue cells was reproducibly comparable in vector and SCC-S2 transfectants (data not shown). The increase in number of cells in sub-G1 phase has been used as an indicator of apoptosis (40). Our data show that TNF-α treatment of vector or SCC-S2 transfectants led to an increase in the number of cells in sub-G1 as compared with the untreated counterpart (Fig. 6, right panel). Interestingly, however, expression of exogenous SCC-S2 resulted in a significant decrease in number of cells in sub-G1 phase, in the presence or absence of TNF-α, as compared with vector transfectants (p < 0.001). These data suggest that SCC-S2 overexpression per se is a negative mediator of apoptosis.

The molecular genetic factors that negate cell death and contribute to tumor progression can be attractive targets for therapeutic intervention (41, 42). Current investigations in our laboratory are designed to examine the functional significance of SCC-S2 in cancer progression.
