Identification of SNF1/AMP Kinase-related Kinase as an NF-κB-regulated Anti-apoptotic Kinase Involved in CD95-induced Motility and Invasiveness*

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The death receptor CD95 (APO-1/Fas) induces apoptosis in many tissues. However, in apoptosis-resistant tumor cells, stimulation of CD95 induces up-regulation of a defined number of mostly anti-apoptotic genes, resulting in increased motility and invasiveness of tumor cells. The majority of these genes are known NF-κB target genes. We have identified one of the CD95-regulated genes as the serine/threonine kinase (SNF1/AMP kinase-related kinase (SNARK)), which is induced in response to various forms of metabolic stress. We demonstrate that up-regulation of SNARK in response to CD95 ligand and tumor necrosis factor α depends on activation of NF-κB. Overexpression of SNARK rendered tumor cells more resistant, whereas a kinase-inactive mutant of SNARK sensitized cells to CD95-mediated apoptosis. Furthermore, small interfering RNA-mediated knockdown of SNARK increased the sensitivity of tumor cells to CD95 ligand- and TRAIL-induced apoptosis. Importantly, cells with reduced expression of SNARK also showed reduced motility and invasiveness in response to CD95 engagement. SNARK therefore represents an NF-κB-regulated anti-apoptotic gene that contributes to the tumor-promoting activity of CD95 in apoptosis-resistant tumor cells.

CD95 (APO-1/Fas) is best known as a death receptor, inducing apoptosis in various tissues by recruiting initiator caspases to the death-inducing signaling complex leading to their activation (1, 2). It has been known for many years that CD95 can also activate nonapoptotic pathways such as the NF-κB, the mitogen-activated protein kinase pathways Erk1/2, JNK1/2, and p38, and the phosphatidylinositol 3-kinase/Akt pathway (3). Recently, it has become clear that activation of a combination of these pathways in apoptosis-resistant tumor cells can increase their motility and invasiveness (4), providing an explanation for experiments that identified CD95 as a tumorigenic factor when expressed on certain tumor cells grown in vivo (5). We recently identified a defined number of genes that were up-regulated in response to CD95 stimulation that could be involved in the nonapoptotic activities of CD95 (4). However, the nature of most of the genes that mediate the tumor-promoting activity of CD95 remains unknown.

Members of the AMP kinase family are serine/threonine kinases that play a role in tumorigenesis (6). This activity is believed to be due to their activation by various forms of metabolic stress such as glucose deprivation, a condition to be expected within solid tumors. Currently, five members of this family are known: AMK-α1, AMPK-α2 (7), MELK (8), SNARK (9, 10), and ARK5 (11). The fourth member of the family, SNARK, was originally cloned from a rat kidney library but was recently isolated from a human testis cDNA library after identification through BLAST searches (10). SNARK is activated by glucose starvation and under these conditions induces acute cell-cell detachment (10). We have recently identified human SNARK in a gene screen as one of only 17 genes that were up-regulated more than 2-fold in CD95-stimulated MCF7-Fas-Bcl-xL(FB) cells (4).

We now show that SNARK is up-regulated by CD95 stimulation on the mRNA as well as the protein level and requires the activation of NF-κB. SNARK was found to have anti-apoptotic activities, as it protected cells from CD95- and TRAIL-induced apoptosis, a kinase-dead mutant of SNARK sensitized cells to CD95-mediated apoptosis. Down-modulation of SNARK using SNARK-specific siRNAs demonstrated that SNARK is also involved in the CD95-induced increase in motility and invasiveness of MCF7-FB cells. SNARK is therefore one of the anti-apoptotic genes that are induced in apoptosis-resistant tumor cells triggered through CD95, which causes increased invasiveness of tumor cells, identifying SNARK as a possible target for cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents—** MCF7-FB cells were cultured as described (13). The embryonic kidney cell line HEK293T was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). The breast tumor cell line MCP7, the cervical carcinoma cell line, HeLa, and the renal adenocarcinoma cell lines, CAKI-1 and ACHN, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). For transfection, cells were seeded into a 6-well plate at 2 × 10^5/well, and 2 μg of DNA or 100 pmol of siRNA were transfected with Lipofectamine 2000 (Invitrogen). HEK293T cells were transfected with the calcium phosphate method. The anti-CD95 monoclonal antibody, anti-APO-1,

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¶ The abbreviations used are: AMPK, AMP kinase; SNARK, SNF1/AMPK-related kinase; TNF, tumor necrosis factor; siRNA, small interfering RNA; siSNARK, small interfering SNARK; FB, Fas-Bcl-xL; Z, benzoxycarbonyl; fmk, fluoromethyl ketone; RT, reverse transcription; HA, hemagglutinin; GST, glutathione S-transferase; LcCD95L, leucine zipper-tagged CD95 ligand; uPA, urokinase plasminogen activator; huSNARK, human SNARK.
has been described previously (14). Leucine zipper-tagged CD95 ligand (LcCD95L) was generated as described previously (12). TNFα was purchased from Peprotech (Rocky Hill, NJ) and used at a concentration of 1000 units/ml. The caspase inhibitors Z-VD-fmk and Z-IETD-fmk were purchased from Calbiochem. All other chemicals were of analytical grade and purchased from Sigma.

Semiquantitative RT-PCR and Real Time PCR—Total RNA was prepared from different stimuli-induced cells using TRIzol reagent (Invitrogen). An equal amount of total RNA (2 μg) isolated from cells at each of the stimulated conditions was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). For each PCR target, only the dilution that yielded visible products within the linear amplification range for the untreated cDNA was shown. 10⁷ MCF7-FB cells were preincubated with or without 40 μM Z-IETD-fmk and then treated or untreated for 8 h with anti-APO-1 (1 μg/ml). Real time PCR was performed as described (4). Briefly, reverse-transcribed cDNA was made from 1 μg of each RNA sample and used for all subsequent real time PCR reactions.² Cycles were run on an ABI 7700, and SYBR® green was used for fluorescent detection of double-stranded DNA, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Normalization of the initial RNA composition was calculated from concurrent runs with primers specific for actin. Triplicates were performed with each primer set for each RNA sample. The mid-linear range was used to establish the threshold for each oligonucleotide set. After normalization and the calculation of differences within each time point experiment subset, all data from different time point were pooled to calculate differences.

SNARK Cloning, Plasmid Construction, Site-directed Mutagenesis, and siRNAs—Human SNARK was isolated from the IMAGE clone (ID: 5088057) (4) using the PCR primer set eggaattcgctgtcagttgcttctgge- gcgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttgcttctggegccgtgctgtcagttg...
SNARK Is a CD95-induced Target Gene

**Fig. 1.** CD95 and TNF receptor-engagement induces up-regulation of huSNARK. *A,* semiquantitative RT-PCR on RNA purified from MCF7-FB cells stimulated with anti-APO-1 or TNFα for the indicated times. *B,* MCF7-FB were treated with anti-APO-1 or left untreated for 8 h, and real time PCR was performed for SNARK. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a second housekeeping gene control, and A20 served as a positive control. *C,* semiquantitative RT-PCR on RNA purified from MCF7 and HeLa cell lines treated (Lz) or untreated (U) for 2 h with LzCD95L. D, MCF7-FB cells were stimulated for the indicated times with either LzCD95L or TNFα. Cells were lysed, and a Western blot analysis was performed with the indicated antibodies. E, autophosphorylation kinase assay of immunoprecipitated SNARK from LzCD95L- or TNFα-stimulated MCF7-FB cells as described under “Experimental Procedures.”

With LzCD95L or TNFα using an affinity-purified rabbit antibody we generated against a peptide located at the C terminus of SNARK in a region with high sequence diversity among different AMP kinase family members (Fig. 1D). This up-regulation of SNARK also caused a parallel increase in SNARK activity in cells, which was determined by performing an in vitro kinase assay on immunoprecipitated SNARK from LzCD95L- or TNFα-stimulated cells (Fig. 1E).

**SNARK Is an NF-κB-regulated Gene**—Induction of SNARK mRNA was direct because it could not be inhibited by treatment with the protein synthesis inhibitor cycloheximide (Fig. 2A). Also, the increase of SNARK mRNA was because of direct transcriptional activation rather than stabilization of mRNA because induction of SNARK could efficiently be blocked by actinomycin D (Fig. 2B). Interestingly, similar to TNFα and CD95 ligand treatment of MCF7-FB cells, TRAIL induced NF-κB activation in MCF7-FB cells (4) but only weakly induced SNARK mRNA (Fig. 2B).

Because 12 of 17 induced genes in the gene screen were known NF-κB-regulated genes, we tested whether inhibition of NF-κB would prevent up-regulation of SNARK (Fig. 2C). MCF7-FB were pretreated with the specific membrane-permeable NF-κB inhibitor, Tat-IκBmut, described recently (4). CD95-induced up-regulation of SNARK was completely abolished by this reagent, confirming that activation of NF-κB is required for CD95-induced transcriptional activation of SNARK. This regulation was similar to a classical NF-κB target gene, ICAM-1. Finally, we found that the caspase-8 activity induced in the apoptosis-resistant MCF7-FB cells (13) does not play a significant role in the induction of SNARK; this is because preincubation of cells with the oligo caspase inhibitor Z-VDAD-fmk did not inhibit up-regulation of SNARK mRNA (Fig. 2D, upper panel), although it blocked processing of caspase-8 in this experiment (Fig. 2D, lower panel). In summary, we have identified SNARK as a transcriptionally induced gene in CD95-stimulated cells. Its activation depends critically on activation of NF-κB, making it a potential mediator of the novel CD95 tumorigenic activities.

**Fig. 2.** CD95-induced SNARK up-regulation is direct and relies on the NF-κB pathway but not on caspase activity. *A,* semiquantitative RT-PCR was performed on the indicated genes with RNA from MCF7-FB cells preincubated with the indicated concentration of cycloheximide (CHX) or actinomycin D (Act. D) for 1 h and left unstimulated or stimulated for 2 h with anti-APO-1. B, RT-PCR of MCF7-FB cells treated for 8 h with either anti-APO-1 (A), LzCD95L (Lz), LzICAM-1 (I), or GST-Tat (T), or TNFα (TNF) or left untreated (U). C, semiquantitative RT-PCR of MCF7-FB cells preincubated for 1 h with 10 μg/ml GST-Tat (Tat) or GST-Tat-IκBmut (Tat-IκBmut) (see Ref. 4) and then stimulated for 8 h with 1 μg/ml anti-APO-1. D, MCF7-FB were preincubated for 30 min with Z-VDAD-fmk (40 μm), left untreated (U), or treated for 4 h with anti-APO-1 (A). Semiquantitative RT-PCR on isolated RNA for the indicated genes (top two panels) and Western blot for caspase-8 on cell lysates (bottom two panels) were carried out.

Kinase-inactive SNARK Sensitizes Cells to CD95-induced Apoptosis—We overexpressed the cloned human SNARK kinase in 293T cells and determined that it is an active kinase with typical autophosphorylation properties and with an apparent size of 70 kDa (Fig. 3A). In this experiment we also...
detected a phosphorylated protein in control-transfected cells that comigrated with immunoprecipitated untagged SNARK, which was likely corresponding to active endogenous SNARK in these cells (Fig. 3A, lane 5).

To determine the function of endogenous SNARK in apoptosis, we generated a kinase-inactive mutant (Fig. 3B). This mutant was generated by replacing lysine 82, a highly conserved position in the AMP kinase family, with arginine, causing it to act in a dominant negative fashion when over-expressed (17, 18). The kinase function of the K82R mutant of SNARK was inactive, as it did not undergo autophosphorylation (Fig. 3B). It also did not phosphorylate a substrate comprising a fusion protein of GST with the SAMS peptide, which is the optimal sequence for phosphorylation by members of the AMP kinase family (19) (Fig. 3C). We then over-expressed either SNARK or SNARK K82R in the renal cell carcinoma cell line ACHN (12), which expressed endogenous SNARK (data not shown) and induced apoptosis with either anti-APO-1 or LzCD95L. ACHN cells were significantly protected by the expression of SNARK. This inhibition was dependent on the kinase activity of SNARK because SNARK K82R could not protect cells from CD95-mediated apoptosis. In contrast, SNARK K82R rendered cells even more susceptible to CD95-mediated apoptosis, suggesting that endogenous SNARK, which is induced upon triggering these cells through CD95 (data not shown), was inhibited in a dominant negative manner by the overexpressed mutant of SNARK. Transient overexpression of SNARK also protected another renal cell carcinoma cell line, Caki-1 (12), from both CD95 ligand and TRAIL-induced apoptosis (data not shown). Taken together, our data suggested that SNARK is an NF-kB-regulated, CD95-inducible kinase that renders cells resistant to death receptor-mediated apoptosis. This activity is consistent with other CD95-induced genes that we recently found were up-regulated in CD95-stimulated, apoptosis-resistant cells (4). Many of these genes were anti-apoptotic and/or potentially tumorigenic.

SNARK Is a CD95-induced Target Gene

To directly determine the function of SNARK as a CD95-regulated gene, we reduced the expression of SNARK using siRNAs. Using RT-PCR, ACHN cells were found to express endogenous SNARK (Fig. 4A, lane 1); however, expression could be enhanced by treating cells with LzCD95L (Fig. 4, lane 2). Treating the cells with two independent siRNAs (siSNARK and an siRNA SMARTpool) for 24 h resulted in a significant reduction of SNARK mRNA (Fig. 4A, lanes 3 and 4). ACHN cells treated with either of the SNARK siRNAs rendered them more sensitive to CD95-mediated apoptosis (Fig. 4B). SNARK also inhibited TRAIL-induced apoptosis. ACHN cells treated with SNARK siRNAs were significantly more sensitive to TRAIL-induced apoptosis (Fig. 4C). These experiments confirmed the anti-apoptotic activity of endogenous SNARK for death receptor-induced apoptosis.

SNARK Is an Anti-apoptotic Gene Required for CD95-induced Increase of Motility and Invasiveness of CD95 Apoptosis-resistant Tumor Cells

To directly determine the function of SNARK as a CD95-regulated gene, we reduced the expression of SNARK using siRNAs. Using RT-PCR, ACHN cells were found to express endogenous SNARK (Fig. 4A, lane 1); however, expression could be enhanced by treating cells with LzCD95L (Fig. 4, lane 2). Treating the cells with two independent siRNAs (siSNARK and an siRNA SMARTpool) for 24 h resulted in a significant reduction of SNARK mRNA (Fig. 4A, lanes 3 and 4). ACHN cells treated with either of the SNARK siRNAs rendered them more sensitive to CD95-mediated apoptosis (Fig. 4B). SNARK also inhibited TRAIL-induced apoptosis. ACHN cells treated with SNARK siRNAs were significantly more sensitive to TRAIL-induced apoptosis (Fig. 4C). These experiments confirmed the anti-apoptotic activity of endogenous SNARK for death receptor-induced apoptosis.

SNARK was one of only 17 CD95-induced genes in MCF7-FB cells, which respond to CD95 triggering with increased motility and invasiveness. We therefore tested whether the loss of SNARK would inhibit the CD95-mediated migration and invasiveness characteristics of these cells. As reported previously, MCF7-FB cells responded to stimulation with LzCD95L with up-regulation of typical NF-kB target genes such as urokinase plasminogen activator (uPA), A20, and SNARK (Fig. 4D, lanes 1 and 2). Treating the cells with either of the siRNAs directed at the SNARK mRNA resulted in a significantly reduced up-regulation of SNARK without affecting induction of uPA or A20 (Fig. 4D, lanes 3 and 4). We then subjected siRNA SNARK-treated MCF7-FB cells to motility and invasiveness assays, as described recently (4). Both CD95-induced motility and invasiveness were severely reduced in siSNARK-treated cells without affecting the general viability of these cells (data not shown), identifying SNARK as a gene that regulates this novel non-apoptotic activity of CD95.

Stress induced by treatment with AMP or glucose deprivation activates human and rat SNARK (9, 10). We incubated
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MCF7 cells with 200 μM AMP but did not observe activation of SNARK (data not shown). Both AMPK and another member of the AMPK family, ARK5, which shows 55% overall homology with SNARK, including 84% identity within the N-terminal kinase domain, were recently described as having anti-apoptotic activity (11, 16). Overexpression of ARK5 rendered cells more resistant to TNFα, TRAIL, and glucose deprivation. It was therefore postulated that ARK5 could promote tumor cell survival during nutrient starvation. The recent recognition that CD95 has a tumorigenic activity on tumor cells both in vitro (4) as well as in vivo (5) suggests the existence of protumorigenic genes that are induced in response to CD95 stimulation. SNARK induction requires activation of NF-κB, which is critical for the CD95 protumorigenic activities (4). We recently demonstrated that uPA, one of the NF-κB-regulated genes induced by CD95, is critical for the CD95-induced motility and invasiveness of apoptosis-resistant tumor cells. Blocking the activity of uPA blocked the ability of cells to invade in response to CD95 stimulation (4). SNARK is the second of the recently identified CD95-induced genes tested, and siRNA-induced down-modulation of SNARK demonstrated that SNARK, too, is important for the novel CD95-dependent tumorigenic activities.

Knocking down SNARK seemed to render cells more sensitive to TRAIL-induced apoptosis then apoptosis triggered through CD95. Although SNARK is induced by CD95 stimulation, it may not play its role as an anti-apoptotic factor predominantly in CD95-mediated apoptosis. CD95 apoptosis resistance can be achieved in many ways by tumor cells. Our data suggest that stimulating CD95 on tumor cells that are resistant to CD95-mediated apoptosis with whatever mechanism can up-regulate tumorigenic genes, such as SNARK, may give these cells further protection to CD95-mediated apoptosis. This stimulation can also give tumor cells resistance to other apoptosis inducers (e.g., TRAIL) and induce tumorigenic pathways that can cause increased motility and invasiveness.

Reducing the expression of SNARK did not affect the expression of uPA, suggesting that SNARK is not required for the induction of uPA. The fact that CD95 activates at least five different nonapoptotic signaling pathways independently (4), resulting in the induction of multiple genes, suggests that the regulation of these activities is complex. Future work will have to determine how many genes and pathways are involved in the regulation of these activities, ultimately giving a better understanding of the nonapoptotic functions of CD95 in cancer cells.

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