Loss of Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein α (αSNAP) Induces Epithelial Cell Apoptosis via Down-regulation of Bcl-2 Expression and Disruption of the Golgi

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Background: Vesicle trafficking proteins play important roles in regulating cell survival.

Results: Inhibition of a key membrane fusion protein, αSNAP, induced epithelial cell apoptosis, decreased Bcl-2 level, and impaired ER-Golgi communications.

Conclusion: αSNAP promotes cell survival by controlling ER/Golgi-dependent expression of Bcl-2.

Significance: Uncovering mechanisms that link vesicle trafficking and apoptosis is critical for understanding the regulation of cell fate.

Intracellular trafficking represents a key mechanism that regulates cell fate by participating in either prodeath or prosurvival signaling. Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein α (αSNAP) is a well known component of vesicle trafficking machinery that mediates intermembrane fusion. αSNAP increases cell resistance to cytotoxic stimuli, although mechanisms of its prosurvival function are poorly understood. In this study, we found that either siRNA-mediated knockdown of αSNAP or expression of its dominant negative mutant induced epithelial cell apoptosis. Apoptosis was not caused by activation of the major prodeath regulators Bax and p53 and was independent of a key αSNAP binding partner, NSF. Instead, death of αSNAP-depleted cells was accompanied by down-regulation of the antiapoptotic Bcl-2 protein; it was mimicked by inhibition and attenuated by overexpression of Bcl-2. Knockdown of αSNAP resulted in impairment of Golgi to endoplasmic reticulum (ER) trafficking and fragmentation of the Golgi. Moreover, pharmacological disruption of ER-Golgi transport by brefeldin A and eeyarestatin 1 or siRNA-mediated depletion of an ER/Golgi-associated p97 ATPase recapitulated the effects of αSNAP inhibition by decreasing Bcl-2 level and triggering apoptosis. These results reveal a novel role for αSNAP in promoting epithelial cell survival by unique mechanisms involving regulation of Bcl-2 expression and Golgi biogenesis.

Apoptosis is the major pathway of regulated cell death that has evolved to efficiently eliminate aged and damaged cells without compromising tissue architecture and functions. Apoptosis is crucial for normal organismal homeostasis, and dysregulation of this cell death mechanism has profound pathophysiologic consequences by modulating inflammatory responses and tumor progression (1–3). Either external or internal signaling pathways activate the apoptotic cascade. The external pathway is triggered by death receptors at the plasma membrane (4), whereas the internal pathway is typically instigated from mitochondria or the endoplasmic reticulum (ER)3 (5, 6). Both pathways activate a caspase-based proteolytic cascade (7, 8) leading to profound biochemical and morphological changes in intracellular organelles and the plasma membrane (9).

Intracellular vesicle trafficking represents an emerging regulator of apoptosis that can determine cell fate by participating in transduction and execution of prodeath signals (10, 11). For example, stimulation of the extrinsic apoptotic pathway requires assembly of the death-inducing signaling complex in the endosomal compartment, which involves endocytosis of death receptors and exocytosis of their adaptor proteins (12, 13). Furthermore, several cytotoxic agents can trigger apoptosis by yet to be defined mechanisms, depending on activation of vesicle fluxes to endosomes and lysosomes (14, 15). Trafficking events culminate with docking and fusion of cytoplasmic vesicles with target membranes (16–18). Efficient intermembrane fusion requires assembly of multiprotein soluble N-ethylmaleimide-sensitive factor-associated receptor (SNARE) com-

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plexes (19–21). SNARE proteins are located on both vesicle and target membranes, and by participating in specific trans-interactions, they bring two membranes into close opposition and create a fusion pore (19–21). Recent studies have revealed unanticipated connections between the SNARE machinery and apoptosis. For example, expression of several SNARE components was found to be regulated by key apoptotic molecules, such as p53 (22) and caspases (23). On the other hand, SNARE proteins themselves can either suppress or accelerate apoptosis (22, 24, 25).

Soluble N-ethylmaleimide-sensitive factor-attachment proteins (SNAPs) are key regulators of SNARE-mediated vesicle fusion (17, 19, 20, 26). Mammalian cells ubiquitously express two SNAP isoforms, α and γ, whereas expression of the third isoform, βSNAP, is limited to the brain (27). αSNAP acts as an adaptor protein that physically links SNAREs to a hexameric ATPase, N-ethylmaleimide sensitive factor (NSF), thereby mediating disassembly and recycling of SNARE complexes (28, 29). A number of additional binding partners of αSNAP have been identified (27), which may explain pleiotropic biological functions of this membrane fusion protein. Interestingly, αSNAP was recently implicated in regulation of cell survival because its overexpression protected cells from apoptosis induced by cytotoxic drugs (30, 31). However, mechanisms underlying such antiapoptotic activity of αSNAP remain unknown. The present study was designed to dissect molecular events that mediate a prosurvival role of αSNAP in model epithelia. We report that loss of αSNAP functions induces epithelial cell apoptosis by mechanisms involving decreased expression of antiapoptotic Bcl-2 protein, impaired ER-Golgi communications, and disintegration of the Golgi.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following primary monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) were used to detect signaling and vesicle trafficking proteins: anti-αSNAP, p97, and GFP mAbs (Abcam); anti-cleaved poly(ADP-ribose) polymerase (PARP), total PARP, active caspase-3, procaspase-3, active caspase-7 Bak, and Bid pAbs and anti-GAPDH mAb (Cell Signaling Technology, Danvers, MA); anti-NSF, GM130, Bnip1, GRP78, FAS ligand, syntaxin-5, and E-cadhein mAbs (BD Biosciences); anti-Bcl-2, p53, and CHOP mAbs and Fas-associated death domain protein and p47 pAbs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-giantin pAB (Covance, Princeton, NJ); anti-Bax pAB (Millipore, Billerica, MA); anti-Bak and Noxa mAbs (EMD Chemicals, Gibbstown, NJ); anti-KDEL receptor mAb (Enzo Life Sciences, Farmingdale, NY); anti-E-cadherin ECCD1 rat mAb (Invitrogen). Anti-junctional adhesion molecule A (JAM-A) mAb J10.4 was provided by Dr. Charles Parkos (Emory University, Atlanta, GA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa-488 or Alexa-555 dye-conjugated donkey anti-rabbit and goat anti-mouse secondary antibodies were obtained from Invitrogen. Z-VAD-fmk was purchased from MP Biomedical (Santa Ana, CA); salubrinal, MG-132, and epoxomicin were obtained from EMD Chemicals; and HA 14-1 was purchased from Tocris Bioscience (Ellisville, MO). All other reagents were obtained from Sigma-Aldrich.

**Cell Culture**—SK-CO15 (a gift from Dr. Enrique Rodriguez-Boulan (Weill Medical College of Cornell University) (32), DU145, and PC3 epithelial cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% of fetal bovine serum. HCT116, Bax null and HCT116 p53 null cells (derivatives of HCT116 in which Bax or p53 genes were somatically knocked out (33, 34)) were cultured in McCoy medium supplemented with 10% of fetal bovine serum. These cells and their appropriate controls were provided by Dr. Bert Vogelstein (John Hopkins University School of Medicine, Baltimore, MD). PC-3 cells with stable overexpression of Bcl-2 were generated as described previously (35). For biochemical and immunolabeling experiments, the cells were cultured on 6-well plastic plates and collagen-coated cover slips, respectively.

**Immunoblotting**—Cells were homogenized in radioimmune precipitation assay lysis buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4), containing a protease inhibitor mixture (1:100; Sigma) and phosphatase inhibitor mixtures 2 and 3 (both at 1:200; Sigma). Lysates were cleared by centrifugation, diluted with 2× SDS sample buffer, and boiled. SDS-PAGE and immunoblotting were conducted by standard protocols with an equal amount of total protein (10 or 20 μg) per lane. Protein expression was quantified by densitometry of three immunoblot images, each representing an independent experiment, with a Kodak Image Station 2000R and Kodak Molecular Imaging software version 4.0 (Eastman Kodak Co.). Data are presented as normalized values assuming the expression levels in control siRNA-treated groups were at 100%. Statistical analyses were performed with raw densitometric data using the Microsoft Excel program.

**Immunofluorescence Labeling**—Epithelial cell monolayers were fixed/permeabilized in 100% methanol and were double-immunolabeled for the Golgi and plasma membrane markers as described previously (36–38). The effect of NSF depletion on junctional protein exocytosis was analyzed by using a calcium switch model as described elsewhere (39). Briefly, confluent SK-CO15 cell monolayers were subjected to overnight incubation in a low calcium medium (calcium-free Eagle’s minimum essential medium for suspension culture [Invitrogen]) to internalize/deplete E-cadherin and JAM-A at the plasma membrane. To induce E-cadherin and JAM-A exocytosis, the cells were returned to normal cell culture medium for 5 h (referred to hereafter as “calcium repletion”). Calcium-repleted cells were fixed with 4% paraformaldehyde without permeabilization in 100% methanol and were double-immunolabeled for the Golgi and plasma membrane markers as described previously (36–38). The effect of NSF depletion on junctional protein exocytosis was analyzed by using a calcium switch model as described elsewhere (39). Briefly, confluent SK-CO15 cell monolayers were subjected to overnight incubation in a low calcium medium (calcium-free Eagle’s minimum essential medium for suspension culture [Invitrogen]) to internalize/deplete E-cadherin and JAM-A at the plasma membrane. To induce E-cadherin and JAM-A exocytosis, the cells were returned to normal cell culture medium for 5 h (referred to hereafter as “calcium repletion”). Calcium-repleted cells were fixed with 4% paraformaldehyde without permeabilization, and surface-exposed E-cadherin and JAM-A were visualized by immunolabeling with ECCD1 and J10.4 monoclonal antibody, respectively, which specifically recognized extracellular domains of these proteins. Labeled cells were examined using an Olympus Fluoview 1000 confocal microscope (Olympus America, Center Valley, PA). The Alexa Fluor 488 and 555 signals were imaged sequentially in frame interlace mode to eliminate cross-talk between channels. The images were processed using the Olympus FV10-ASW 2.0 Viewer software and Adobe Photoshop. Images shown are representative of at least three experiments, with multiple images taken per slide.
RNA Interference and αSNAP cDNA Expression—Small interfering RNA (siRNA)-mediated knockdown of αSNAP, NSF, BNIP1, GRP78, and p97 was carried out as described previously (36–38). Individual siRNA duplexes GAAGGUCGUGGUUACGCU (duplex 1) and CAGAGUUGGUGGA-CAUCGA (duplex 2; DHarmaco, Lafayette, CO) were used to down-regulate αSNAP expression, whereas knockdown of other targets was performed by using gene-specific siRNA SmartPools (Dharmacon). A non-coding siRNA duplex-2 (Dharmacon) served as a control. SK-CO15 cells were transfected using the DharmaFECT 1 reagent (Dharmacon) in Opti-MEM I medium (Invitrogen) according to the manufacturer’s protocol with a final siRNA concentration of 50 nM. For dual knockdown, cells were first transfected with either BNIP1 or GRP78 siRNAs and then cultured for 12 h in complete DMEM followed by the second transfection with αSNAP siRNA, duplex 1. Cells were analyzed 72 h after the second transfection. For the rescue experiment, HCT116 cells were simultaneously co-transfected with αSNAP siRNA duplex 1 (50 nM) and pEGFP-C1 plasmid bearing a coding sequence of bovine αSNAP (100 ng; gift from Dr. Reinhard Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)). The bovine αSNAP transcript has 4 nucleotide mismatches in the corresponding sequence and is not targeted by the human αSNAP siRNA duplex 1. Transfection was performed by using a DharmaFECT Duo reagent (Dharmacon, Lafayette, CO), and cells were analyzed 48 h later. A full-length bovine αSNAP was cloned in phCMV3-HA vector (Genelantis, San Diego, CA) as a HindIII, BamHI fragment. L294A αSNAP mutant was created by using PhusionTM site-directed mutagenesis kit (Finzymes, Espoo, Finland) with the forward primer GAG GAA GAC GCC GCN GCC GGG GTT GTT TT and the reverse primer GTT ACC CGT GAT GGT TCC TTT CTG. The mutant construct was verified by sequencing and was transfected into DU145 cells by using Attractene reagent (Qiagen, Valencia, CA).

Flow Cytometry Analysis—Control and αSNAP-depleted cells were trypsinized, pooled together with spontaneously detached cells, and stained for Annexin-V and propidium iodide using a BD Biosciences apoptosis detection kit according to the manufacturer’s instructions. Labeled cells were analyzed using the Epics XL-MCL flow cytometer (Beckman Coulter, Brea, CA) and Expo 32 ADC XL 4 Color software.

Quantitative Real-time RT-PCR—Total RNA was isolated using the RNeasy minikit (Qiagen) followed by DNase treatment to remove traces of genomic DNA. Total RNA (1 μg) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with 2 μg of cDNA/reaction using IQ SYBR Green Supermix (Bio-Rad) and the DNA Engine OpticonTM thermocycler (MJ Research, Boston, MA). The following primers were used: human β-actin (NM_001101.3) forward (CGAGGCCCCAGAAGCAAGAGAG) and reverse (CGTGGTGGCCT- TAGGGTTCCG); human Bcl-2 (NM_000633.2) forward (TGTGGAGAGCGTCAAAGGGGAAG) and reverse (CAAG-CTCCACCAGGGGCAACT). The threshold cycle number for the gene of interest was calculated based on the amplification curve representing a plot of the fluorescent signal intensity versus the cycle number.

Statistics—Numerical values from individual experiments were pooled and expressed as means ± S.E. of three independent experiments throughout. Obtained numbers were compared by two-tailed Student’s t test, with statistical significance assumed at p < 0.05.

RESULTS

Inhibition of αSNAP Functions Induces Apoptosis in Human Epithelial Cells—In order to examine the role of αSNAP in epithelial cell survival, we first down-regulated its expression in SK-CO15 human colonic epithelial cells using RNA interference. Cell treatment with two different αSNAP siRNAs resulted in a more than 90% decrease in its protein level that became evident at 24 h and lasted for at least 96 h post-transfection (Fig. 1A) (data not shown). Such αSNAP depletion was accompanied by cell rounding/detachment and profound cleavage of PARP at 48–72 h post-transfection (Fig. 1A), which was indicative of apoptosis-like cell death. To better define the nature of the death pathway induced in αSNAP-depleted cells, we analyzed additional apoptotic markers and determined whether this process can be reversed by caspase inhibition. Along with PARP cleavage, αSNAP knockdown caused cleavage (activation) of caspase-3 (Fig. 1B) and caspase-7 (data not shown) as well as a significant increase in cell labeling with Annexin-V and propidium iodide (PI) (Fig. 1, C and D). Importantly, induction of all apoptotic markers was prevented by incubation of αSNAP-depleted SK-CO15 cells with a pan-caspase inhibitor, Z-VAD-fmk (Fig. 1, B–D).

To ensure that apoptosis triggered by αSNAP knockdown did not represent an off-target effect of siRNA expression, we performed a rescue experiment in HCT116 colonic epithelial cells. In these cells, siRNA duplex 1 efficiently down-regulated αSNAP expression and induced apoptotic PARP cleavage (supplemental Fig. 1). However, PARP cleavage was dramatically reduced in cells co-transfected with αSNAP siRNA and GFP-tagged bovine αSNAP lacking complementation to the human siRNA sequence (supplemental Fig. 1). This rescue effect validates the observed apoptosis as a specific consequence of αSNAP knockdown. We also investigated if prosurvival activity of αSNAP depends on its ability to regulate vesicle trafficking by transfecting epithelial cells with an L294A point mutant of αSNAP, which is known to inhibit exocytosis (28). Overexpression of the dominant negative αSNAP mutant but not of wild type protein or control plasmid in DU145 epithelial cells resulted in marked apoptosis at 48 h post-transfection (Fig. 1, E and F). The described experiments provide strong evidence that inhibition of αSNAP activity triggers the apoptotic death pathway in different types of cultured epithelial cells.

Cell Death in αSNAP-depleted Epithelia Is Not Due to Induction of Proapoptotic Signaling—We next sought to elucidate mechanisms that are responsible for the apoptosis induction in αSNAP-depleted epithelial cells. Generally, this can be caused either by activation of proapoptotic signaling or by the decrease in antiapoptotic defense mechanisms. Given a recent report that αSNAP knockdown potentiated cisplatin cytotoxicity by up-regulating expression of proapoptotic Bax and p53 proteins (31), we asked if similar events mediate apoptosis in αSNAP-depleted epithelial cells. Surprisingly, knockdown of αSNAP
resulted in decreased expression of Bax in SK-CO15 (Fig. 2A) and HCT116 (Fig. 2B) cells. Furthermore, immunofluorescence labeling with a conformation-specific Bax antibody did not detect the appearance of activated Bax in H9251 SNAP-depleted SK-CO15 cells (data not shown). Finally, analyses of major apoptotic markers, such as cleaved PARP, procaspase-3, and Annexin-V/PI labeling did not show significant attenuation of apoptosis in H9251 SNAP-depleted Bax null HCT116 cells as compared with their parental controls (Fig. 2, B–D).

Similarly, loss of αSNAP failed to up-regulate p53 expression in SK-CO15 and HCT116 cells (Fig. 3, A and B). Furthermore, p53 null HCT116 cells responded to αSNAP depletion with a similar magnitude of apoptosis compared with their parental controls (Fig. 3, B–D). We also examined expression of other important proapoptotic proteins, such as Bak, Bid, Bim, Noxa, Fas ligand, and Fas-associated death domain protein and did not find their significant up-regulation in SK-CO15 cells at 48 and 72 h post-transfection with two different αSNAP siRNAs (supplemental Fig. 2). Taken together, these data suggest that activation of major prodeath pathways is not responsible for the induction of apoptosis in αSNAP-depleted epithelial cells.

Down-regulation of Antiapoptotic Bcl-2 Protein Mediates Decreased Survival of αSNAP-depleted Cells—In order to examine the role of antiapoptotic defense mechanisms in the...
diminished survival of αSNAP-depleted cells, we first analyzed expression of a key antiapoptotic protein, Bcl-2. In SK-CO15 cells, the Bcl-2 level was decreased by ~40% and 80% at 48 and 72 h of αSNAP knockdown, respectively (Fig. 4A). Interestingly, overexpression of the dominant negative αSNAP mutant in DU145 cells also caused an ~70% decrease in Bcl-2 expression as compared with the empty plasmid transfected control (data not shown). To examine whether suppression of Bcl-2 activity is sufficient to induce apoptosis, SK-CO15 cells were incubated for 24 h with a small molecule Bcl-2 inhibitor HA 14–1 (50 μM). Such Bcl-2 inhibition resulted in a marked apoptotic death, which according to Annexin-V/PI staining encompassed more than 70% of cells (Fig. 4, B and C). To strengthen a causal connection between the decreased Bcl-2 level and increased cell death following αSNAP knockdown, we next investigated the effects of Bcl-2 overexpression on αSNAP-depleted PC3 human prostate epithelial cells. Loss of αSNAP expression triggered a typical apoptotic response in control PC3-neo cells at 72 h post-transfection, which was manifested by the increase in PARP cleavage and Annexin-V/PI labeling (Fig. 4, D–F). Remarkably, induction of all apoptotic markers was significantly attenuated in αSNAP-depleted PC3 cells with Bcl-2 overexpression (Fig. 4, D–F). Collectively, these data strongly suggest that decreased expression of antiapoptotic Bcl-2 protein plays a key role in triggering the cell death program following αSNAP depletion.

We next sought to determine mechanisms by which loss of αSNAP expression decreases Bcl-2 protein level in model epithelia. A quantitative RT-PCR analysis did not show significant differences in Bcl-2 mRNA level between control and αSNAP-depleted SK-CO15 cells (supplemental Fig. 3A), thereby pointing to post-transcriptional mechanisms of Bcl-2 regulation. One such mechanism may involve mistargeting Bcl-2 for degradation due to impaired protein trafficking/sorting caused by αSNAP knockdown. This idea was tested by using inhibitors of the two major protein degradation pathways, specifically targeting either lysosomes or proteosomes. SK-CO15 cells were transfected with control or αSNAP-specific siRNAs followed by a 48-h treatment with either vehicle or with either of two different inhibitors of lysosomal protein degradation (chloroquine (100 μM) or concanamycin A (100 nM)) or with either of two proteosomal inhibitors (MG-132 (25 μM) or epoxomicin (1 μM)). However, neither lysosomal nor proteosomal inhibition restored the normal protein levels of Bcl-2 in αSNAP-depleted cells (supplemental Fig. 3B). These results indicate that mechanisms other than accelerated degradation mediate the observed decrease in Bcl-2 protein level.
Proapoptotic Effect of αSNAP Depletion Is Associated with Impaired ER to Golgi Trafficking—Although αSNAP can regulate vesicle fusion with different cellular membranes, the majority of its binding partners are located at the Golgi and the ER (30, 41–44). On the other hand, ER and Golgi are known generators of proapoptotic signaling because pharmacological disruption of these compartments results in profound cell death (10, 11). Based on these data, we hypothesized that loss of αSNAP can initiate proapoptotic signaling by interfering with the normal ER-Golgi vesicle trafficking. To test this hypothesis, we examined localization of KDEL receptor (KDEL-R), a protein that is known to constantly cycle between the Golgi and the ER (45) and whose mislocalization is a reliable indicator of the disrupted ER-Golgi transport (46, 47). In control SK-CO15 cells, KDEL-R accumulated in a set of condensed elongated structures surrounding the nucleus (Fig. 5A, arrows) representing a predominant Golgi-resident fraction of the protein (45). By contrast, in αSNAP-depleted cells distinguishable by the diffuse intracellular staining of a cell-cell adhesion protein β-catenin, the perinuclear KDEL-R labeling was transformed into a diffuse dotlike staining scattered throughout the cell (Fig. 5A, arrowheads). Furthermore, a dramatic disruption of Golgi morphology in αSNAP-depleted epithelial cells was revealed by immunolabeling of a Golgi marker, GM130 (supplemental Fig. 4). Importantly, dispersion of normal Golgi structure represented an early effect of αSNAP knockdown detectable at 24 h post-transfection before the appearance of morphological changes in epithelial monolayers (supplemental Fig. 4) and biochemical markers of apoptosis (Fig. 1A).

To investigate the causal link between disrupted ER-Golgi communications and cell death, we used pharmacological agents, brefeldin A (BFA) and eeyarestatin 1 (Eer1) that block vesicle cycling between the ER and the Golgi by two unrelated mechanisms. BFA is known to inhibit guanine nucleotide exchange factors for Golgi-resident ARF GTPases (48, 49), whereas Eer1 targets several ER proteins, most notably p97 ATPase (50, 51). Exposure of SK-CO15 cells to either BFA (2 μM) or Eer1 (50 μM) phenocopied all major effects of αSNAP depletion by inducing massive apoptosis (Fig. 5, B and C), decreasing Bcl-2 expression (Fig. 5B), and disrupting KDEL-R labeling (Fig. 5D). Apoptosis appeared to be a delayed effect of the ER-Golgi-disrupting drugs, being detectable only after 6 and 12 h of continuous exposure to Eer1 and BFA, respectively (supplemental Fig. 5A). Importantly, both drugs disrupted KDEL-R labeling within 3 h, which places the impaired ER-Golgi trafficking as an upstream event for apoptosis induction. Together, these findings suggest that the loss of αSNAP down-regulates antiapoptotic Bcl-2 signaling and triggers epithelial

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**FIGURE 3. Apoptosis in αSNAP-depleted epithelial cells is p53-independent.** A, SK-CO15 cells were transfected with either control or αSNAP duplex 1 siRNAs. Expression of p53 protein in total cell lysates was determined by immunoblotting at different times post-transfection. B–D, p53 null HCT116 cells and their wild-type controls were transfected with either non-targeted or αSNAP duplex 1 siRNAs. Induction of apoptotic markers as well as αSNAP and p53 expression were determined by immunoblotting and flow cytometry analyses at 72 h post-transfection. *, p < 0.01 compared with the corresponding control groups. Error bars, S.E.
cell apoptosis by mechanisms involving disruption of the ER-Golgi trafficking and fragmentation of the Golgi.

Apoptosis in αSNAP-depleted Epithelia Does Not Depend on NSF but Can Be Associated with Inhibition of p97 ATPase—Because αSNAP is known to regulate vesicle fusion in association with NSF ATPase, we next asked if these binding partners also cooperate in promoting epithelial cell survival. NSF was dramatically (>90%) down-regulated in SK-CO15 cells by a specific siRNA SmartPool without affecting αSNAP protein level (Fig. 6A). Such NSF depletion attenuated calcium-dependent delivery of E-cadherin and JAM-A to intercellular junctions (supplemental Fig. 6), thereby indicating defects of protein exocytosis. Surprisingly, loss of NSF did not result in significant SK-CO15 cell apoptosis (Fig. 6, A and B); it also had no effect on Bcl-2 expression (Fig. 6A) and cellular localization of KDEL-R or GM130 (Fig. 6C). Collectively, these experiments indicate that unlike αSNAP, NSF is dispensable for the steady-state ER-Golgi vesicle cycling and epithelial cell survival.

p97 is another oligomeric ATPase, which along with NSF regulates vesicle trafficking between the ER and the Golgi compartments (50, 52, 53), and the activity of which can be modulated by αSNAP (43). We next sought to examine whether p97 can be involved in the decreased survival of αSNAP-depleted epithelial cells. Remarkably, siRNA-mediated knockdown of p97 in SK-CO15 cells phenocopied all major effects of αSNAP depletion, including induction of apoptosis (Fig. 7, A and B), decreased expression of Bcl-2 protein (Fig. 7A), and abnormal localization of ER/Golgi markers (Fig. 7C). Apoptosis of p97-deficient cells was consistent with the observed prodeath effects of Eer1 (Fig. 5B), which is a potent pharmacological

FIGURE 4. Down-regulation of Bcl-2 mediates induction of apoptosis in αSNAP-depleted epithelial cells. A, expression of Bcl-2 protein was analyzed in control and αSNAP-depleted SK-CO15 cells at different times after siRNA transfection. †, p < 0.05 compared with the control siRNA-treated groups. B and C, SK-CO15 cells were treated for 24 h with Bcl-2 inhibitor, HA 14-1 (50 μM), followed by examination of apoptosis by immunoblotting and flow cytometry. *, p < 0.01 compared with the vehicle-treated control. D–F, PC3 cells stably overexpressing Bcl-2 and their controls were transfected with either non-targeting or αSNAP duplex 1 siRNAs, and apoptosis was analyzed by immunoblotting and flow cytometry at 72 h post-transfection. †, p < 0.01 compared with control siRNA-transfected cells; †, p < 0.05 compared with Bcl-2-overexpressing αSNAP-depleted cells. Error bars, S.E.
inhibitor of p97 (51). Although αSNAP knockdown did not affect p97 expression, it perturbed a balance between key p97-binding partners. Specifically, expression of syntaxins 5 and 18 was significantly decreased, whereas the protein level of p47 was substantially elevated in αSNAP-depleted cells (supplemental Fig. 7A). Furthermore, loss of αSNAP disrupted a characteristic perinuclear labeling of p97 in SK-CO15 cells (supplemental Fig. 7B). Collectively, these data suggest that inhibition of p97, but not NSF ATPase can be associated with ER/Golgi disruption and apoptosis in αSNAP-depleted epithelial cells.

**FIGURE 5.** The proapoptotic effect of αSNAP depletion is associated with impaired vesicle cycling between the Golgi and the ER. A, control and αSNAP-depleted SK-CO15 cells were subjected to dual immunofluorescence labeling for the marker of Golgi/ER vesicle trafficking, KDEL receptor (red), and the plasma membrane marker, β-catenin, (green) at 72 h post-transfection. B–D, SK-CO15 cells were treated for 24 h with either BFA (2 µM) or Eer1 (50 µM), followed by examination of apoptosis, Bcl-2 expression, and localization of KDEL receptor. *, p < 0.01 compared with the vehicle-treated control. Scale bar, 20 µm. Error bars, S.E.

**BNIP1 and ER Stress Play No Roles in Death of αSNAP-deficient Cells**—To gain additional insights into signaling mechanisms that link the αSNAP-dependent Golgi disruption with initiation of apoptosis, we next analyzed possible involvement of BNIP1 and the ER stress. BNIP1 is a member of the proapoptotic BH3-only protein family (54) that is known to directly interact with αSNAP (30) and to participate in the ER to Golgi trafficking (44). Because αSNAP binding was shown to suppress BNIP1-dependent apoptosis (30), we hypothesized that loss of αSNAP could stimulate apoptotic activity of BNIP1 by
either increasing its expression or by promoting prodeath signaling. However, immunoblotting analysis did not show a significant increase of BNIP1 expression in \( \text{H9251} \) SNAP-depleted SK-CO15 cells at different times post-transfection (Fig. 8A).

Furthermore, a dual siRNA-mediated depletion of \( \text{H9251} \) SNAP and BNIP1 did not attenuate apoptosis as compared with SK-CO15 cells subjected to \( \text{H9251} \) SNAP knockdown only (Fig. 8, B–D). Together, these data strongly suggest that BNIP1 activity plays no role in mediating death of \( \text{H9251} \) SNAP-depleted epithelial cells.

Another possible mechanism of apoptosis induction in \( \text{H9251} \) SNAP-deficient cells may involve accumulation of misprocessed/mistargeted proteins in the ER that triggers the so-called “unfolded protein response” or ER stress (55, 56). Uncontrolled and prolonged ER stress eventually leads to apoptosis (57). To test this mechanism, we first examined whether \( \text{H9251} \) SNAP knockdown induces ER stress in SK-CO15 cells by measuring expression of known markers of this response, such as GRP78 chaperone and a transcriptional regulator CHOP. A significant increase in GRP78 expression was observed only at the later times (72 h) of \( \text{H9251} \) SNAP depletion, when apoptosis was already well under way, whereas the level of CHOP remained unchanged (Fig. 9A). To further examine possible causal relationships between induction of ER stress and apoptosis in \( \text{H9251} \) SNAP-depleted cells, we used two different experimental approaches. One approach was to enhance the ER stress-related prodeath cascade by decreasing expression of protective GRP78 (58, 59); the other approach was to pharmacologically inhibit ER stress (60). Immunoblotting and flow cytometry analyses showed the same extent of apoptosis in SK-CO15 cells with dual \( \text{H9251} \) SNAP and GRP78 knockdown as in cells where \( \text{H9251} \) SNAP was depleted alone (Fig. 9, B and C). Furthermore, ER stress inhibition with salubrinal (50 \( \mu \)M for 48 h) did not affect the magnitude of \( \text{H9251} \) SNAP-dependent cell death (Fig. 9, D and E). These results suggest that induction of the ER stress does not play a significant role in epithelial cell apoptosis caused by \( \text{H9251} \) SNAP knockdown.

**DISCUSSION**

\( \alpha \text{SNAP} \) is a key component of intracellular vesicle trafficking that mediates turnover of SNARE complexes, thereby ensuring the continuity of vesicle fusion (17, 27). The present study has revealed a novel role of this protein, which acts as an important positive regulator of epithelial cell survival. Such prosurvival
activity was uncovered by siRNA-mediated depletion of αSNAP or expression of its dominant negative mutant, which triggered a marked apoptosis in different types of cultured epithelial cells (Figs. 1, 2, and 4). This phenomenon is consistent with previous reports that αSNAP overexpression increased epithelial cell resistance to the cytotoxic drugs staurosporin (30) and cisplatin (31) in vitro. Furthermore, some indirect evidence points to a possible prosurvival role of αSNAP in vivo. For example, genetic deletion of its homolog in Drosophila results in embryonic lethality (61). Additionally, αSNAP hypomorphic hyh mice (62) are characterized by a significant loss of neuroepithelial cells (63), which can be a consequence of increased apoptosis.

The life cycle of the cell depends on a delicate balance between proapoptotic and antiapoptotic mechanisms. Disruption of such balance by either increasing proapoptotic signaling or decreasing antiapoptotic defense results in cell death (64). Our data suggest that the former mechanism plays no role in the decreased viability of αSNAP-depleted epithelial cells. Indeed, αSNAP knockdown did not stimulate expression of major prodeath proteins (Figs. 2 and 3 and supplemental Fig. 2), and Bax null and p53 null HCT116 cells retained the ability to respond to αSNAP depletion with a robust apoptosis (Figs. 2 and 3). Interestingly, a recent study has found that loss of αSNAP sensitized HEK 293 cells to cisplatin cytotoxicity by increasing cellular levels of Bax and p53 (31). This indicates that αSNAP can modulate proapoptotic signaling in response to some external stimuli, but it does not serve as an obligate suppressor of steady-state expression and functions of Bax or p53 proteins.

By contrast, our results highlight the decreased prosurvival Bcl-2 signaling as a crucial mechanism of epithelial cell death triggered by αSNAP knockdown. First, loss of αSNAP significantly decreased expression of Bcl-2 protein at the onset of apoptosis (Fig. 4A). Second, Bcl-2 inhibition alone induced a pronounced caspase-dependent death of SK-CO15 cells (Fig. 4, B and C). Finally, Bcl-2 overexpression attenuated αSNAP depletion-induced apoptosis (Fig. 4, D–F). To our best knowledge, this is the first evidence that implicates vesicle trafficking machinery in regulating expression and antiapoptotic activity of Bcl-2. Interestingly, the progressive loss of Bcl-2 after αSNAP knockdown cannot be explained by its diminished mRNA transcription or accelerated protein degradation (supplemental Fig. 3). We believe that this phenomenon reflects inhibited translation of Bcl-2 protein. Indeed, depletion of epithelial αSNAP resulted in a dramatic down-regulation of protein translational machinery, which involved key regulators of both 5’-m7G-cap-dependent translation initiation and an alternative initiation from so-called internal ribosome entry sites (data not shown). On the other hand, functionally active internal ribosome entry sites were found in the 5’-untranslated region of the Bcl-2 transcript (65), and internal ribosome entry-site-mediated translation of Bcl-2 was shown to be essential for cancer cell survival (66). Further studies are required to shed light on the regulation of Bcl-2 translation in αSNAP-depleted cells.

FIGURE 8. Epithelial cell apoptosis induced by αSNAP knockdown is independent of BNIP1. A, SK-CO15 cells were transfected with either control or αSNAP duplex 1 siRNAs. Expression of BNIP1 protein in total cell lysates was determined by immunoblotting at different times post-transfection. B–D, SK-CO15 cells were subjected to dual transfection with either BNIP1 plus αSNAP or control plus αSNAP siRNAs. BNIP1 and αSNAP expression and induction of apoptosis were determined at 72 h post-transfection. *, p < 0.01 compared with control siRNA-transfected cells; NS, non-significant difference. Error bars, S.E.
We also found that disruption of the ER-Golgi trafficking and Golgi fragmentation represent important upstream events that mediate the decreased Bcl-2 expression and apoptosis in αSNAP-depleted cells. Thus, knockdown of αSNAP impaired normal cycling of the KDEL-R between these organelles (Fig. 5A) and caused a profound loss of the compact Golgi structure that preceded the onset of apoptosis (supplemental Fig. 4). Furthermore, pharmacological interruption of the Golgi-ER vesicle cycling by either BFA or Eer1 phenocopied the effects of αSNAP depletion on cell survival and Bcl-2 expression (Fig. 5, B and C). Although our study provides the first evidence that αSNAP is essential for the Golgi integrity in living cells, it is consistent with several published reports implicating αSNAP in the regulation of the Golgi and ER homeostasis. Indeed, αSNAP was previously identified as a molecular constituent of two major SNARE complexes controlling the bidirectional vesicle trafficking between the Golgi and the ER (41–44). One complex involving syntaxin-5 mediates the direct (anterograde) ER to Golgi transport, whereas the other complex involving syntaxin-18 regulates the opposite retrograde vesicle trafficking from the Golgi to the ER (67). Moreover, αSNAP was shown to be essential for fusion of ER-derived vesicles with the Golgi in a cell-free system (68). Collectively, these data explain why the loss of αSNAP severely disrupts the vesicle cycling between these two organelles, thereby impairing structure and functions of both the Golgi and the ER.

Surprisingly, the prosurvival activity of αSNAP appeared to be independent of its major binding partner, NSF, because...
down-regulation of NSF neither decreased Bcl-2 expression nor induced epithelial cell apoptosis (Fig. 6). These results are consistent with a recent report that siRNA-mediated knockdown of NSF does not affect survival of HeLa cells (69) but contradict earlier observations of the defective ER-Golgi trafficking and induction of cell death after overexpression of an ATP hydrolysis-deficient (E329Q) NSF mutant (52, 70). However, the E329Q NSF mutant was shown to act as a “substrate trap” due to its tight binding to αSNAP (52). Hence, it is reasonable to suggest that overexpression of this dominant-negative form of NSF induces cell death by sequestering and inactivating αSNAP rather than by inhibiting NSF activity per se.

In contrast to NSF, our results implicate its homolog, p97 ATPase, in regulating Golgi integrity, Bcl-2 expression, and epithelial cell survival (Figs. 5 and 7). This opens a possibility that apoptosis in αSNAP-depleted cells can be dependent on p97 inactivation. Although this hypothesis lacks direct proof, it is supported by much indirect evidence. For example, αSNAP and p97 are known to have common binding partners, syntaxin-5 and syntaxin-18 (43, 71); therefore, they may modulate each other’s activity by competing for these interactions. Interestingly, loss of αSNAP significantly affected expression of key p97-binding proteins and altered normal localization of this ATPase (supplemental Fig. 7), all of which is likely to affect p97 activity.

Although the exact sequence of events that links disruption of the ER-Golgi vesicle cycling and activation of the proapoptotic program in αSNAP-depleted cells remains to be investigated, the decreased Bcl-2 synthesis is likely to be a key step of this process (Figs. 5 and 7). This notion is consistent with previous reports revealing the functional interplay between membrane trafficking and protein synthetic machinery. Thus, stimulation of exocytosis by overexpression of certain components of the SNARE or exocyst complexes resulted in increased protein synthesis (72, 73), whereas BFA-dependent disruption of the vesicle trafficking severely impaired this process (74). Regulators of membrane trafficking can modulate several steps of polypeptide chain processing at the ER, including ribosomal attachments to the ER membrane, translation initiation, and polypeptide translocation into the ER lumen (72, 73).

It is noteworthy that besides down-regulation of Bcl-2 expression, other Golgi-dependent mechanisms may contribute to the diminished survival of αSNAP-depleted epithelial cells. Thus, these cells were characterized by the decreased expression of Golgi BFA-sensitive guanidine nucleotide exchange factors GBF1, BIG1, and BIG2, whereas selective depletion of GBF1 in SK-CO15 cells induced Bcl-2-independent apoptosis (data not shown). On the other hand, our study ruled out the role of several known mechanisms responsible for generation of the proapoptotic signaling from the ER and the Golgi. For example, a component of the Golgi-ER SNARE machinery, BNIP1, was previously identified as a proapoptotic protein, whose activity can be neutralized by αSNAP binding (30). However, our data argue against the involvement of BNIP1 in apoptosis triggered by knockdown of αSNAP (Fig. 8). Furthermore, ER-derived apoptotic events are usually associated with induction of the ER stress due to abnormal accumulation of unprocessed proteins in the ER (6, 57). Although αSNAP-depleted SK-CO15 cells demonstrated some signs of the ER stress (Fig. 9A), our experiments with the enhancement or inhibition of this stress response do not support its involvement in apoptosis following αSNAP knockdown (Fig. 9, B–E). Finally, we did not find any evidence that loss of αSNAP triggers cell death by some other known mechanisms, such as cleavage of Golgi proteins p115, golgin-160, or GRASP65, activation of Golgi-resident caspase-2, increase in cytosolic calcium concentration, or enhancement of ceramide signaling (data not shown).

In summary, our study reveals a novel role of αSNAP in regulating epithelial cell survival. This role is based on the αSNAP-dependent vesicle cycling between the Golgi and the ER, which is critical for normal structure of these compartments and their ability to generate the antiapoptotic defense. Such a novel role for αSNAP can be essential for human diseases where loss or dysfunctions of this membrane fusion regulator may result in excessive cell death and disruption of normal tissue architecture.

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