Novel Mechanism of Apoptosis Resistance in Cancer Mediated by Extracellular PAR-4

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Abstract

Tumor suppressor PAR-4 acts in part by modulating sensitivity to apoptosis, but the basis for its activity is not fully understood. In this study, we describe a novel mechanism of antiapoptosis by NF-κB, revealing that it can block PAR-4–mediated apoptosis by downregulating trafficking of the PAR-4 receptor GRP78 from the endoplasmic reticulum to the cell surface. Mechanistic investigations revealed that NF-κB mediated this antiapoptotic mechanism by upregulating expression of UACA, a proinflammatory protein in certain disease settings. In clinical specimens of cancer, a strong correlation existed between NF-κB activity and UACA expression, relative to normal tissues. UACA bound to intracellular PAR-4 in diverse cancer cells, where it prevented translocation of GRP78 from the endoplasmic reticulum to the cell surface. This pathway of antiapoptosis could be inhibited by suppressing levels of NF-κB or UACA expression, which enhanced endoplasmic reticulum stress and restored GRP78 trafficking to the cell surface, thereby sensitizing cancer cells to apoptosis by extracellular PAR-4 or GRP78 agonistic antibody. In summary, our results identify a novel intracellular pathway of apoptosis mediated by NF-κB through UACA elevation, which by attenuating endoplasmic reticulum stress and GRP78 translocation to the cell surface can blunt the sensitivity of cancer cells to apoptosis. Cancer Res; 73(2); 1011–9. ©2012 AACR.

Introduction

The tumor suppressor Par-4 is expressed ubiquitously across various tissue types and is localized at various cell compartments, including the endoplasmic reticulum, cytosol, and nucleus (1). Endogenous Par-4 is essential for the apoptotic function of diverse cytotoxic agents (2). Nuclear translocation and the apoptotic function of Par-4 is activated by chemotherapeutic agents, such as vincristine, that cause phosphorylation of Par-4 (2). Activated Par-4 is capable of nuclear entry, NF-κB inhibition, caspase activation, and induction of apoptosis (2).

Par-4 protein is secreted in response to endoplasmic reticulum stress, and extracellular Par-4 protein produces autocrine and paracrine effects by binding to its cell surface receptor GRP78 (1). Apoptosis by extracellular Par-4 acting via GRP78 receptor requires intracellular Par-4 function for trafficking GRP78 to the membrane, and endoplasmic reticulum stress for activation of the caspase-8/caspase-3 pathway (1). Furthermore, the ability to systemically express Par-4 can be transferred from cancer-resistant mice to cancer-sensitive mice by bone marrow transplantation, implying a role for systemic Par-4 in suppression of tumor growth and metastasis (3).

As susceptibility to extracellular Par-4 is dependent on cell surface GRP78 (1), we sought to identify cell survival factors that may broadly regulate GRP78 trafficking to the cell surface. Our studies indicated that cell survival NF-κB activity (4) suppressed GRP78 translocation to the cell surface and rendered cancer cells resistant to apoptosis by extracellular Par-4.

Materials and Methods

Cell culture

All the cancer cell lines and HEL cells were obtained from American Type Culture Collection in the past 2 years and authenticated (February 29, 2012 and August 16, 2012) at Genetica DNA Laboratories using short tandem repeats profiling of DNA.

Coimmunoprecipitation and Western blot analysis

Precleared cell lysates were subjected to immunoprecipitation with 4 μg of antibody conjugated to 50 μL of protein G-Sepharose beads. The immunoprecipitates were washed with lysis buffer and subjected to Western blot analysis as described (1).

Immunohistochemistry, immunocytochemistry, proliferation, and apoptosis

These standard procedures have been previously described (1, 2), and additional details are presented in the Supplementary section.
**NF-κB reporter assays and FACS analysis**

NF-κB transcription activity in the cells was determined using transfection with NF-κB-luc construct as reporter and β-galactosidase expression construct to normalize luc activity, as previously described (1, 2). Cell surface GRP78 analysis was conducted (without fixing the cells to allow the detection of GRP78 at their surface) by fluorescence-activated cell sorting (FACS) analysis using either GRP78 primary antibody (N-20, SantaCruz Biotechnology, Inc.) or no primary antibody as control, and R-phycocerythrin–conjugated secondary antibody (R&D Systems; ref. 1).

**Quantitative real-time PCR analysis**

To determine the expression of UACA, total RNA was prepared from cells using Trizol reagent (Invitrogen Corp.) and subjected to quantitative real-time PCR (qRT-PCR; see Supplementary section).

**Statistical analysis**

All experiments were carried out in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System software (SAS Institute) and P values were calculated using the Student t test. The effect of interaction between 2 different treatments was analyzed using a 2-way ANOVA model with data normality and equality of variance assumptions. Mean of 3 experiments ± SD bars are shown.

All other cell lines, reagents, and procedural details are presented in the Supplementary section.

**Results**

**NF-κB activity regulates apoptosis by extracellular Par-4**

Constitutively activated NF-κB pathway is one of the most commonly activated mechanisms that promotes antiapoptosis and therapeutic resistance in diverse human cancers (5). To determine whether NF-κB activity regulates apoptosis by extracellular Par-4, we used 2 different approaches to block NF-κB activity: (i) the IκB-suppressor repressor (IκB-SR) S32A/S36A mutant of IκB, and (ii) treatment with PS-1145, an inhibitor of IκB kinase (IKK) activation. Various cancer cells were infected with IκB-SR or control GFP adeno virus and then treated with recombinant control protein thioredoxin (TRX) or TRX-Par-4. TRX-Par-4, but not thioredoxin, produced apoptosis in a dose-dependent manner, and reporter assays confirmed inhibition of NF-κB activity by IκB-SR (Supplementary Fig. S1A–S1C). The apoptotic sensitivity of PC-3 and H460 cells, which are intrinsically sensitive to TRX-Par-4, was further enhanced by IκB-SR (Fig. 1A). Moreover, A549 cells, which are resistant to the action of TRX-Par-4, were rendered highly susceptible to TRX-Par-4 by IκB-SR (Fig. 1A). These findings suggest that elevated NF-κB activity in cancer cells mitigates susceptibility to extracellular Par-4.

We next examined whether NF-κB activity also regulated GRP78 levels at the cell surface. Increased expression of GRP78 at the cell surface was noted in response to IκB-SR adenoviral expression (Fig. 1B). These findings were corroborated by cell surface biotinylation studies (Supplementary Fig. S1D).

![Figure 1](image-url)

**Figure 1.** Apoptosis by extracellular Par-4 is regulated by NF-κB. A–C, IκB-SR upregulates cell surface GRP78 and sensitizes cancer cells to apoptosis. Cells were transduced with IκB-SR or control GFP adeno virus, and then fixed cells were subjected to FACS analysis for cell surface GRP78 (B), or the cells were treated with TRX-Par-4 or thioredoxin (100 nmol/L of each protein; A and C) or GRP78 C-terminal agonistic antibody or PTEN control antibody (1 µg/mL; C) for 24 hours. The cells were subjected to immunocytochemistry for active caspase-3 and apoptotic cells were scored under a fluorescent microscope. Expression of IκB-SR was confirmed by Western blot analysis (A, inset). D, PS-1145 upregulates cell surface GRP78 and sensitizes cells to apoptosis. Cells were cotreated with PS-1145 (10 µmol/L) and thioredoxin or TRX-Par-4 protein (100 nmol/L), or GRP78 agonistic antibody or PTEN antibody (1 µg/mL) for 24 hours and subjected to FACS analysis for cell surface GRP78 (right) or to apoptotic assays (left). Asterisk (*) indicates statistically significant (P < 0.001) difference by the Student t test, and †† indicates that the effect is significantly (P < 0.001) higher based on 2-way ANOVA with data normality and equality of variance assumptions.

To further confirm the upregulation of cell surface GRP78, we tested the effect of IκB-SR on cellular response to the GRP78 (carboxyl-terminal) agonistic antibody (6). Cells infected with IκB-SR- but not GFP–producing adenovirus showed enhanced apoptosis of cancer cells with GRP78 agonistic antibody (Fig. 1C). Moreover, PS-1145 significantly elevated GRP78 expression.
at the cell surface, and increased the susceptibility of the cells to apoptosis by TRX-Par-4 or the GRP78 antibody (Fig. 1D and Supplementary Fig. S1E and S1F). TRX-Par-4 protein by itself did not inhibit NF-kB activity (Supplementary Fig. S1G). Collectively, these findings indicate that NF-kB activity negatively regulates the expression of cell surface GRP78 and apoptosis by extracellular Par-4 in cancer cells.

**Par-4 interacts with UACA**

Because intracellular Par-4 function is essential for translocation of GRP78 to the cell surface (1), we hypothesized that an NF-kB–regulated protein may bind to Par-4 and thereby prevent Par-4 from translocating GRP78 to the cell surface. We screened for Par-4 binding proteins: (i) that were upregulated by NF-kB, and (ii) that inhibited GRP78 translocation to the cell surface. We conducted a yeast-two hybrid (Y2H) screen using a human lung cDNA library and full-length Par-4 as bait. The surface. We conducted a yeast-two hybrid (Y2H) screen using a human lung cDNA library and full-length Par-4 as bait. The interactions were further confirmed by a one-on-one Y2H analysis. UACA (7) emerged as a strong binding partner of Par-4 in the presence of highly selective growth medium (Fig. 2A).

The interaction between Par-4 and UACA was validated by coimmunoprecipitation (co-IP) studies using various human cell lines. The Par-4 antibody immunoprecipitated Par-4 and coimmunoprecipitated UACA in all the cell lines (Fig. 2B). The Par-4 antibody also coimmunoprecipitated UACA from whole-tissue extracts from the prostate and lung of C57/BL6 mice (Supplementary Fig. S2A). The UACA antibody immunoprecipitated UACA, and coimmunoprecipitated Par-4 protein (Supplementary Fig. S2B). Neither UACA nor Par-4 was coimmunoprecipitated with the p65/NF-kB control antibody. As expected from our previous studies (1), the Par-4 antibody also coimmunoprecipitated GRP78 (Fig. 2B). These findings indicate that Par-4 binds to UACA in mammalian cells and tissues.

The interaction between Par-4 and UACA was further confirmed by immunocytochemical analysis in cancer cells. UACA colocalizes with Par-4, as well as with calnexin, an endoplasmic reticulum–resident protein (Fig. 2C). Par-4 also colocalizes with calnexin (1). These findings indicate that Par-4 and UACA colocalize with each other. Consistently, Par-4 and UACA showed coexpression within the cytoplasmic compartment of cancer tissues (Supplementary Fig. S2C). Importantly, cell fractionation studies indicated that Par-4 and UACA were present in both the endoplasmic reticulum- and nonendoplasmic reticulum subcellular fraction of cells, and the Par-4 antibody coimmunoprecipitated UACA from the endoplasmic reticulum-fraction but not from the nonendoplasmic reticulum fraction (Supplementary Fig. S2D). Together, these studies provide evidence that Par-4 and UACA bind to each other.

To test whether the basal level of UACA expression is regulated by NF-kB in unstimulated mouse and human cells, IKKβ−/− and IKKβ−/− mouse embryonic fibroblasts (MEF) were tested for UACA expression. IKKβ+/− MEFs showed UACA expression, but IKKβ−/− MEFs showed severely diminished expression of UACA (Fig. 2D). Lack of NF-kB activity in IKKβ−/− MEFs was confirmed by NF-kB reporter assays (Supplementary Fig. S2E). IκB-8R inhibited the expression of UACA in NIH 3T3/RasG12V cells or PC-3 cells (Fig. 2D). Similarly, PS-1145 and Bay-11-7082 inhibited UACA expression in diverse cell lines (Fig. 2D and Supplementary Fig. S2F). UACA expression was regulated by NF-kB at the RNA level (Supplementary Fig. S2G). Collectively, these findings indicate that UACA is upregulated by NF-kB activity and binds to Par-4 in mouse and human cells.

**Expression of UACA in normal and tumor tissues**

We next examined UACA levels and p65/NF-kB nuclear expression in human tissues. UACA expression showed 3-fold or more elevation in prostate cancer specimens compared with normal counterpart tissues, as judged by immunohistochemistry (IHC; Figs. 3A and Supplementary Fig. S3A). Similarly, UACA expression was markedly higher in lung adenocarcinoma and in squamous cell carcinoma relative to normal lung specimens (Fig. 3B). Upregulation of UACA was seen in all the grades of cancer and there was no further increase with tumor grade (Fig. 3A). We consistently noted a correlation between nuclear expression of p65/NF-kB and high levels (≥3+) of UACA in the tissues (Fig. 3C, left and Supplementary Fig. S3C). These data suggest that UACA expression and nuclear expression of p65/NF-kB are particularly elevated in diverse cancers. Moreover, GRP78 levels were overall higher in cancer tissues than in normal tissues (Supplementary Fig. S3D). A majority of the cancer tissues that coexpressed nuclear p65 and high levels of UACA showed 2+ (rather than ≥3+) levels of GRP78 (compare Fig. 3C, left and right).

Western blot analysis indicated that UACA and XIAP (a surrogate marker for NF-kB activity) were upregulated in prostate and lung cancer tissues relative to the corresponding normal tissues (Fig. 3D). On the other hand, Par-4 levels were similar in the normal and cancer tissue pairs. These findings indicated that an increase in NF-kB activity correlates with an increase in UACA expression in cancer tissues. Similarly, we noted an increase in NF-kB activity and UACA expression in prostate cancer or lung cancer cell lines relative to corresponding normal cells (Supplementary Fig. S3E), and in NIH 3T3/RasG12V–transformed cells relative to genetically matched control cells (Supplementary Fig. S3F).

**UACA blocks apoptosis by Par-4**

We investigated whether the interaction of intracellular Par-4 with UACA regulated the apoptotic effects of extracellular Par-4. To address this question, we knocked down the expression of UACA with siRNA in PC-3, H460, and A549 cell lines and assessed the apoptotic effects of extracellular TRX-Par-4 protein added to the medium. UACA knockdown rendered A549 cells sensitive to extracellular TRX-Par-4 relative to thioridoxin control (Fig. 4A). PC-3 and H460 cells were rendered further sensitive to TRX-Par-4 protein following UACA knockdown with multiple RNA interference (RNAi) approaches (Fig. 4A and Supplementary Fig. S4, data not shown). These findings indicate that UACA inhibits apoptosis by extracellular Par-4.

As UACA knockdown resulted in sensitization to apoptosis by extracellular Par-4, we investigated whether UACA prevents intracellular Par-4 from translocating GRP78 to the cell surface. UACA knockdown in PC-3 cells resulted in a significant (P < 0.001) increase in the number of GRP78 receptors at
Figure 2. UACA binds to Par-4 and is regulated by NF-κB. A, UACA binds to Par-4 in one-on-one Y2H assay. Yeast cells were cotransformed with various constructs. 1, positive control (pB27-TCF4 and pP7β-catenin); 2, negative control (pB27empty, which contains only the DNA binding domain and pP7empty, which contains only the TA domain and is identical to pP6); 3, negative control (Par-4 + pP7empty); 4, negative control (pB27empty + Sfrs11); 5, Par-4 + Sfrs11; 6, negative control (pB27empty + UACA); 7, Par-4 + UACA. The cells were then cultured in a 96-well plate, and the indicated serial dilutions were spotted on solid medium lacking tryptophan, leucine, and histidine, and containing 0, 5, or 10 mmol/L 3-amino-1,2,4-triazole (3AT). Sfrs11 is another prospective, albeit weaker, binding partner of Par-4. Interaction for 2 independent yeast clones is shown. B, UACA binds to Par-4 in human cell lines. Whole-cell lysates from the indicated cell lines were subjected to co-IP with Par-4 or p65/NF-κB (control) antibody. The immunoprecipitates were subjected to Western blot analysis. Lysates from each cell line were included as input samples. UACA, approximately 160 kDa; p65, 65 kDa; Par-4, approximately 40 kDa; GRP78, approximately 78 kDa. C, UACA colocalizes with Par-4. H460 cells were subjected to ICC analysis for UACA (green fluorescence) and Par-4 (red fluorescence) or for calnexin (red fluorescence) and UACA (green fluorescence), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The images were overlayed to determine colocalization (yellow fluorescence). More than 70% cells showed colocalization of Par-4 and UACA. D, NF-κB activity is essential for UACA expression. Western blot (WB) analysis for the indicated proteins was conducted on whole-cell lysates from: MEFs from IKKβ−/− or IKKβ+/+ mice (left); NIH 3T3/Ras G12V or PC-3 cells infected for 18 hours with hxB-SR adenovirus or GFP adenovirus (middle); or various cell lines treated with PS-1145 or dimethyl sulfoxide (DMSO) for 24 hours (right).
NF-κB Regulates Cell Surface GRP78

Figure 3. UACA expression is elevated in cancer tissues. A–C, expression of UACA, p65/NF-κB, or GRP78 in normal and cancer tissues. Sections of normal prostate (n = 20) or grade 1 prostate adenocarcinoma (n = 24; A and C) and normal lung (n = 10), lung adenocarcinoma (n = 38) or squamous cell lung carcinoma (n = 39; B and C) were subjected to IHC with either a control antibody or UACA antibody (A–C), p65/NF-κB antibody (C, left), or GRP78 antibody (C, right) using 3,3′-diaminobenzidine (DAB; brown), and nuclei were counterstained blue with hematoxylin. As expected, no staining was observed with the control antibody (Supplementary Fig. S3A), and detection of UACA with the UACA antibody was neutralized by corresponding UACA peptide (Supplementary Fig. S3B). Representative sections are shown at ×100 (A and B). UACA or GRP78 expression in the normal and cancer tissues was scored as ≤1+, 2+, or ≥3+. Percentage of tissues coexpressing nuclear p65 expression and high (≥3+) UACA (C, left), or nuclear p65 expression/high (≥3+) UACA and ≤1+, 2+, or ≥3+ levels of GRP78 (C, right) is shown. D, UACA upregulation correlates with NF-κB activation. Western blot (WB) analysis was conducted on lysates prepared from freshly frozen prostate cancer or lung cancer tissues and corresponding matched normal tissue controls.

the cell surface (Fig. 4B). These results imply that UACA modulates sensitivity to extracellular Par-4 preventing translocation of GRP78 to the cancer cell surface.

Primary prostate epithelial cells PrE, on the other hand, were resistant to cell surface GRP78 translocation and apoptosis by TRX-Par-4 after knockdown of UACA (Fig. 4C). Moreover, consistent with our previous observation that GRP78 cell surface trafficking is associated with an endoplasmic reticulum stress response (1), we noted that expression of the endoplasmic reticulum stress response proteins, GRP78, phospho-PERK, and CHOP/GADD153 was elevated after UACA knockdown in the cancer cells but not in the normal cells (Fig. 4B and C). These findings indicate that UACA regulates endoplasmic reticulum stress and GRP78 cell surface translocation in cancer cells but not in normal cells.

Our previous studies (1) have indicated that normal fibroblasts secrete Par-4 in their conditioned medium. To confirm that Par-4 secreted by mammalian cells shows results similar to those seen with recombinant TRX-Par-4, we tested the conditioned medium from NIH 3T3 cells for
Figure 4. UACA knockdown induces apoptosis by Par-4. A and B, UACA knockdown affects cancer cells. The indicated cell lines were transfected with UACA siRNA or control siRNA duplexes. As indicated in Supplementary Fig. S4, transfection with 2 different siRNA duplexes and 1 shRNA was used to confirm the findings. B, upregulation of GRP78 at the cell surface was determined by FACS analysis of unfixed cells. A, the cells were treated with thioredoxin or TRX-Par-4 (100 nmol/L) for 24 hours and subjected to immunocytochemistry for active caspase-3 and scored for apoptosis. Knockdown of UACA was confirmed by Western blot (WB) analysis. C, UACA knockdown does not affect normal cells. PrE cells were transfected with siRNA duplexes, and upregulation of GRP78 at the cell surface was determined by FACS analysis of unfixed cells (left). Knockdown of UACA was confirmed by Western blot (WB) analysis (left) or the cells were treated with thioredoxin or TRX-Par-4 (100 nmol/L) for 24 hours and scored for apoptosis (right). D, effects of Par-4 protein secreted by mammalian cells. PC-3 cells were treated with conditioned medium (CM) from NIH 3T3 fibroblasts or with RPMI control medium, in the presence of PS-1145 or DMSO, and no antibody, PTEN antibody or Par-4 antibody (left), or GRP78 N-terminal antibody or control antibody (right). Moreover, the cells were transfected with UACA siRNA or control siRNA duplexes, then treated with conditioned medium from NIH 3T3 fibroblasts or with RPMI control medium for 24 hours and scored for apoptosis (middle). Par-4 in the conditioned medium was confirmed by Western blot analysis using albumin in the Coomassie blue (CB)-stained gel to normalize loading (left, inset). Each treatment used 5 nmol/L secreted Par-4 protein, as judged by quantitative Western blot analysis (not shown). Asterisk (*) indicates statistically significant (P < 0.001) difference by the Student t test; and ** indicates that the effect is significant (P < 0.001) based on 2-way ANOVA with data normality and equality of variance assumptions.
apoptosis. The conditioned medium from NIH 3T3 cells, but not RPMI-1640 growth medium, produced apoptosis of PC-3 cells (Fig. 4D, left). The action of the conditioned medium was neutralized by the Par-4 antibody, indicating that the apoptotic activity of the conditioned medium was induced by Par-4. PS-1145 sensitized the cells to Par-4 in the conditioned medium (Fig. 4D, left). The action of Par-4 in the conditioned medium was also enhanced by siRNA-mediated knockdown of UACA (Fig. 4D, middle). The action of Par-4 in the conditioned medium was abrogated by an amino-terminal GRP78 antibody, which blocks binding of extracellular Par-4 to the amino-terminus of cell surface GRP78 (1), but not with control PTEN antibody (Fig. 4D, right). Collectively, these findings indicate that NF-κB activity and UACA negatively regulate apoptosis by Par-4 secreted naturally by mammalian cells.

We then interrogated whether UACA suppression following NF-κB inhibition was essential for enhanced apoptosis. A549 cells were transfected with red fluorescent protein (RFP)-UACA or RFP expression constructs driven by the cytomegalovirus (CMV)-promoter, then infected with IκB-SR or GFP adenovirus and treated with TRX-Par-4 or thioredoxin protein. RFP-UACA, but not RFP control vector, rescued the cells from the apoptotic effect of IκB-SR plus TRX-Par-4 (Fig. 5A) or IκB-SR plus the GRP78 carboxyl-terminal antibody (Fig. 5B). RFP-UACA inhibited cell surface GRP78 levels that were induced by IκB-SR (Fig. 5C). Together, these results indicate that suppression of UACA following inhibition of NF-κB activity is necessary for enhancing cell surface expression of GRP78 and apoptosis by Par-4.

We next tested whether binding of UACA to intracellular Par-4 was necessary to rescue the cells from apoptosis by extracellular Par-4. These experiments used full-length UACA
and a deletion mutant of UACA (1-630aa), which could bind to endogenous Par-4, or another deletion mutant of UACA (631-1413aa) that failed to bind to endogenous Par-4 (Supplementary Fig. S5). Consistently, full-length UACA and 1-630aa mutant, but not 631-1413aa mutant, prevented PS-1145-inducible GRP78 translocation to the cell surface (Fig. 5D, right), and rescued the cells from the apoptotic action of naturally secreted Par-4 (±PS-1145) in the conditioned medium from NIH 3T3 fibroblasts (Fig. 5D, left). Collectively, these findings indicate that UACA binding to intracellular Par-4 is essential for inhibition of GRP78 translocation to the cell surface and apoptosis by extracellular Par-4.

**Discussion**

GRP78 is an endoplasmic reticulum–resident protein involved in cell survival and autophagy (8–10). GRP78 is selectively expressed at the plasma membrane of cancer cells owing to increased endoplasmic reticulum stress caused by cumulative genetic aberrations and altered metabolic dependency (8). Par-4, localized at the endoplasmic reticulum, binds and promotes the translocation of GRP78 to the cancer cell surface (1). The present study uncovered UACA as a Par-4–binding protein, which sequesters Par-4 and prevents it from translocating GRP78 to the cell surface. Accordingly, suppression of UACA expression by RNAi led to GRP78 translocation to the cell surface and sensitization of the cells to apoptosis by extracellular Par-4 protein or GRP78 agonistic antibody. Thus, UACA is a functional regulator of sensitivity to extracellular inducers of apoptosis in cancer cells. Importantly, UACA expression is regulated by NF-κB activity, which is elevated in cancer cells, and NF-κB–specific inhibitors cause suppression of UACA expression to promote GRP78 translocation to the cell surface. The endogenous NF-κB-UACA-Par-4-GRP78 pathway was recapitulated by using vincristine, which activates endogenous Par-4 by PKA-mediated phosphorylation and thereby inhibits NF-κB activity (Supplementary Fig. S6). Because cell surface GRP78 also responds to apoptosis by the GRP78 agonistic antibody and several other growth inhibitory proteins and small molecules (6, 11), our findings imply that inhibition of the NF-κB pathway or UACA may be an effective strategy for sensitization and selective targeting of cancer cells. The NF-κB pathway is known to promote cell survival by induction of proteins (Bcl2, BclXL, A1/BFL1, c-FLIP, and IAPs) that inhibit progression of apoptotic pathways at the downstream steps involving intracellular changes in mitochondrial membrane potential or caspase activation. This study indicates that NF-κB activity inhibits apoptosis by naturally secreted/extracellular Par-4 at the proximal step of receptor-ligand interaction by downregulating the translocation of GRP78 to the cell surface (Supplementary Fig. S7).

NF-κB activation plays a pivotal role in antiapoptosis, tumor promotion and progression, and therapeutic resistance in diverse cancers, and NF-κB inhibitors are considered promising candidates in cancer therapy (4, 5). We noted that UACA protein levels were elevated in cancer tissues and cell lines relative to counterpart normal tissues and cells, and elevated UACA expression correlated with p65/NF-κB nuclear localization/activity. These observations are in striking contrast with the reduced expression of UACA reported recently in lung tumors, in which NF-κB activity was not revealed (12). Furthermore, a majority of cancer tissues that coexpressed nuclear p65 and high levels of UACA showed relatively lower (i.e., 2+ rather than $\geq3+$) levels of GRP78. Inhibition of UACA expression by RNAi led to an endoplasmic reticulum stress response, elevated total and cell surface levels of GRP78 and sensitization to apoptosis by extracellular Par-4 in cancer cells (Fig. 4B). Consistently, all the treatments that cause UACA downregulation result in increased levels of total GRP78 in cancer cells (Supplementary Fig. S6E). GRP78 is a regulator of the unfolded protein response (UPR) pathway and is also induced by UPR signaling. An overall increase in total GRP78 may contribute to elevated cell surface expression of GRP78. However, intracellular Par-4 function is essential for translocation of GRP78 to the cell surface (Supplementary Fig. S6E and ref. 1). Increased levels of C/EBP homologous protein (a downstream proapoptotic UPR signaling component) in cancer cells in response to UACA inhibition (Fig. 4B) may contribute to the proapoptotic effect of extracellular Par-4. The precise molecular link between UACA inhibition and increased phosphorylation of PERK (Fig. 4B), a proximal sensor of UPR, is currently being investigated.

The levels of GRP78 were overall lower in normal cells and tissues relative to cancer cells and tissues, and although the NF-κB pathway may regulate UACA in normal cells, UACA knockdown in normal cells did not result in GRP78 translocation to the cell surface or sensitization to apoptosis by extracellular Par-4. This implies that the NF-κB–UACA–endoplasmic reticulum stress–GRP78 translocation pathway is truncated in normal cells but intact and fully functional in cancer cells. Together with the finding that a knockout mouse for UACA lacks a clear phenotype (13), these data indicate that in normal cells either UACA is dispensable or other redundant gene(s) compensate for loss of UACA function. UACA suppression combined with extracellular molecules that function via cell surface GRP78 signaling may therefore be an effective strategy for selective elimination of cancer cells.

In summary, our findings indicated that inhibition of GRP78 translocation to the cell membrane is a downstream effect of NF-κB activation. The action of NF-κB is mediated through UACA, which sequesters Par-4 and prevents it from translocating GRP78 to the cell surface. Importantly, this pathway can be targeted to overcome apoptosis-resistance in cancer cells. Thus, this study suggests a novel link between NF-κB activity, UACA levels, and resistance to extracellular inducers of apoptosis that act via cell surface GRP78 in cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** R. Burikhanov, S. Qiu, S.M. Lele, C. Horbinski

**Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis):** R. Burikhanov, S. Qiu, C. Horbinski

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