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A Novel Extrinsic Pathway for Apoptosis by Tumor Suppressor Par-4

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Summary

Prostate apoptosis response-4 (Par-4) is a pro-apoptotic protein with intracellular functions in the cytoplasm and nucleus. Unexpectedly, we noted Par-4 protein is spontaneously secreted by normal and cancer cells in culture, and by Par-4 transgenic mice that are resistant to spontaneous tumors. Short exposure to endoplasmic reticulum (ER) stress-inducing agents further increased cellular secretion of Par-4 by a brefeldin A-sensitive pathway. Secretion occurred independently of caspase activation and apoptosis. Interestingly, extracellular Par-4 induced apoptosis by binding to the stress response protein, glucose-regulated protein-78 (GRP78), expressed at the surface of cancer cells. The interaction of extracellular Par-4 and cell surface GRP78 led to apoptosis via ER stress and activation of the FADD/caspase-8/caspase-3 pathway. Moreover, apoptosis inducible by TRAIL, which also exerts cancer cell-specific effects, is dependent on extracellular Par-4 signaling via cell surface GRP78. Thus, Par-4 activates a novel extrinsic pathway involving cell surface GRP78 receptor for induction of apoptosis.

Keywords

Par-4; cell surface GRP78; apoptosis

Introduction

Prostate apoptosis response-4 (Par-4) is a leucine zipper domain protein identified in cells undergoing apoptosis in response to exogenous insults (Sells et al., 1994). Par-4 is expressed ubiquitously among the various tissue types, and resides in both the cytoplasm and the nucleus (Sells et al., 1997; El-Guendy et al., 2003). Although endogenous Par-4 is largely inactivated, and does not produce extensive apoptosis by itself, it is essential for the apoptotic function of diverse cytotoxic agents (Gurumurthy et al., 2005). Interestingly, Par-4 over-expression is sufficient to induce apoptosis in most cancer cells, but not in normal or immortalized cells, and this apoptotic action of Par-4 does not require its leucine zipper domain (El-Guendy et al.,

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2003). Deletion analyses have identified the core domain (aa137–195) of Par-4, designated SAC (selective for apoptosis in cancer cells), as the effector domain of Par-4. Like Par-4, the SAC domain is capable of nuclear entry, caspase activation, inhibition of NF-κB activity, and induction of apoptosis. The SAC domain is 100% conserved in rat, mouse, and human Par-4, and does not resemble other pro-apoptotic proteins (El-Guendy et al., 2003). Transgenic mice ubiquitously expressing the SAC domain of Par-4 are resistant to the growth of spontaneous and inducible tumors (Zhao et al., 2007). Consistent with its tumor suppressor function in mice (Garcia-Cao, et al, 2003), Par-4 is mutated or silenced in endometrial cancer (Moreno-Bueno et al., 2007), down-regulated in renal cell carcinoma (Cook et al., 1999), and in a non-functional state in prostate cancer due to inactivation by the cell survival kinase, Akt1 (Goswami et al., 2005). Our recent studies suggest Par-4 serves as an intracellular repressor of topoisomerase 1 (TOP1) catalytic activity, and regulates DNA topology to suppress cellular transformation by a SAC domain-independent mechanism (Goswami et al., 2008).

All Par-4 studies thus far have analyzed the role of intracellular (i.e., cytoplasmic or nuclear) Par-4. Distinct partner proteins of intracellular Par-4 have been confirmed, including ζPKC (Diaz-Meco et al., 1996), WT1 (Johnstone et al., 1997), ZIP kinase (Page et al., 1999), Akt1 (Goswami et al., 2005), and TOP1 (Goswami et al., 2008); each of these partner interactions requires the leucine zipper domain. Par-4 interactions with ζPKC or TOP1 in the cytoplasm or nucleus, respectively, impede NF-κB activity (Diaz-Meco et al., 1996; Goswami et al., 2008), and Par-4 interactions with WT1 inhibit the Bcl-2 promoter (Cheema et al., 2003); these interactions reveal distinct, cell compartment-specific roles for the Par-4 partners in growth regulation.

Rather unexpectedly, our recent studies suggest Par-4 and SAC domain are secreted by both normal/immortalized and cancer cells, and that these secreted proteins selectively inhibit the growth of cancer cells. We have identified a novel regulatory loop that involves the interaction of extracellular Par-4, via its SAC domain, with the cell surface receptor GRP78, and that is dependent on intracellular Par-4 in order to selectively induce apoptosis in cancer cells.

Results

Par-4 is secreted and induces apoptosis of cancer cells via its SAC domain

Although ectopic Par-4 and its SAC domain are well-known to exert their apoptotic effects in the nucleus of the cell in which they are produced, we have noted bystander activity in tumor regression studies wherein a single intratumoral injection of an adenoviral-Par-4 construct (with a 50–60% transduction efficiency) resulted in apoptosis in over 80% cells of the tumor (Chakraborty et al., 2001). To further characterize this bystander effect of Par-4, we transfected PC-3 cells in culture with GFP, Par-4-GFP, or SAC-GFP expression constructs, and subjected the cell populations to immunocytochemical (ICC) analysis for active caspase-3 to detect apoptosis. Intriguingly, apoptosis occurred not only in the green fluorescent Par-4-GFP or SAC-GFP transfectants, but also in neighboring cells that did not express the green-fluorescent Par-4-GFP protein (Figure 1A, right panel). Quantification of the cultures for apoptotic cells indicated a significantly large number of non-transfected cells underwent apoptosis with Par-4-GFP or SAC-GFP transfection, but not with the GFP construct (Figure 1A, left panel).

Further studies revealed the conditioned medium (CM) derived from Par-4-GFP- or SAC-GFP-transfected PC-3 and BPH-1 cells contained Par-4-GFP and SAC-GFP proteins, respectively (Figure 1B). The CM did not contain detectable levels of actin or ERK1/2 (Figure 1B), implying the GFP fusion proteins are not released as a result of apoptotic cell death. Significantly, CM from the GFP (control) and SAC-GFP transfectants also contained endogenous Par-4 (Figure 1B), indicating endogenous Par-4 is secreted by PC-3 cells. Moreover, immortalized, non-transformed BPH-1 cells, which do not undergo apoptosis with ectopic Par-4, were transfected

with either a pCB6+/Par-4 construct (lacking the GFP-tag) or a GFP control construct. CM from these cells showed secretion of both endogenous Par-4 and ectopic Par-4 (Figure 1B), demonstrating secretion of Par-4 is neither dependent on the GFP-tag, nor on apoptosis of the transfectants. Secretion of endogenous Par-4 was not limited to prostatic cells; immortalized human embryonic lung fibroblasts HEL, human embryonic kidney epithelial cells HEK293, and mouse fibroblasts NIH 3T3 showed secretion of Par-4 (Supplemental Figure S1A). Overall, mammalian cells secrete endogenous Par-4, as well as ectopic Par-4 and SAC protein.

To determine whether secreted Par-4 was functionally active, PC-3 cells and BPH-1 cells were treated with the CM prepared from PC-3 transfectants, and their response was examined by ICC for caspase-3 activation. As seen in Figure 1C, the CM from the Par-4-GFP and SAC-GFP transfectants (but not from the GFP control transfectants) induced apoptosis in PC-3 cells, but not in the BPH-1 cells. Importantly, when the CM was pre-incubated with neutralizing antibodies for GFP, Par-4, or PTEN (control), the apoptotic activity in the CM was neutralized by antibodies against Par-4/SAC and the GFP-tag, but not by the control PTEN antibody.

Because CM may contain other mammalian cell secreted proteins, we used recombinant Par-4 and SAC (TRX-Par-4 and TRX-SAC, respectively) as an alternate approach to determine whether extracellular Par-4 and SAC induce apoptosis. Exposure of PC-3 cells to TRX-Par-4 and TRX-SAC, but not TRX control protein, produced apoptosis in a dose- and time-dependent manner (Figure S1). When cancer cells PC-3, H460, and HeLa were treated with these proteins and scored for apoptosis, we noted that TRX-Par-4 and TRX-SAC, but not TRX, induced apoptosis in each cell line (Figure 1D). Conversely, TRX-Par-4 or TRX-SAC did not induce apoptosis in the non-transformed cells BPH-1 (Figure 1D). Indeed, TRX-Par-4 and TRX-SAC induced apoptosis in diverse other cancer cell lines, but not non-transformed cell lines (data not shown). We conclude recombinant Par-4 is competent to cause apoptosis in diverse cancer cell lines, and that the SAC domain of Par-4 is sufficient to support apoptosis.

Par-4 secretion occurs by a BFA-sensitive pathway and is not dependent on apoptosis

We also examined whether treatment of cells with pro-apoptotic proteins, such as TRAIL, potentiated the secretion of endogenous Par-4. PC-3 cells were treated with TRAIL for short time intervals (30–180 minutes), which were of insufficient duration to produce apoptosis, and the harvested CM and whole-cell lysates were subjected to Western blot analysis for endogenous Par-4 and actin. TRAIL did not increase the levels of intracellular Par-4 in the cell lysates, yet caused increased secretion of Par-4 in the CM (Figure 2A). To determine whether Par-4 is released via the conventional cell secretory pathway, we inhibited secretory transport with brefeldin A (BFA), which blocks anterograde ER export to the Golgi resulting in obstruction of secretion. Following pre-treatment with BFA, TRAIL caused a negligible increase in Par-4 in the CM, yet the amount of intracellular Par-4 was unaltered (Figure 2A). As BFA blocks protein trafficking from the ER to the Golgi, a characteristic step in cell secretion by the classical pathway, these data indicate secretion of endogenous Par-4 occurred by the classical pathway involving the ER-Golgi network.

We also determined whether Par-4 secretion in the presence of TRAIL was associated with activation of apoptosis. PC-3/DN-FADD cells stably expressing dominant-negative FADD, which inhibits TRAIL-inducible apoptosis, were treated with TRAIL for short time interval (Figure 2A). As seen in Figure 2A, TRAIL-induced Par-4 secretion in these cells. These findings confirm secretion of Par-4 is not dependent on apoptosis.

Par-4 secretion is associated with ER stress

As Par-4 induction is not dependent on apoptosis, we examined whether secretion of Par-4 is a cellular response to ER-stress. PC-3 cells were treated with prototypical inducers of ER-stress

(Marchand et al., 2006), such as thapsigargin (TG; an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) and tunicamycin (TU; an inhibitor of N-linked glycosylation), for short time intervals, and CM prepared from the cultures was examined for secreted Par-4 protein. As seen in Figure 2B (upper panel), TG and TU induced Par-4 secretion, which was severely attenuated by the presence of BFA. To confirm induction of ER-stress, PC-3 cells were treated with TG, TU, or TRAIL for short time intervals, and up-regulation of GRP78 and CHOP/GADD153, key indicators of ER-stress was examined. As seen in Figure 2B, TG, TU, and TRAIL increased expression of intracellular GRP78 and CHOP/GADD153 relative to untreated cells. As a whole, these findings imply secretion of Par-4 is associated with induction of ER-stress.

Par-4 and SAC bind to GRP78

To determine whether Par-4 induces apoptosis by binding to specific proteins, we performed GST-pull down assays on whole cell extracts from PC-3 cells, using either GST-Par-4 or GST as bait. GST-Par-4, but not GST, bound a number of proteins, and mass spectrometry identified the ~80 kDa band (Figure S2A) as GRP78 (mass spectrometry data are shown in Figure S2C and Table S1). GRP8 is a typical stress protein that is a member of the HSP70 superfamily of heat shock proteins (Lee, 2007). For verification, the proteins pulled down from the PC-3 lysates were subjected to Western blot analysis for GRP78; His-SAC-GFP, but not the His-GFP control, pulled down GRP78 protein from PC-3 cells (Figure S2B).

Seeking confirmation that endogenous GRP78 binds Par-4 in mammalian cells, we used PC-3 whole-cell lysates for co-immunoprecipitations with GFP, Par-4, or GRP78 antibodies, followed by immunoblotting for Par-4 or GRP78. The data demonstrate Par-4 antibody co-immunoprecipitated GRP78, and the GRP78 antibody co-immunoprecipitated Par-4 (Figure 3A), whereas the GFP antibody co-immunoprecipitated neither Par-4 nor GRP78. These findings indicate endogenous Par-4 is bound to GRP78 protein.

Although the above data indicate Par-4 binds GRP78, it was unclear if the SAC domain and GRP78 interact in the same manner. We transfected PC-3 cells with GFP, Par-4-GFP, or SAC-GFP expression constructs, and subjected the cell lysates to immunoprecipitation with either GFP or Par-4 antibodies. As seen in Figure 3B, GRP78 co-immunoprecipitated with Par-4-GFP (Figure 3B) when we used the Par-4 antibody with lysates from Par-4-GFP transfectants. In co-immunoprecipitations using Par-4 antibody and lysates from cells that were either untransfected or transfected with GFP, we co-immunoprecipitated GRP78 with endogenous Par-4. Likewise, when we used GFP antibodies with lysates from the SAC-GFP transfectants, GRP78 co-immunoprecipitated with SAC-GFP, whereas co-immunoprecipitations using GFP antibodies with lysates from either GFP-transfected cells or untransfected cells (as controls) did not yield GRP78. These data indicate Par-4 antibody co-immunoprecipitates endogenous Par-4 and ectopic Par-4-GFP with endogenous GRP78, and GFP antibody co-immunoprecipitates ectopic SAC-GFP with endogenous GRP78 (Figure 3B). Accordingly, the SAC domain is sufficient for interactions with GRP78.

As recent studies suggest cell surface GRP78 serves as a receptor for soluble ligands to induce either apoptosis or growth stimulation (Misra, et al., 2002; Davidson et al., 2005; Gonzalez et al., 2006), we explored whether (1) Par-4 co-localizes with GRP78 at the plasma membrane, and (2) cell surface GRP78 is essential for apoptosis by extracellular Par-4 and extracellular SAC. Treatment of PC-3 cells with TRAIL, TRX-Par-4, and TRX-SAC, but not TRX, caused up-regulation of ER stress proteins GRP78 and CHOP/GADD153 (Figure S3A). FACS analysis performed on PC-3 cells, which were not chemically fixed in order to allow detection of cell surface proteins, indicated a significant increase ($P < 0.001$ by Student t test) in GRP78 expression on the cell surface upon treatment with TRAIL, TRX-Par-4, or TRX-SAC (Figure 3C and Figure S3B). Similarly, TRAIL treatment caused an increase in the expression of

GRP78 and Par-4 protein in the membrane fraction, and GRP78 and Par-4 were co-immunoprecipitated from this fraction (Figure S3F). To further confirm the cell surface interaction of endogenous GRP78 and Par-4, PC-3 cells were treated with TRAIL, then intact cells were biotinylated to label cell surface proteins. Whole-cell extracts were subjected to immunoprecipitation with GRP78, Par-4, or PTEN control antibody, and biotinylated proteins were detected with avidin-HRP. The GRP78 antibody, as well as the Par-4 antibody, co-immunoprecipitated biotinylated Par-4 and biotinylated GRP78 (Figure 3D). Moreover, TRX-Par-4 but not TRX control protein, pulled-down biotinylated GRP78 (Figure 3D). These findings support Par-4 binding GRP78 at the cell membrane.

To examine co-localization of Par-4 and GRP78, we exposed PC-3 cells and BPH-1 cells (which do not respond to TRAIL; see Figure S3A) to TRAIL or vehicle, then performed ICC analysis for Par-4 and GRP78. As seen in Figure 3E, PC-3 and BPH-1 cells treated with vehicle showed co-localization of Par-4 and GRP78 in the ER. However, upon treatment with TRAIL, PC-3 cells showed membrane translocation of GRP78, whereas BPH-1 cells retained GRP78 in the ER (Figure 3E). Notably, in TRAIL-treated PC-3 cells, but not TRAIL-treated BPH-1 cells, Par-4 co-localized with GRP78 at the plasma membrane (Figure 3E and Figure S3E), indicating Par-4 co-localizes with GRP78 at the plasma membrane in response to TRAIL exposure. We further confirmed co-localization of GRP78 and Par-4 with the ER marker, calnexin, and plasma membrane marker, Na^+/K^+-ATPase, in PC-3 cells and BPH-1 treated with either TRAIL or vehicle (Figure S3C).

GRP78 cell surface interaction is essential for apoptosis by extracellular Par-4

We next determined whether cell surface GRP78 is essential for apoptosis by extracellular Par-4. PC-3 cells were pre-treated with GRP78 or control GST protein, subsequently treated with TRX-Par-4, and finally scored for apoptotic cells. TRX-Par-4 induced apoptosis in the presence of GST protein, but failed to induce apoptosis in the presence of GRP78 protein (Figure 4A).

To further elucidate the interaction between extracellular Par-4 and cell surface GRP78, we pre-incubated PC-3 cells with neutralizing antibodies against various regions of GRP78, and then treated the cells with TRX-Par-4 or TRX. As seen in Figure 4B, apoptosis induced by TRX-Par-4 was blocked by the N-terminal-GRP78 antibody (NT-GRP78/N-20), but not by antibodies (H-129 for amino acids 525–653, or CT-GRP78/C-20) targeting the C-terminus of GRP78. Similarly, in lung cancer cells (H460) and cervical cancer cells (HeLa) apoptosis by TRX-Par-4 was blocked by the N-terminal GRP78 antibody, but not the C-terminal GRP78 antibodies (Figure S4A). These findings indicate the N-terminal region of cell surface GRP78 is essential for interaction with, and apoptosis by, extracellular Par-4. Apoptosis induced by TRX-Par-4 or TRX-SAC was abrogated by pre-incubation of PC-3 cells with NT-GRP78 neutralizing antibody, but not with several other antibodies (Figure 4C). Interestingly, when PC-3 cells were transfected with Par-4-GFP, SAC-GFP, or GFP constructs, and incubated with neutralizing antibody for GRP78 (NT-GRP78), Par-4, or PTEN (control), antibody-mediated inhibition of cell surface GRP78 prevented extracellular-Par-4 or -SAC from inducing apoptosis in the primary transfectants and non-transfected cells (Figure S4C). In addition, RNAi-mediated knock-down of GRP78 in PC-3 cells resulted in diminished expression of GRP78, both in whole-cell lysates and at the plasma membrane, and these cells were resistant to apoptosis by TRX-Par-4 (Figure S5). Collectively, these results suggest extracellular-Par-4 and -SAC induce apoptosis in PC-3 cells (transfected or non-transfected) by a GRP78-dependent mechanism.

Because we noted increased secretion of Par-4 by PC-3 cells in response to TRAIL (Figure 2B), and because PC-3 cells, but not BPH-1 cells, are sensitive to apoptosis induced by TRAIL (Figure S3A), extracellular-Par-4, and extracellular-SAC (Figure 1D), we asked whether (1)

extracellular-Par-4 is essential for apoptosis by TRAIL, and (2) extracellular-Par-4/GRP78 interaction is required to induce apoptosis by TRAIL. PC-3 cells were incubated with neutralizing antibody for GRP78, Par-4, or PTEN (for control), and next treated with either TRAIL or vehicle. As seen in Figure 4D, TRAIL-inducible apoptosis was inhibited by the Par-4 antibody, as well as by the GRP78 antibody, but not by the PTEN antibody. These data imply secreted Par-4 and cell surface GRP78 are requisite for TRAIL-induced apoptosis.

Given these *in vitro* observations, we explored Par-4 secretion and biological activity *in vivo*. Our laboratory recently described SAC-transgenic mice that are resistant to the growth of spontaneous tumors, whereas GFP-transgenic mice and littermate control mice develop spontaneous tumors (Zhao et al., 2007). By extension, Par-4 transgenic mice are also resistant to the growth of spontaneous tumors (Table S2). The bone marrow cells and serum of GFP- and Par-4-transgenic animals, as well as littermate control mice, were tested for expression of GFP and Par-4 protein. As seen in Figure 4E, the bone marrow cells from the transgenic animals expressed GFP or Par-4-GFP, while the serum from the Par-4-transgenic mice, but not from the GFP and littermate control mice, contained detectable levels of Par-4-GFP protein.

Next, we tested the biological activity of the serum from Par-4 transgenic mice against normal/immortalized cells and cancer cell lines. The serum from Par-4-GFP-transgenic mice, but not from GFP-transgenic mice and littermate control mice, induced apoptosis in PC-3 cells, but not in BPH-1 cells (Figure 4E, bottom left panel). To verify the apoptotic activity of the serum from the Par-4-transgenic mice was indeed due to the Par-4-GFP protein, serum samples were pre-treated with GFP or Par-4 antibody to neutralize Par-4-GFP protein, or with GRP78 antibody to impede binding of Par-4-GFP to cell surface GRP78, and applied to PC-3 cells in culture. As seen in Figure 4E (bottom right panel), GFP and Par-4 antibodies, but not the control PTEN antibody, prevented the serum from Par-4-GFP mice from inducing apoptosis in the PC-3 cells. Significantly, GRP78 antibody also negated the apoptotic effects of the serum from the Par-4-GFP mice (Figure 4E, bottom right panel). These results indicate the Par-4 protein is secreted in mice, and effects apoptosis via cell surface GRP78 binding, thereby inhibiting the growth of tumor cells.

Role of intracellular Par-4 in regulation of apoptosis by extracellular Par-4

As intracellular Par-4 is essential for apoptosis in cancer cells (Goswami et al., 2007), we investigated its role in the apoptotic action of extracellular Par-4. In this effort, we knocked-down the expression of endogenous Par-4 by RNA-interference, and then exposed the cells to recombinant Par-4, SAC, or control protein. Cells transfected with control siRNA underwent apoptosis upon treatment with TRX-Par-4 or TRX-SAC, whereas cells transfected with Par-4 siRNA did not (Figure 5A). Likewise, endogenous Par-4 knock-down did not alter GRP78 levels in GFP control protein treated cells, but inhibited induction of GRP78 by TRX-Par-4 or TRX-SAC (Figure 5A). We conclude endogenous Par-4 expression is essential for ER-stress dependent induction of GRP78 and for apoptosis by extracellular Par-4.

As cell surface GRP78 is essential for apoptosis induced by extracellular Par-4, we sought to determine whether intracellular Par-4 regulates GRP78 expression at the cell surface. PC-3 cells were incubated with Par-4 or control siRNA duplexes, treated with TRX or TRX-Par-4, and examined for cell surface GRP78 expression by FACS analysis. Knock-down of endogenous Par-4 by siRNA resulted in severe reduction of cell surface GRP78 expression (Figure 5B). However, total GRP78 protein levels in the cell lysates were unaltered by Par-4 knock-down (Figure 5B), implying that Par-4 was essential for trafficking GRP78 to the plasma membrane.

Next, we restored expression of cell surface GRP78 by transfecting the PC-3 cells with membrane-directed full-length GRP78 (mGRP78) or its N-terminal mutant (mΔN-GRP78;

lacking 66 amino acids at the N-terminus), following Par-4 knock down, and tested the cells for sensitivity to extracellular Par-4. TRX-Par-4 induced apoptosis in cells transfected with mGRP78, but not mΔN-GRP78 (Figure 5C). Similar observations were made in H460 cells and HeLa cells (Figures S6 and S7A). We also tested immortalized BPH-1 cells, which do not respond to extracellular Par-4, for apoptosis in response to extracellular Par-4 after transfection with mGRP78 or mΔN-GRP78. Cell membrane-directed full-length mGRP78, but not mΔN-GRP78, rendered BPH-1 cells sensitive to apoptosis by TRX-Par-4 (Figure S7B). As expected, Par-4 binds to full-length mGRP78, but not mΔN-GRP78 (Figures S7C). Collectively, these findings indicate intracellular Par-4 is essential for trafficking of GRP78 to the plasma membrane, and that extracellular Par-4 induces apoptosis by binding to the N-terminal region of cell surface GRP78.

Discussion

We have uncovered a novel extracellular role for Par-4 protein. Par-4 is spontaneously secreted by cultured cells in conditioned medium, and Par-4 transgenic mice secrete Par-4 in their serum. Spontaneous secretion of Par-4 was noted in both normal and cancer cell cultures, and was further enhanced by exposure to ER stress-producing agents, such as TG, TU, and TRAIL. Secretion of Par-4 occurred by a BFA-sensitive pathway, and was not dependent on apoptosis of the cells. Importantly, extracellular Par-4 was functionally active, and bound to cell surface GRP78 protein to induce apoptosis in cancer cells. The SAC domain of Par-4 also interacts with GRP78, therefore implying Par-4 binds to GRP78 independently of its carboxy-terminal leucine zipper domain. Extracellular Par-4/SAC binding to cell surface GRP78 produces a robust ER stress response loop involving up-regulation and trafficking of GRP78 to the cell membrane, in an intracellular Par-4-dependent manner (Figure 6). Moreover, extracellular Par-4 activates caspase-8 and caspase-3 in a FADD-dependent manner (Figures S8 A-E). Consistent with a recent report suggesting ER-stress causes activation of PERK (PKR-like ER kinase), a proximal component of the ER stress pathway, which is known to activate FADD and caspase-8 dependent apoptosis (Park et al., 2008), we noted inhibition of PERK expression by RNAi led to inhibition of caspase-8 dependent apoptosis by extracellular Par-4 and TRAIL (Figure S8 F). This extracellular function of Par-4/SAC constitutes a paradigm shift in our interpretation of the mechanism of Par-4 action, and uncovers a broader therapeutic significance for this cancer cell-selective apoptotic molecule.

Secretion of Par-4

Our studies indicate intracellular Par-4 co-localizes with GRP78 in the ER, and is secreted spontaneously via the classical BFA-sensitive pathway involving the ER/Golgi network. Par-4 secretion is further enhanced by TG, TU, and TRAIL. Interestingly, all three agents caused up-regulation of GRP78 and CHOP/GADD153, two key molecular indicators of ER stress/unfolded protein response. Although TG and TU are known to produce ER stress (Marchand et al., 2006), and 3,3′-diindolylmethane induces apoptosis by ER stress signaling via activation of caspase-8 and -3 (Abdelrahim et al., 2006), the role of ER stress in the action of TRAIL was not recognized previously. Similar to many other proteins that are secreted into the extracellular environment, Par-4 does not have a readily discernible signal peptide sequence. Par-4 may, however, contain atypical short signal peptide sequence(s), and this possibility is currently being investigated.

Apoptotic signaling via GRP78 cell surface receptor

In this report we provide several lines of evidence to confirm a physical and functional interaction between Par-4 and GRP78 at the cell surface. These include: (a) Par-4 and GRP78 co-localize at the plasma membrane, as judged by ICC studies using markers for the ER and plasma membrane; (b) Par-4 and GRP78 co-immunoprecipitate from the membrane fraction

using either Par-4 or GRP78 antibody; (c) Par-4 and GRP78 were biotinylated using cell-impermeable sulfo-NHS-LC-biotin, and co-immunoprecipitated by Par-4 or GRP78 antibody; (d) neutralizing antibodies against Par-4 or the N-terminus of GRP78 inhibit apoptosis by TRAIL or Par-4 in intact cells; (e) RNAi-mediated inhibition of Par-4 or GRP78 results in diminished GRP78 expression at the cell surface and resistance to apoptosis by exogenous Par-4; (f) cell membrane-directed GRP78 co-immunoprecipitates with Par-4; and (g) cell membrane-directed GRP78 can restore sensitivity to exogenous Par-4. Collectively, these data demonstrate Par-4 interacts with GRP78 at the cell surface.

GRP78 exists primarily as an ER protein with intracellular chaperone functions, and is up-regulated in response to ER stress in order to diminish growth inhibitory signals, and promote cell survival (Pootrakul et al., 2006; Lee, 2007). However, GRP78 is also expressed on the surface of most cancer cells and a few non-transformed cells, and serves as a receptor for extracellular ligands, such as alpha-2 macroglobulin (promotes cell proliferation) and Kringle 5 (induces apoptosis). It is also reported that VDAC may serve as a co-receptor for Kringle 5-mediated apoptosis of endothelial cells (Gonzales et al., 2006). Also, synthetic peptides have been specifically designed to bind cell surface GRP78, and induce apoptotic signaling (Arap et al., 2004). Although Kringle 5 has been reported to induce caspase-7 activation following GRP78 binding (Davidson et al., 2005), extrinsic Par-4 and its SAC domain induce activation of caspase-8 and -3, but not caspase-7 or -9 following cell surface GRP78 binding (Figures S8 A-F and S9). This indicates ligand-specific activation of distinct caspases prompts a similar cellular outcome following binding to cell surface GRP78. Consistent with the role of ER stress in the action of TRAIL and exogenous Par-4, cancer cells (such as DU145) that are resistant to apoptosis by TRAIL or exogenous Par-4 can be rendered sensitive to the action of these proteins by bortezomib (Velcade), which induces ER stress (Figure S10). We are currently investigating the precise role of the ER components phospho-PERK and phospho-eIF2Eα, which play a role in c-FLIP suppression following CD95 activation (Park et al., 2008), in activation of FADD and caspase-8 by exogenous TRAIL and Par-4. It is also noteworthy that, unlike all of the previously described protein-protein interactions of Par-4 that are mediated by its carboxy-terminal leucine zipper domain, binding of Par-4 to GRP78 is uniquely mediated by the SAC domain. Further deletion/mutational analysis will uncover the specific residues or sub-domains within the SAC sequence that promote binding and/or apoptotic signaling.

Mutual dependency of extracellular and intracellular Par-4 for induction of apoptosis

In the present study, ectopic over-expression of Par-4 and SAC induced apoptosis not only in transfected cells, but also in non-transfected cells within the same cell culture. Apoptosis in the transfected and non-transfected cells was inhibited by antibodies targeting either extracellular Par-4 or GRP78, implying both paracrine and autocrine effects for secreted Par-4. Consistently, inhibition of intracellular Par-4 expression by RNAi negates caspase 3 activation and apoptosis by extracellular Par-4. These findings reveal a mutual dependency between extracellular and intracellular Par-4 in activating apoptosis.

In contrast to intracellular GRP78, which may generally serve anti-apoptotic functions (Lee 2007), cell surface GRP78 promotes apoptosis by Par-4. Furthermore, intracellular Par-4 regulates the expression of GRP78 at the cell surface, as Par-4 knock-down does not alter total GRP78 levels in whole-cell lysates, yet the amount of cell surface GRP78 is severely down-modulated. As exogenous Par-4/SAC-inducible expression of GRP78 is inhibited upon knock-down of endogenous Par-4, we infer inadequate GRP78 expression at the cell surface precludes an ER stress-dependent increase in GRP78 in response to exogenous Par-4/SAC. These results are consistent with our observation that endogenous Par-4 is essential for cell surface expression of GRP78.

Although both non-transformed and cancer cells express GRP78 at the cell surface, only cancer cells exhibit ER stress and apoptosis in response to extracellular Par-4. Par-4 binds to the cell surface GRP78 molecules that are initially available on cancer cells, thereby causing ER stress that further enhances both translocation of GRP78 molecules to the cell surface and apoptosis. Non-transformed cells neither respond to extracellular Par-4 by ER stress, nor do they allow such trafficking of GRP78 to the cell surface. However, artificial over-expression of GRP78 at the cell membrane can promote apoptosis of the non-transformed cells. Therefore, differential ER stress response may contribute to the selective increase in cell surface trafficking of GRP78, thus leading to Par-4-induced apoptosis in cancer cells (also see Supplemental Discussion).

Similar to Par-4 and its SAC domain, TRAIL is known to selectively induce apoptosis in cancer cells only. This study reveals TRAIL causes secretion of Par-4, and that secreted Par-4 regulates apoptosis by TRAIL. Consequently, inhibition of either extracellular Par-4 or cell surface GRP78 impedes apoptosis by TRAIL. Our results further reveal intracellular Par-4 is essential for translocation of GRP78 from the ER to the cell surface in response to TRAIL, thereby triggering the binding of Par-4 and GRP78 at the cell surface to boost ER stress, and leading to activation of PERK and caspase-8. Inhibition of intracellular Par-4 by RNAi blocked all of these responses, as well as apoptosis by TRAIL (data not shown). These findings indicate extracellular and intracellular Par-4 are both essential for apoptosis by TRAIL.

In summary, the present study identified a novel extrinsic pathway for apoptosis by Par-4 acting via its SAC domain. This pathway is essential for apoptosis by TRAIL, which, similar to Par-4, induces cancer-specific apoptosis. The identification of an extracellular role for Par-4 and SAC significantly broadens their therapeutic potential for primary and metastatic tumors.

Experimental Procedures

Cell culture, plasmids, recombinant proteins, and chemical reagents

Human immortalized epithelial cells BPH-1, prostate cancer cells PC-3, lung cancer cells H460, cervical cancer cells HeLa, human primary lung fibroblasts HEL, human embryonic kidney cells HEK293, and mouse fibroblasts NIH 3T3 were from American Type Culture Collection, MD. The preparation of recombinant proteins and plasmid constructs is detailed in Supplemental Experimental Procedures.

The control siRNA and siRNA for Par-4 were from Dharmacon, Inc. and SantaCruz Biotechnology, Inc. TRAIL was purchased from R. & D. Systems. The antibodies for active caspases and ERK1/2 were from Cell Signaling, Inc. The monoclonal antibody for β-actin, purified GRP78 protein, and BFA were from Sigma Corp. All other antibodies (including those for GRP78) were from SantaCruz Biotechnology, Inc. (Santa Cruz, CA). GRP78 antibodies C-20, N-20, and H-129, were tested for inhibition of cell surface GRP78 interaction with Par-4; H-129 or 76-E6 was used for ICC and Western blot analysis; and H-129 was used for immunoprecipitation and FACS analysis.

Cell surface biotinylation

Cell surface proteins were biotinylated and visualized by treatment with avidin-horseradish peroxidase (avidin-HRP) as described in the Supplemental Methods.

GST-pull down assay, co-immunoprecipitation and Western blot analysis

Purified GST (6 μg) or GST-Par-4 (3 μg) protein was incubated with glutathione beads for 30 min, and subsequently washed and incubated with cell extracts (10 μg) for 18 h at 4°C. Protein eluted from the beads was resolved by SDS-PAGE, and subjected to Coomassie blue staining

and mass spectrometry at the Protein Core Facility of the Columbia University Medical Center, NY. Proteins were co-immunoprecipitated and detected by Western blot analysis as described (Goswami et al, 2005).

Immunocytochemistry, apoptosis, and FACS analysis

Immunocytochemical analysis and apoptosis procedures have been described (Goswami et al., 2005). For fluorescence-activated cell sorting (FACS) analysis, PC-3 cells were treated with recombinant proteins for 6 h and then, without fixing them to allow detection of GRP78 at the cells surface, the cells were subjected to FACS analysis using either GRP78 primary antibody (H-129) or no primary antibody as control, and R-phytoerythrin-conjugated secondary antibody (from Invitrogen, Inc.).

Statistical analysis

All experiments were performed in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System software (SAS Institute, Cary, NC) and *P* values were calculated using the Student *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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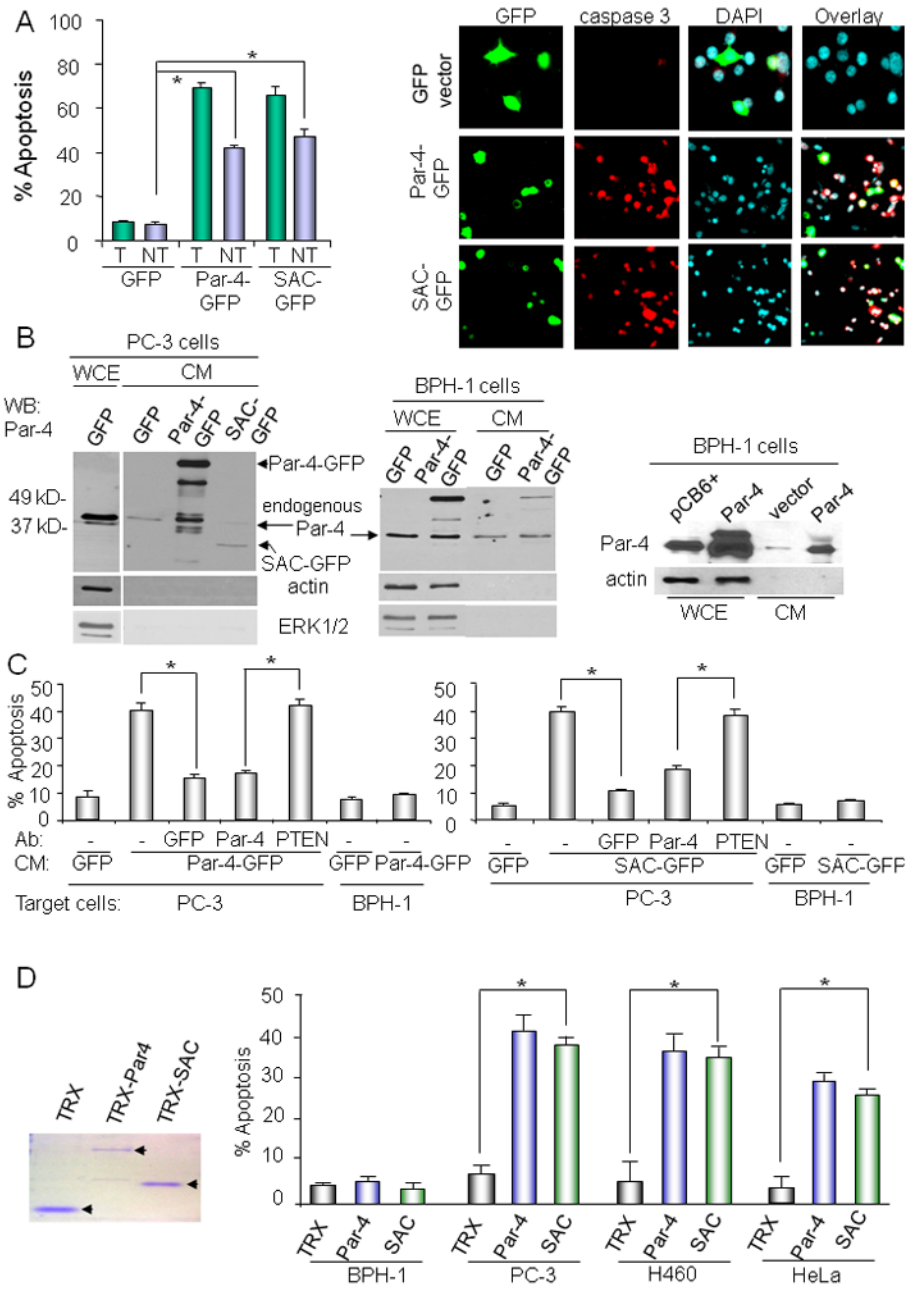

Figure 1. Par-4 and the SAC protein are secreted and induce apoptosis

A. Bystander effect of Par-4 and SAC domain. PC-3 cells were transiently transfected with the indicated expression constructs, and subjected to ICC for active caspase-3 to detect apoptosis, and counterstained with DAPI (right panel). Quantification of apoptosis in transfected (T) and non-transfected (NT) cells is presented (left panel).

B. Conditioned medium (CM) from transfected cells contains secreted Par-4-GFP and SAC-GFP protein. The CM from cells transfected with the indicated expression constructs or pCB6+ vector was subjected to Western blot (WB) analysis with the Par-4, ERK1/2, or actin antibody. Appropriate whole-cell extracts (WCE) were used as controls.

C. The secreted fusion proteins induce apoptosis in target PC-3 cells. Cells were treated with the CM from the indicated PC-3 transfectants (GFP, Par-4-GFP, or SAC-GFP) or with

the CM that was pre-incubated for 30 min with neutralizing antibody (Ab) for GFP, Par-4, or PTEN. After 24h, the cells were scored for apoptosis by caspase-3 activation using ICC analysis.

D. Recombinant Par-4 and SAC protein induce apoptosis in cancer cells. Recombinant protein was subjected to SDS-PAGE and Coomassie blue (CB) staining (left panel). Various cancer or non-transformed cells were treated with 100 nM amounts of each protein for 24 h (right panel). The cells were scored for apoptosis by ICC for active caspase-3. Panels C & D: Mean values (+ standard deviation bars) of three separate experiments are shown. Asterisk (*) indicates the difference is statistically significant ($P < 0.001$) by the Student's *t* test.

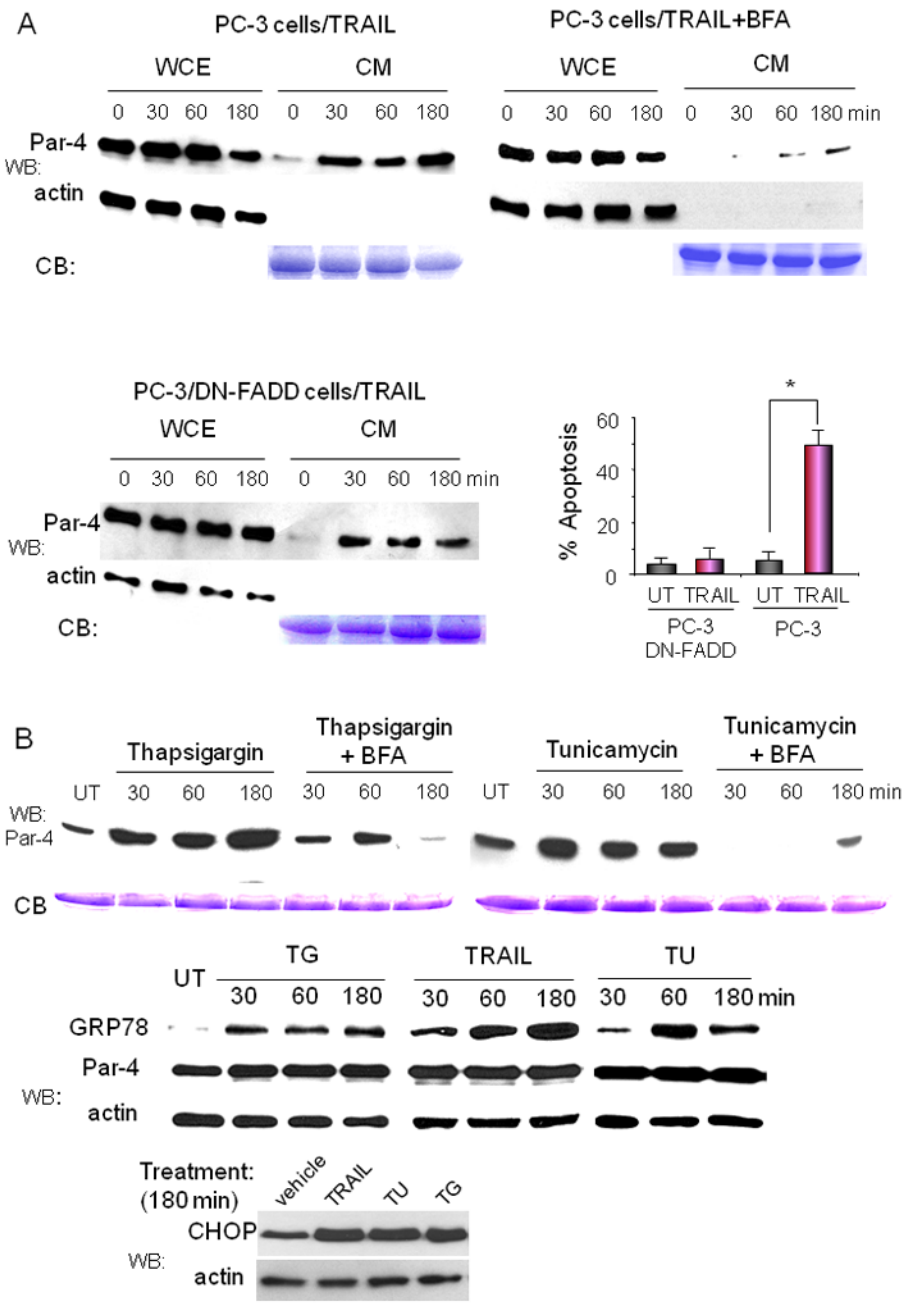

Figure 2. Par-4 protein is secreted by an ER-stress inducible pathway

A. Par-4 protein is secreted by a BFA-sensitive pathway independently of apoptosis. Cells were left untreated (UT) or pre-treated with BFA (1 μM) for 30 min as indicated, then exposed to TRAIL (100 ng/ml) for 30, 60, and 180 min. Whole-cell extracts (WCE) or CM were prepared, and subjected to Western blot (WB) analysis for Par-4 or actin. The CM was also subjected to SDS-PAGE followed by Coomassie blue (CB) staining to reveal the 68 kDa BSA band for loading control (upper panels and lower left panel). PC-3 or PC-3/DN-FADD cells were also treated with TRAIL or left untreated (UT) for 24 h, and scored for apoptosis by ICC for active caspase 3 (lower right panel). Mean values (+ standard deviation bars) of three separate experiments are shown. Asterisk (*) indicates the difference is statistically significant ($P < 0.001$) by the Student's *t* test.

B. Par-4 protein is secreted by an ER-stress inducible pathway. PC-3 cells were left untreated (UT) or treated with thapsigargin (TG,100 nM) or tunicamycin (TU, 5 μM) in the presence or absence of BFA (1 μM) for the indicated time intervals. CM was prepared, and subjected to SDS-PAGE followed by staining with Coomassie blue (CB; upper panel). Also, whole-cell extracts from untreated (UT) PC-3 cells or PC-3 cells treated with vehicle, TG, TU, or TRAIL for various time intervals were subjected to Western blot (WB) analysis for Par-4, GRP78, and actin (middle panel), or CHOP/GADD153 and actin (lower panel).

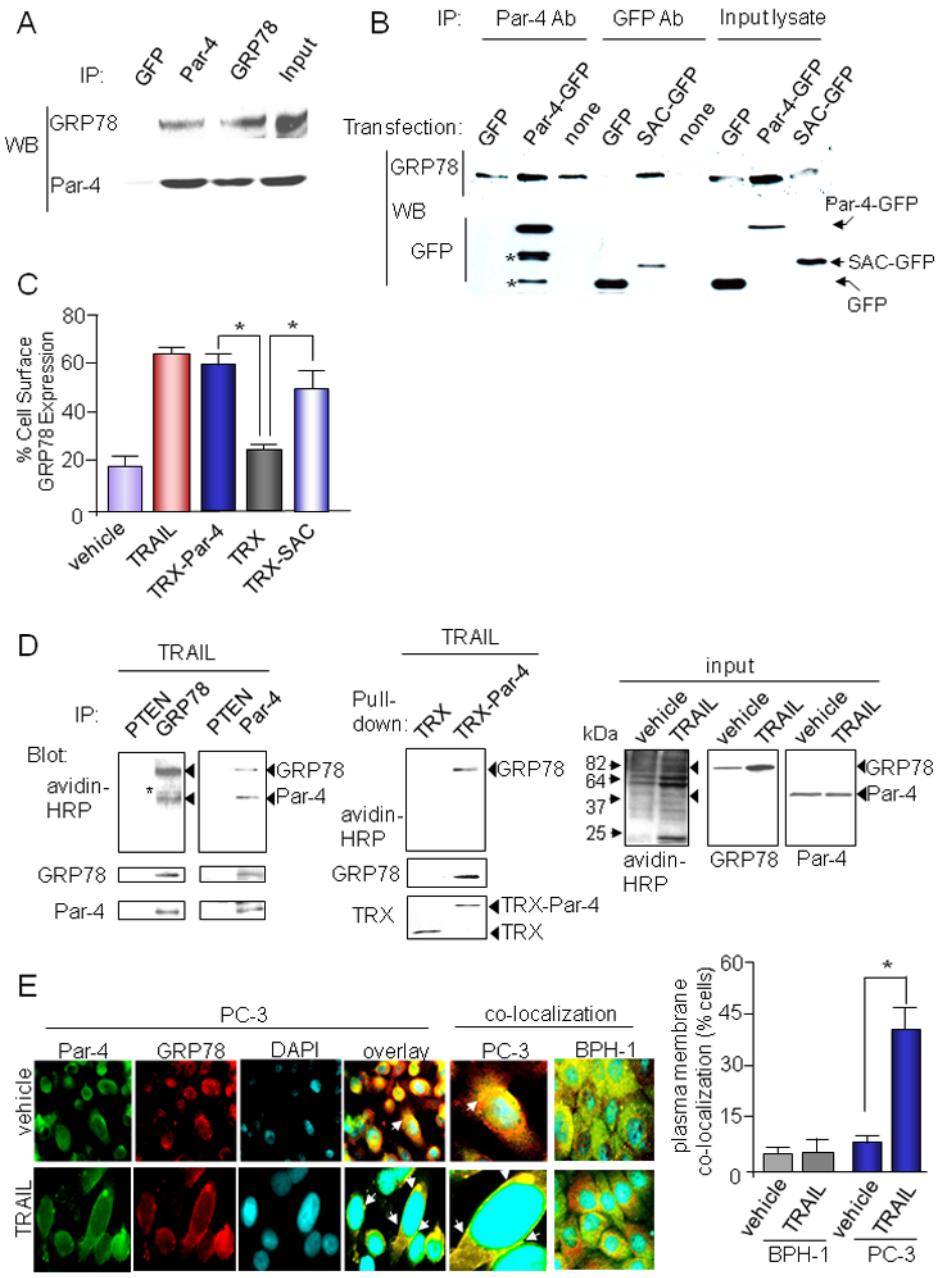

Figure 3. Par-4, via its SAC domain, binds to GRP78 and co-localizes with GRP78 in the ER and at the cell membrane

A. Par-4 binds to GRP78. Whole-cell extracts from PC-3 cells were subjected to immunoprecipitation (IP) with antibodies for Par-4, GRP78, or GFP, and the immunoprecipitated complexes were resolved by SDS-PAGE and subjected to Western blot (WB) analysis for GRP78 and Par-4. PC-3 whole-cell extracts were used as input.

B. Par-4 binds to GRP78 via its SAC domain. PC-3 cells were transfected with GFP, Par-4-GFP, or SAC-GFP expression constructs or left untransfected, and the cell lysates were subjected to immunoprecipitation (IP) with Par-4 or GFP antibody. The immunoprecipitated complexes were subjected to Western blot (WB) analysis for GRP78 or GFP. Whole-cell extracts from the transfectants were used as input.

C. Recombinant Par-4, SAC, and TRAIL individually increase GRP78 expression at the cell surface. PC-3 cells were treated with TRAIL (100 ng/ml), TRX-Par-4 (100 nM), TRX-SAC (100 nM), or TRX (250 nM) for various time intervals. The cells were collected after 6 h (unfixed, so the antibody could detect only cell surface GRP78) and subjected to FACS analysis for GRP78. Mean values (+ standard deviation bars) of three separate experiments are shown. Asterisk (*) indicates the difference is statistically significant ($P < 0.001$) by the Student's *t* test.

D. GRP78 binds Par-4 at the cell surface. Cells were treated with TRAIL (100 ng/ml) or vehicle for 6 h, and then labeled with cell-impermeable sulfo-NHS-LC-biotin. Whole-cell extracts (WCE) of the TRAIL-treated/biotinylated samples were subjected to immunoprecipitation (IP) with the indicated antibodies (left panels). For the pull-down of biotinylated GRP78 from TRAIL-treated cells, the corresponding WCE (10 μg) were incubated with polyhistidine-tagged recombinant proteins TRX-Par-4 or TRX control (2 μg of each) for 2 h at 4°C. Bound complexes were purified with metal affinity resin beads (middle panels). The immunoprecipitated proteins (left panels), bound complexes (middle panels), and also the corresponding WCE (indicated as 'input', right panels) were subjected to SDS-PAGE, and blotted with avidin-HRP or with GRP78, Par-4, or TRX antibody. The ~50 kDa band indicated by the asterisk is unidentified.

E. Par-4 co-localizes with GRP78 in the ER and in the cell membrane. Cells were treated with TRAIL (100 ng/ml) or vehicle for 6 h, and subjected to ICC for Par-4 (green fluorescence), GRP78 (red fluorescence), and nuclei were revealed by DAPI (cyan fluorescence). White arrows show co-localization of Par-4 and GRP78 in merged images (yellow fluorescence). A total of 400 cells were scored for co-localization of Par-4 and GRP78 at the cell membrane in each experiment, and mean values (+ standard deviation bars) of three separate experiments are shown in the right panel. Asterisk (*) indicates the difference is statistically significant ($P < 0.0001$) by the Student's *t* test. Magnification: For co-localization images- PC-3/TRAIL (200x); PC-3/vehicle (120x); and BPH-1 (80x); for all other BPH-1 images (40x) and PC-3 images (80x).

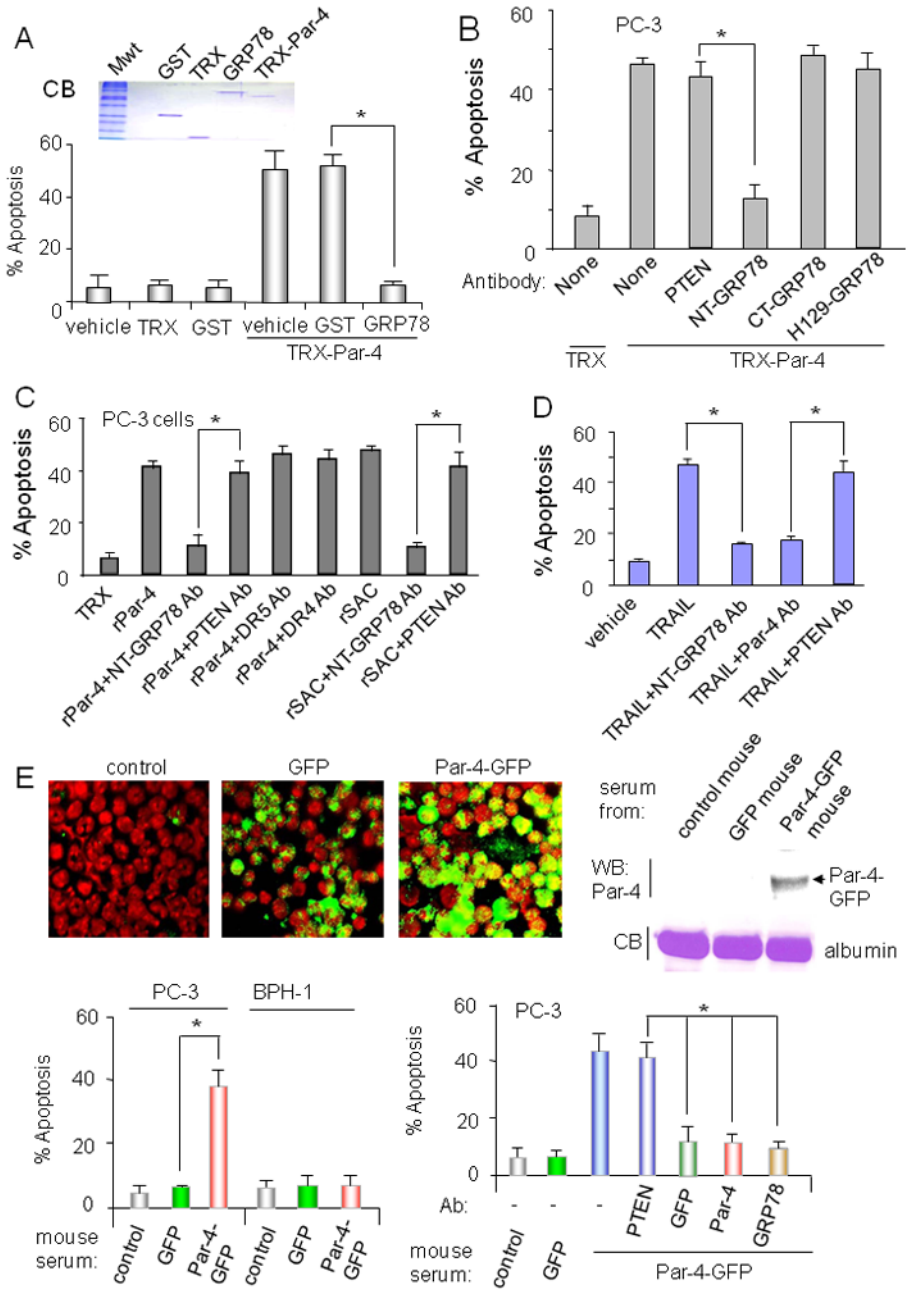

Figure 4. Extracellular Par-4, SAC, and TRAIL are dependent on cell surface GRP78 for induction of apoptosis

A. Extracellular GRP78 neutralizes the apoptotic action of TRX-Par-4. PC-3 cells were pre-treated with vehicle, GST, or GRP78 protein, and then treated with TRX-Par-4, TRX protein, or vehicle. After 24 h, cells were subjected to ICC for active caspase-3 (green fluorescence, see Supplemental Figures S4B), using DAPI (pseudo-colored red, lower panel) to reveal nuclei, and apoptosis was quantified. The integrity of the purified proteins was verified by SDS-PAGE and Coomassie blue (CB) staining using protein molecular weight markers (upper panel).

B. Neutralizing antibody (NT-GRP78) directed toward the N-terminal region of GRP78 inhibits apoptosis by extracellular Par-4. Cells were pre-incubated with the indicated

antibodies for 30 minutes, and then treated with TRX-Par-4 or TRX (100 nM of each) for 24 h. Apoptotic cells were scored by ICC for active caspase-3.

C. NT-GRP78-antibody neutralizes the apoptotic action of exogenous TRAIL, Par-4, and SAC. Cells were pre-treated with PTEN, NT-GRP78, DR4, or DR5 antibody for 30 min, and then treated with TRX-Par-4 (rPar-4), TRX-SAC (rSAC), or TRX. After 24 h, apoptosis was quantified by ICC for active caspase-3.

D. Par-4- and NT-GRP78-antibodies individually neutralize the apoptotic action of TRAIL. PC-3 cells were pre-treated with PTEN, NT-GRP78, or Par-4 antibody, then treated with TRAIL (100 ng/ml) or vehicle. After 24 h, apoptosis was quantified by ICC for active caspase-3.

E. Par-4, secreted in the serum of Par-4-transgenic mice, induces GRP78-mediated apoptosis in PC-3 cells. Bone marrow cells from littermate control, GFP-, and Par-4-GFP-transgenic mice were analyzed by ICC for GFP (upper left panel), and the serum from these mice was examined by Western blot for secreted Par-4 (upper right panel). Loading was verified by CB staining for serum albumin (upper right panel). Cells were treated with the serum samples (20% final concentration) either in the absence or presence of neutralizing antibodies, and, after 24 h, apoptosis was quantified by ICC for active caspase-3 (lower panel). In panels A-E, mean values (+ standard deviation bars) of three separate experiments are shown. Asterisk (*) indicates the difference is statistically significant ($P < 0.001$) by the Student's *t* test.

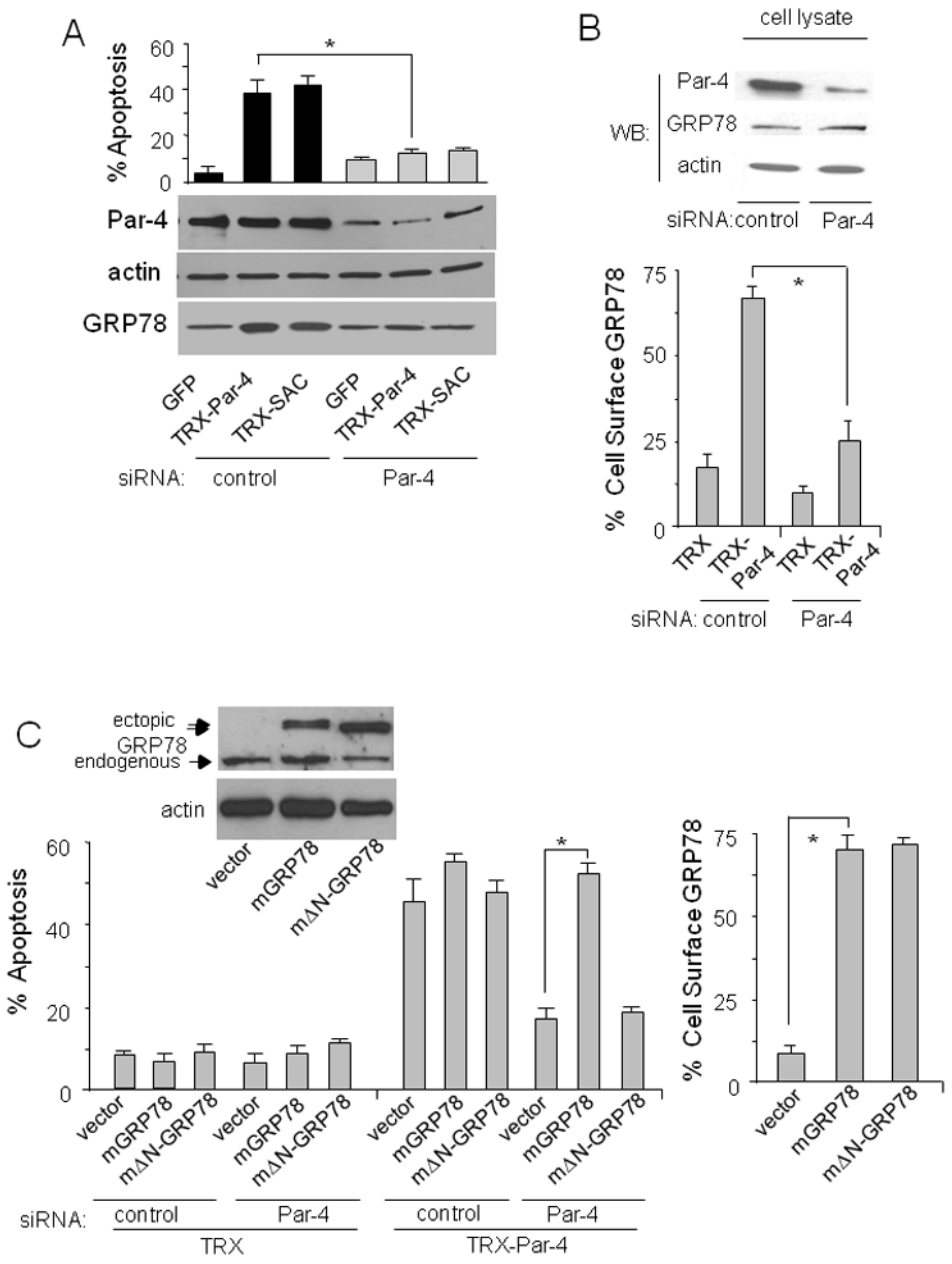

Figure 5.

Cell membrane-directed GRP78 overrides the effect of intracellular Par-4 knock-down and restores sensitivity to TRX-Par-4

A. Endogenous Par-4 expression is essential for apoptosis by extracellular Par-4. PC-3 cells were transfected with siRNA duplexes for Par-4 or control siRNA. The cells were treated with TRX-Par-4, TRX-SAC, or GFP protein for control for 24 h, and apoptotic cells were scored by evaluating active caspase-3 by ICC (upper panel). Western blot analysis (lower panel) confirmed both knock-down of Par-4 expression by siRNA and its effect on GRP78 expression.

B. Intracellular Par-4 regulates trafficking of cell surface GRP78. PC-3 cells were transfected with siRNA duplexes for Par-4 or control siRNA, treated with TRX or TRX-Par-4, and processed as intact, unfixed cells for expression of cell surface GRP78 by FACS analysis

(lower panel). Knock-down of intracellular Par-4 by siRNA was confirmed by Western blot analysis of corresponding whole-cell lysates (upper panel).

C. Expression of membrane-targeted GRP78 restores sensitivity to TRX-Par-4 following intracellular Par-4 knock-down. PC-3 cells were transfected with siRNA duplexes for Par-4 or control siRNA, and knock-down of intracellular Par-4 was confirmed as indicated in Panel B. The cells were then transfected with vector, cell membrane-directed full-length GRP78 (mGRP78), or ΔN mutant of GRP78 (mΔN-GRP78), and expression of endogenous and ectopic GRP78 in whole-cell lysates was confirmed by Western blot analysis (left panel inset). Finally, the transfectants were subjected to treatment with TRX or TRX-Par-4 for 24 h, and apoptotic cells were scored by evaluating active caspase-3 by ICC (left panel). Expression of cell surface GRP78, as determined by FACS analysis, is shown for Par-4 knock-down cells transfected with the mGRP78 construct or vector and subsequently treated with TRX-Par-4 (right panel). In panels A-C, mean values (+ standard deviation bars) of three separate experiments are shown. Asterisk (*) indicates the difference is statistically significant ($P < 0.001$) by the Student's *t* test.

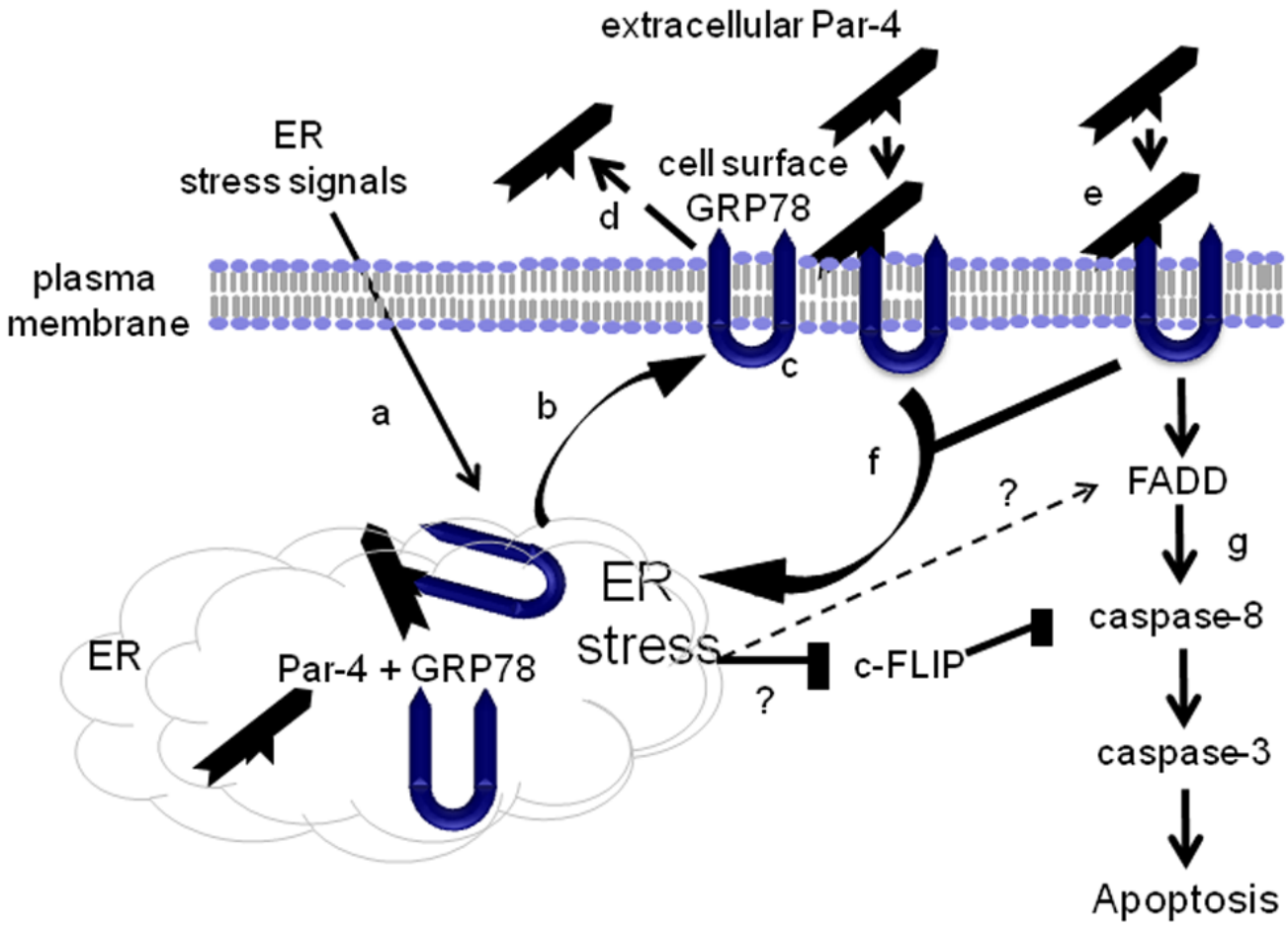

Figure 6.

Model for apoptosis by extracellular Par-4

ER stress (a), induced by extracellular insults such as TRAIL, causes translocation of the Par-4-GRP78 complex from the ER to the plasma membrane (b). This leads to elevated expression of GRP78 and Par-4 at the plasma membrane (c), and secretion of Par-4 (d). Extracellular Par-4 binds to cell surface GRP78 (e), and induces yet more ER stress (f), thereby activating the loop for translocation of ER GRP78 to the plasma membrane, in an intracellular Par-4-dependent manner. Finally, ER stress involves up-regulation of the ER component phospho-PERK, and activation of caspase-8 in a PERK-dependent manner (g). Activation of caspase-8 is also dependent on activation of FADD. Collectively, these events lead to activation of caspase-3 and apoptosis. This model is not restricted to prostate cancer cells, as the key features were recapitulated in lung and cervical cancer cells (Figure S11).