Phosphorylated Galectin-3 Mediates Tumor Necrosis Factor-related Apoptosis-inducing Ligand Signaling by Regulating Phosphatase and Tensin Homologue Deleted on Chromosome 10 in Human Breast Carcinoma Cells*1

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Nachman Mazurek1,†, Yun Jie Sun1,†, Kai-Feng Liu1, Michael Z. Gilcrease3, Wendy Schober1, Pratima Nangia-Makker1, Avraham Raz1, and Robert S. Bresalier1,2

From the Departments of 1Gastrointestinal Medicine and Nutrition, 2Pathology, and 3Blood and Marrow Transplantation, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 and the 4Tumor Progression and Metastasis Program, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

Galectin-3 (GAL3), a β-galactoside-binding lectin, confers chemoresistance to a wide variety of cancer cell types. It may exhibit anti- or pro-apoptotic activity depending on the nature of the stimulus. We report here that introducing phosphorylated galectin-3 (P-GAL3) into GAL3-null, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant human breast carcinoma cells promotes TRAIL-induced apoptotic cell death by stimulating the phosphorylation/inactivation of the pro-apoptotic molecule Bad resulting in the inhibition of mitochondrial depolarization and the release of cytochrome c. Exposure of the transfectant cells to TRAIL leads to the recruitment of the initiator caspase-8 followed by activation of the effector caspase-9, independent of cytochrome c, and subsequently the processing of the executioner caspase-3. P-GAL3 and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) were coordinately expressed, with concomitant dephosphorylation of Akt in TRAIL-sensitive cells. In contrast, overexpression of phospho-mutant GAL3 (incapable of phosphorylation) failed to elicit similar responses. Depletion of PTEN using small interference RNAs reinstalled Akt phosphorylation and conferred TRAIL resistance. In addition phosphatidylinositol 3-kinase inhibitors rendered the phospho-mutant GAL3-resistant cells sensitive to TRAIL. These findings suggest a pivotal role for P-GAL3 in promoting TRAIL sensitivity through activation of a nonclassic apoptotic pathway and identify P-GAL3 as a novel regulator of PTEN.

Galectins are a family of carbohydrate-binding proteins that have been well conserved through evolution and are abundant in epithelial and immune cells of animals (1, 2). These proteins have sequence similarities in the carbohydrate recognition domain and specificity for β-galactoside moieties found on both N- and O-linked glycans (3, 4). Galectins play a significant role in apoptosis, and galectins such as galectin-1 and -9 have been implicated in the promotion of apoptosis of immune and melanoma cells (5, 6), whereas galectin-7 induces apoptosis of colon cancer cells (7).

In contrast, galectin-3 (GAL3)3 may exert anti- or pro-apoptotic activity depending on the cell type and the nature of the stimulus. For example, GAL3 inhibits Fas-induced T-cell apoptosis (8) as well as epithelial cell apoptotic cell death induced by staurosporine, cisplatin, and genistein (9). Overexpression of GAL3 in breast carcinoma cells inactivates Akt and sensitizes them to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (10) but renders them resistant to chemotherapeutic drugs (11, 12). In contrast, when GAL3 is introduced into bladder carcinoma cells, it activates Akt and confers resistance to TRAIL (13). The mechanisms by which GAL3 regulates apoptosis are not fully understood. A portion of its anti-apoptotic activity may be attributed to the anti-death motif (NEGR) that is conserved in the BH1 domain of the Bcl-2 family (11). In a manner similar to Bcl-2, phosphorylation may play an important role in controlling the anti-apoptotic function of GAL3 (14). GAL3 undergoes phosphorylation at Ser-6 by casein kinase 1 (15, 16) and in response to an apoptotic insult P-GAL3 is exported from the nucleus to the cytoplasm, where it maintains mitochondrial homeostasis and blocks cytochrome c release (17).

TRAIL, a member of the tumor necrosis factor family, transmits death signals through death domain-containing receptors (18). The death signal triggered by TRAIL can be classified into

3 The abbreviations used are: GAL3, galectin-3; P-GAL3, phosphorylated galectin-3 at Ser-6; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; PTEN, phosophatase and tensin homologue deleted on chromosome 10; TUNEL, terminal deoxynucleotidyl-transferase dUTP nick end labeling; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P-GAL3, phosphorylated galectin-3; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; PI3K, phosphatidylinositol 3-kinase; PIP2, inositol 1,4,5-bisphosphate; PIP3, inositol 1,4,5-trisphosphate; cisplatin, cis-diaminedichloroplatinum (II); ERK, extracellular signal-regulated kinase; PARP, poly(ADP-ribose) polymerase; CMV, cytomegalovirus; siRNA, small interference RNA; FMK, fluoromethyl ketone.
two categories. In the first category, the signal is independent of mitochondrial activation and cannot be blocked by Bcl-2 and Bcl-XL (19). In this pathway, TRAIL initiates signaling via the formation of a death-inducing signaling complex (DISC) that recruits and activates procaspase-8. Activated caspase-8 then processes directly the executioner caspase-3 (20). In the second category, the signal requires activation of effector caspase-9 by the mitochondria and can be blocked by Bcl-2 and Bcl-XL (21). In this pathway, activation of caspase-8 results in Bid cleavage and subsequent mitochondrial depolarization and release of apoptogenic factors (22). One such factor, cytochrome c, binds Apaf1 and promotes oligomerization and recruitment of apical caspase-9 into a high molecular weight complex termed the apoptosome. Assembly of the apoptosome induces processing of caspase-9 and subsequent activation of caspase-3 (23). In addition to its direct apoptosis-inducing effect, TRAIL also plays an important role in immune surveillance against tumor initiation, development, and metastasis, suggesting a potential application to cancer therapy (24, 25). Resistance to TRAIL-mediated apoptosis in cancer cells may, however, limit the successes of TRAIL therapy. Resistance to TRAIL can occur at different points in the signaling pathways triggered by TRAIL-induced apoptosis such as dysfunction of the death receptors DR4 and DR5 (26), defects in the Fas-associated death domain (FADD) (27) and caspase-8 (28), and activation of the survival PI3K/Akt pathway (29). The mechanisms of acquired TRAIL resistance in most cancer cell types are largely unknown.

Akt is a serine-threonine kinase that has potent anti-apoptotic effects by directly phosphorylating and inactivating proteins involved in apoptosis, including GSK-3, Bad, Forkhead, and procaspase-9 (30, 31). Because its activation promotes cell survival, sustained up-regulation of Akt activity has been reported to contribute to development of cancers such as breast, ovarian, prostate, and gastric adenocarcinomas (32). Activation of Akt by growth factors and cytokine treatment generally occurs via the PI3K pathway. Growth factor treatment leads to the recruitment of PI3K to the plasma membrane, where it catalyzes the conversion of PIP2 to PIP3 (33). The accumulation of PIP3 creates docking sites for Akt, which binds PIP3 via its pleckstrin homology domain. Another kinase that is recruited to the plasma membrane by the same phospholipids is phosphoinositide-dependent kinase-1, which phosphorylates Akt at Thr-308. Full activation of Akt requires its phosphorylation at a second site, Ser-473 (34). Although activation of PI3K leads to the generation of PIP3, this can be counterbalanced by the action of phosphatase and tension homologue deleted on chromosome 10 (PTEN) phosphatase.

PTEN is a dual phosphatase that mainly dephosphorylates PIP3. Because PI3K catalyzes the production of PIP3, PTEN antagonizes this PI3K function and negatively regulates Akt activities (35). Loss of PTEN function or expression increases the concentration of PIP3 and induces Akt hyperactivation, which leads to the protection of cells from various apoptotic stimuli (36). In contrast, overproduction of PTEN induces growth suppression via cell cycle arrest and/or induction of apoptosis (37). Despite the obvious importance of PTEN, only a handful of molecules are known to control its expression. Intracellular molecules reported to regulate PTEN transcription include p53 (38), Egr-1 (39), and DJ-1 (40).

In this study, we examined the role of P-GAL3 in the acquisition of TRAIL sensitivity by breast carcinoma cells and sought to identify the molecular mechanisms underlying P-GAL3-mediated TRAIL signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Human breast carcinoma BT549 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1-glutamine (2 mm), penicillin (100 units/ml), and streptomycin (50 units/ml) in a 95% air/5% CO2 incubator at 37 °C. The establishment of a stable neoresistant control vector-transfected BT549 clone (BT549/V), two wild-type galectin-3-transfected BT549 clones (gal25A and gal25B), and two phospho-mutant galectin-3 BT549 transfected clones (SA7 and SA9) was previously described (41). Galectin-3 is phosphorylated at Ser-6 (15, 16), Ser-6 → Ala mutation results in the inability of GAL3 to be phosphorylated at this site, retention of carbohydrate binding (normally lost with phosphorylation) (16), and loss of tumorigenicity (41). Mutation of Ser to Glu mimics constitutive Ser phosphorylation in certain proteins. In contrast, however, it has been demonstrated that Ser → Glu mutation in GAL3 mimics dephosphorylated Ser rather than phosphorylated Ser. Cells expressing the Ser → Glu mutation retain carbohydrate binding, are not tumorigenic and are resistant to cis-diaminedichloroplatinum (cisplatin), mimicking dephosphorylated GAL3 in vitro and in vivo (14, 16, 17, 41). Therefore, phosphomutant galectin-3 was generated by substitution of the Ser-6 residue by alanine (SA clones) only using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (41). The stable transfected clones were maintained in complete RPMI 1640 medium containing G418 (200 μg/ml).

**Immunofluorescence Staining and Confocal Microscopy**—Cells were fixed, permeabilized, and blocked in flow cytometry buffers (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer’s instructions. Cells were double stained with a 1:100 dilution of rat anti-GAL3 monoclonal antibody (TIB166, 0.26 mg/ml, American Type Culture Collection, Manassas, VA) and a 1:500 dilution of rabbit anti-P-GAL (Ser-6, 0.88 mg/ml) for 1 h at room temperature. Anti-P-GAL3 antibody was established in conjunction with Zymed Laboratories (South San Francisco, CA) by immunizing rabbits against a synthetic phosphopeptide comprising of 14 N-terminal amino acids of galectin-3 phosphorylated at Ser-6 (DNF(pS)LHALSGSGN(C)). The antibody was affinity-purified on an immobilized “hot” peptide followed by affinity absorption on an immobilized “cold” peptide. The specificity of the antibody was determined by a differential enzyme-linked immunosorbent assay using hot and cold peptides. Western blot analysis of human recombinant GAL3 and phosphorylated protein (produced, purified, and phosphorylated by casein kinase 1 (obtained from New England Biolabs) as previously described (16)) further confirmed specificity of the antibody for P-GAL3.
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(supplemental Fig. S1a). Secondary antibodies used were as follows. Fluorescein isothiocyanate-conjugated goat anti-rat (Chemicon, Temecula, CA), and phycoerythrin-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) were added at 1:200 dilution and incubated for 30 min at room temperature. (Nuclei were stained with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole dihydrochloride (Invitrogen). Expression and localization of the proteins were observed under a confocal microscope system (Olympus FV500, Olympus America, Melville, NY) and analyzed by CellQuest PRO software (BD Biosciences, Franklin Lakes, NJ) at the Flow Cytometry and Image Analysis Core of The M. D. Anderson Cancer Center.)

Western Blotting—Total cell lysate extractions from cells treated with 100 ng/ml TRAIL (R&D Systems, Minneapolis, MN) for 2 h with or without exposure to LY294002 or wortmannin (Cell Signaling, Beverly, MA) and Western blots were performed as previously described (41). Protein concentrations were measured using a DC protein assay reagent (Bio-Rad). Equal amounts of protein were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with the following primary antibodies: anti-galectin-3, anti-P-GAL3, anti-death receptors DR4 and DR5, or anti-FLIP (R&D Systems); anti-adaptor proteins FADD and FAF1; anti-caspase-3 (full-length and cleaved fragments), anti-caspase-8 (full-length procaspase-8 as well as the cleaved intermediate and the active subunit), caspase-9 (pro-caspase-9 as well as cleaved fragments), anti-members of the inhibitor of apoptosis protein family (C-IAP1, X1AP, and survivin); anti-Akt and phosphorylated Akt at Ser-473 and Thr-308; anti-PTEN; anti-ERK and phosphorylated ERK; and anti-Bad and phosphorylated Bad at Ser-112 and Ser-136 (Cell Signaling). Anti-β-actin (Sigma) was used to monitor equal loading in each lane. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) reagents using the ECL detection system (Amersham Biosciences). For densitometric analysis of Western blots, Alpha Image (Alpha Innotech, San Leandro, CA) was used.

Cytotoxicity and Apoptosis Assays—Cells viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma) assay (Sigma). The cells (10,000/well) were seeded into 96-well plates and grown overnight after treatment with either TRAIL or tumor necrosis factor α (TNF-α, R&D Systems) with a combination of cycloheximide (Sigma) or Fas ligand (Kamiya Biomedical, Seattle, WA). MTT solution was added to a final concentration of 1 mg/ml for 3 h at 37 °C, followed by 200 μl of Me,SO (Fisher Biotech, Fairlawn, NY). Colorimetric reading at 570 mm was performed using an MRX Revelation microplate reader (Dynex, Chantilly, VA). The results are expressed as mean cytotoxicity ± S.D. from triplicate cultures. Determinations were made in triplicate.

Apoptosis was assessed by PARP degradation and quantified by using the TUNEL assay. PARP degradation was determined by Western blot using an anti-PARP antibody capable of detecting full-length PARP as well as fragments of cleaved PARP. The TUNEL assay was performed using an apoptosis detection kit (APO-DIRECT, BD Biosciences). In each experiment, 1 × 10^6 cells were processed for flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences) and analyzed by CellQuest PRO software (BD Biosciences).

Caspase Activity and Caspase Inhibition Analyses—Activation of caspases 8, 9, and 3 was assessed by Western blot analysis with anti caspase-8, -9, and -3 antibodies capable of detecting the full-length pro-caspases and the cleaved intermediate fragments and active subunits of caspases 8, 9, and 3. In vitro activity of caspases 8, 9, and 3 was quantified with the APO Alert caspase assay plates (BD Biosciences) using a fluorescence plate reader (Molecular Devices, Menlo Park, CA) with 380 nm excitation and 460 nm emission. Cell-permeable, nonreversible caspase-8 and -9 inhibitors were incubated with 100 ng/ml TRAIL for 2 h, and apoptosis was analyzed by PARP degradation and MTT assay.

Mitochondrial Energization—Changes in the mitochondrial membrane potential were detected by MitoTracker Red CMXRos dye (Molecular Probes). For this experiment, 1 × 10^6 cells were treated with 0.3 μM of the membrane potential-dependent CMXRos dye in combination with 1 μM of the membrane potential independent MitoTracker Green (Molecular Probes) for 1 h at 37 °C and subjected to flow cytometric analysis. The dual labeling was analyzed by CellQuest Pro software. Single color-stained cells were used for instrument compensations.

Detection of Cytochrome c Release from Mitochondria—The release of cytochrome c into the cytoplasm of untreated and TRAIL-treated cells was determined in floating and attached cells combined using the APO Alert Cell Fractionation kit (Invitrogen). The quality of the separation of the mitochondrial and cytosolic fractions was determined by anti-COX4 antibody, and the location of cytochrome c was detected by Western blot using an anti-cytochrome c antibody.

Real-time Quantitative PCR—Total RNA was isolated from parental BT549 cells and galectin-3 transfectants by the TRizol method (Invitrogen) and further purified using the RNeasy kit (Qiagen). Initial experiments were performed to determine the valid range of RNA concentrations and to demonstrate the similarity of PCR efficiencies compared with the endogenous control gene cyclophilin. To determine -fold changes, real-time reverse transcription-PCR was performed on the ABI Prism 7900 using the commercially available gene expression assay for PTEN and the cyclophilin Vic labeled Pre-Developed Assay Reagent (Applied Biosystems, Foster City, CA) without multiplexing. A 50-μl final reaction volume containing 1× TaqMan Universal PCR Master Mix without AmpErase UNG (Applied Biosystems), 1× Multiscribe with RNase Inhibitors, and 1× gene expression assay was used to amplify 50 ng of total RNA with the following cycling conditions: 30 min at 48 °C, 10 min at 95 °C, then 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The 7900 Sequence Detection System 2.2 software automatically determined the -fold change for each gene in each sample relative to the BD Biosciences Human Reference Pool using the ΔΔC_t method. Calculations were performed for the PTEN gene in transfectants relative to parental BT549 cells.

PTEN Luciferase Reporter Assay—A PTEN promoter-luciferase reporter plasmid was kindly provided by Yufang Tang and
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Charis Eng (42) of The Ohio State University. β-Galactoside expression vector pCH110 (Amersham Biosciences) was used to normalize for transfection efficiency. For transient transfection, cells were seeded at a concentration of 8 × 10^5 cells per well in 6-well plates. After overnight culture, the cells in each well were cotransfected with 0.2 μg of PTEN-luciferase reporter plasmid and 0.2 μg of pCH110 using 3 μl of FuGENE 6 (Roche Applied Science) following the manufacturer’s protocol. Cells were cultured for an additional 48 h and harvested for measurement of luciferase activity in relative light units and normalized to β-galactosidase, using the Promega luciferase assay system (Promega Corp., Madison, WI) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Values shown represent the mean and standard deviation of at least three replicate determinations. To examine whether GAL3 overexpression augments transcriptional activity of the PTEN promoter, BT549 parental cells were transiently transfected with various amounts of PBK-CMV-GAL3 (14) and cotransfected with PTEN-luciferase reporter plasmid (0.2 μg) and pCH110 (0.2 μg). After 48 h, the cells were lysed, and activity was measured using a luciferase assay system (Promega) as described above.

Tissue Microarrays and Immunohistochemistry—A tissue microarray containing 48 samples of invasive breast carcinoma was obtained from a commercial source (Imgenex, San Diego, CA). A second tissue microarray was created at M.D. Anderson Cancer Center using tissue blocks from 73 additional breast carcinomas. The tissue microarrays were prepared using a manual tissue puncher/array (Beecher Instruments, Silver Spring, MD). Up to three cores 0.6 mm in diameter were cut from each donor block and aligned within the recipient block in a rectilinear array. Duplicate slides 5 μm apart were cut from each block, subjected to heat-induced antigen retrieval, and incubated with either anti-GAL3 (0.25 μg/ml) or anti-PTEN (1:50 dilution) antibodies. The Vectastain universal ABC-elite kit (Vector Laboratories) and diaminobenzidine (Dako) were used for detection and visualization, respectively. Staining intensity was scored on a scale of 0–3 (0 = no staining, 1 = faint staining, 2 = moderate staining, and 3 = intense staining), and the proportion of each tumor with cytoplasmic and/or membranous staining was scored on a scale of 0–3 (0 = no staining, 1 = < 30%, 2 = 30–70%, and 3 = > 70%). For statistical analysis, tumors with a combined intensity and proportion score of 2 or greater were considered positive. The relationship between GAL3 and PTEN expression was analyzed using Pearson’s Chi-squared test with Yates’ continuity correction.

siRNA Silencing of Akt—The Akt 1/2/3 kinases ShortCut small interfering RNA (siRNA) mix (New England Biolabs) is a heterogeneous mixture of 21–23 bp of siRNA that induces effective silencing of human Akt 1/2/3 kinases in mammalian cell lines. A 1038-bp DNA template derived from mouse Akt cDNA (284–1322) was used to generate double strand RNA, which was processed by ShortCut RNase III to produce the siRNA mix. The mouse Akt 1 DNA template has a 91% sequencing identity to human Akt 1, a 71% sequence identity to human Akt2, and a 74% sequence identity to human Akt3. The siRNA mix is effective against all three Akt proteins. siControl (siCON), which is a nontargeting siRNA from Cell Signaling, was used to illustrate the nonspecific effect of siRNA transfection. BT549 clones were transfected with TransIT-TKO (Mirus) with siCON or siAkt following the manufacturer’s instructions. The degree of Akt protein depletion was determined by Western blot analysis using a pan anti-Akt antibody, recognizing the three forms of Akt (Cell Signaling).

siRNA Knockdown of PTEN—Small interfering RNA oligonucleotide duplexes (siRNA) designed to inhibit the expression of various forms of PTEN and its spliced variants as well as a non-targeted scrambled negative control siRNA were included in the Signal Silence PTEN siRNA kit (Cell Signaling). Twenty-two base sequences targeting the N-terminal conserved domain of the human PTEN gene (Gene Bank accession number NM_000314) were chosen. The PTEN sense siRNA sequence was 5-GUUAGCAGAAAGAAAAGGAGTT; the PTEN antisense siRNA sequence was 5-CUCCUUUGUUCUGCUAACCTT. BLAST analysis of the siRNA sequence against the NCBI data base (shown in supplemental Fig. S2) revealed 100% homology with various forms of PTEN (for example GenBankTM accession number AAC52017 and two spliced variants reported in Ref. 43 as well as another eight spliced variants reported in Ref. 44). The siRNA duplexes were delivered to the cells using transit-TKO transfection reagent (Mirus) according to the manufacturer’s instructions. The degree of PTEN protein depletion was determined by Western blot analysis.

RESULTS

GAL3 Expression and Phosphorylation in Stable BT549 Transfectant—To assess the role of GAL3 phosphorylation at Ser-6 on death receptor ligands-induced apoptosis, we used BT549 stable transfectants previously established in our laboratory as an experimental cellular model (41). Parental (GAL3 null) BT549 cells were transfected with either wild-type (capable of undergoing phosphorylation) or Ser-6 → Ala mutant (incapable of undergoing phosphorylation) GAL3. After selection with G418, several clones were obtained: a control clone expressing a vehicle plasmid (BT549/V), two GAL3 clones (gal25A and gal25B), and two phospho Ser-6 mutant GAL3 clones (SA7 and SA9).

The expression of GAL3 and P-GAL3 in these clones was determined by Western blot analysis (Fig. 1a) and immunofluorescence staining (Fig. 1b). Similar levels of GAL3 protein expression were detected in both wild-type GAL3 (gal25A and gal25B)- and phospho-mutant GAL3 (SA7 and SA9)-expressing clones. P-GAL3 was detected, however, only in gal25A and gal25B cells and was completely absent in SA7 and SA9 cells. In contrast to P-GAL3, which was randomly localized in both nucleus and cytoplasm, phospho-mutant GAL3 was predominantly expressed in the cytoplasm and absent from the nucleus in SA7 and SA9 cells. These data agree with our previous findings showing that over 90% of gal25A and gal25B cells express P-GAL3 by flow cytometry (45) and metabolic labeling with[^32]P and Western blotting with a P-Ser antibody, which show that only the wild-type GAL3 transfectants undergo phosphorylation in vivo (14, 17).
Phosphorylation of GAL3 Is Required for Death Receptor Ligands-induced Apoptosis of BT549 Breast Cancer Cells—To investigate whether the phosphorylation of GAL3 plays a role in death receptor ligand-induced apoptosis we first compared the sensitivity of gal25A and gal25B with SA7 and SA9 clones to three death receptor ligands, TRAIL, TNFα-H9251, and Fas ligand. The response was determined by the MTT assay, which detects mitochondrial activity and hence cell proliferation and viability. Exposure of parental BT549 and control vector BT549 cells to TRAIL resulted in little or no cytotoxicity (Fig. 2a). In contrast, exposure of gal25A and gal25B to TRAIL resulted in a rapid and persistent cell death. It reduced cell viability in a concentration- and time-dependent manner (Fig. 2a). For both clones, 80–85% of the cells died within 2 h, and the cytotoxic effect was confirmed by TUNEL assay (Fig. 2b). In addition, immunoblot analysis of PARP showed the full-length 116- and 89-kDa apoptosis-related cleaved fragments only in gal25A and gal25B clones (Fig. 2c).
of TRAIL reached saturation at a concentration of 100 ng/ml. These results are in accordance with the findings reported in a previous study by Lee et al. (10). On the other hand, TRAIL did not affect the viability of SA7 and SA9 cells expressing phospho-mutant GAL3 even after 24 h of exposure to a higher concentration of TRAIL (200 ng/ml).

Similar to the effects of TRAIL, exposure of the parental BT549, control, SA7, and SA9 clones to TNFα/H9251 resulted in little cell death. Whereas, TNFα (100 ng/ml) in combination with cycloheximide (1 µg/ml) induced a moderate cytotoxic effect in both gal25A and gal25B clones (41.6% and 37.8% cell death, respectively). In contrast, all transfectants were resistant to Fas ligand-induced cell death (data not shown).

Because the effects of P-GAL3 on TRAIL-induced cell death were more significant, we investigated the nature of cell death observed in gal25A and gal25B treated with TRAIL. We tested parameters that are hallmarks of apoptosis, such as DNA fragmentation as monitored by TUNEL assay and caspase-dependent cleavage of PARP. The TUNEL assay (Fig. 2b) demonstrated that TRAIL (100 ng/ml) significantly induced apoptosis in gal25A (71.3%) and in gal25B (59.8%), whereas the SA7, SA9, parental, and BT549/V cells were resistant to TRAIL. The PARP assay shown in Fig. 2c supported the TUNEL findings. TRAIL induced the cleavage of intact PARP (M, 116,000) into the inactive fragment (M, 85,000), which is characteristic of apoptosis, only in gal25A and gal25B. In contrast, PARP remained intact in SA7, SA9, control, and parental cells treated with TRAIL. These findings suggest that phosphorylation of GAL3 is important for the onset of TRAIL-induced apoptotic death of BT549 cells.

TRAIL Mediates the Activation of Caspases 8, 9, and 3 in BT549 Cells Expressing Phosphorylated GAL3 but Not in BT549-expressing Phospho-mutant GAL3—In receptor-mediated apoptosis, initiation of apoptosis is due to the formation of a DISC that involves the physical association and activation of caspase-8 (20). In type I cells, activated caspase-8 directly cleaves the executioner caspase-3, which then acts on final death substrates. However, in type II cells, activated caspase-8 triggers a mitochondria-dependent apoptotic amplification loop by activating Bid, which induces the release of cytochrome c and activation of the effector caspase-9, which subsequently activates caspase-3 (46). All caspases are synthesized as inactive

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**FIGURE 3. P-GAL3 mediates a nonclassic caspases activation cascade.**

- a, pro-caspase-8 and its intermediate doublet and active subunit.
- b, pro-caspase-9 and its cleaved doublet.
- c, pro-caspase-3 and its active subunit were observed only in gal25A and gal25B clones 2 h after exposure to 100 ng/ml TRAIL.
- d, the caspase-8-specific inhibitor 2-IETP-FMK blocks TRAIL-induced processing of caspase-9, caspase-3, and PARP.
- e, the caspase-9-specific irreversible inhibitor 2-LEHD-FMK blocks TRAIL-mediated cleavage of caspase-3 and PARP but not caspase-8.
- f, the caspase-3 inhibitor A-DEVD-FMK failed to protect caspase-8 and caspase-9 from processing but protected the cells from TRAIL-induced apoptosis as demonstrated by PARP cleavage.
zymogens that need to be activated by proteolytic processing. Therefore, in Western blots, caspase activation can be followed by the disappearance of the zymogenic form and the appearance of the cleaved forms that are detected by a specific antibody. In gal25A and gal25B treated with 100 ng/ml TRAIL, processed forms of caspases 8, 9, and 3 were observed (Fig. 3).

The diminishing of procaspase-8 (57 kDa), procaspase-9 (47 kDa), and procaspase-3 (32 kDa) signals and the appearance of a doublet of 41/43 kDa and 35-kDa and 17-kDa proteins corresponding to the cleaved forms of caspases 8, 9, and 3, respectively, were clearly detected in TRAIL-treated gal25A and gal25B (Fig. 3). In contrast, TRAIL did not activate the caspase cascade in SA7 and SA9 or in the BT549 parental or control cells. Similar results were observed in a cell-free enzyme activity assay (data not shown). These results coupled with previous reports, indicate that GAL3 does not alter apoptosis-related protein expression such as death receptors (DR4 and DR5), death-associated proteins (FAF1 and FADD), and inhibitors of apoptosis proteins (C1AP1, XIAP, and Survivin) (10) and suggest that the acquisition of TRAIL resistance in BT549 cells from caspase-8 to caspase-3 is blocked in the GAL3-expressing BT549 cells.

TRAIL Induces Processing of Caspase-9 Independently of Cytochrome c in GAL3-expressing BT549 Transfectants—In the classic type II apoptosis signaling pathways, the ultimate execution of the apoptotic program relies on mitochondrial contribution (46). The mitochondrial network, which can be initiated by activated caspase-8-cleaved Bid, triggers the release of cytochrome c, which binds Apaf1 and promotes the recruitment of caspase-9 into the apoptosome. Assembly of the apoptosome induces autocleavage of caspase-9 and subsequent activation of caspase-3 (22). The findings that phosphorylated GAL3 protects mitochondrial integrity and prevents cytochrome c release in response to cytotoxic drugs (17) led us to ask whether TRAIL-induced apoptosis is accompanied by caspase-9 activation independently of cytochrome c. We next investigated whether cytochrome c is required for caspase-9 processing in gal25A and gal25B exposed to TRAIL. Because the loss of mitochondrial transmembrane potential is often associated with the early stages of apoptosis, we first examined
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FIGURE 5. Akt phosphorylation is required for the acquisition of TRAIL-resistance. a. Western blot analysis of intracellular levels of phosphorylated Akt, ERK, and Bad in parental (P), control (V), P-GAL3 expressing clones (gal25A and gal25B), and phospho-mutant GAL3 clones (SA7 and SA9). b, effects of silencing Akt on the sensitivity of parental and phospho-mutant GAL3 BT549 clones to TRAIL. The Western blot analysis of basal Akt/P-Akt and BAD/P-BAD in BT549 clones transfected with control RNA (si CON) or siRNA targeting constitutive Akt (si Akt). Protein expression levels were compared with β-actin as an internal control. c, MTT assay demonstrates the acquisition of TRAIL sensitivity by Akt knockdown transfectants. Data represent the mean and standard deviations of experiments performed in triplicate.

The effect of TRAIL exposure on the mitochondrial integrity. No apparent dissipation of the mitochondrial transmembrane potential was observed in gal25A or gal25B cells treated with TRAIL (Fig. 4a). Parental cells treated with cisplatin, as a positive control, demonstrated reduced CMXRs accumulation (a Mitoprobe sensitive to membrane potential), indicating depolarization of the mitochondrial membrane potential (Fig. 4a). In keeping with the mitochondrial transmembrane potential experiments, immunoblot analysis of cytosolic cytochrome c showed no significant increase in the level of cytochrome c released from the mitochondria in gal25A and gal25B treated with TRAIL (Fig. 4b). In contrast, a 10-fold increase in cytosolic cytochrome c was detected in BT549 parental cells treated with cisplatin as a positive control (Fig. 4b). Taken together, these results indicate that in GAL-3-expressing breast cancer cells TRAIL triggers a nonclassic caspase activation cascade. It first activates caspase-8 and then caspase-9, independently of cytochrome c, and subsequently caspase-3, which leads to the cleavage of PARP and finally culminates in apoptosis.

Effects of Phosphorylated GAL3 on Akt Activation—It is well known that dephosphorylation/inactivation of Akt sensitizes TRAIL-induced apoptosis (10). We therefore examined the Akt activity in BT549 transfectants. Akt activity was measured by Western blot analysis using an antibody that specifically recognizes the phosphorylated/activated form of Akt. As shown in Fig. 5a, a high expression of constitutively phosphorylated Akt at serine 473 and threonine 308 residues was observed in BT549 parental, control, and phospho-mutant GAL3-expressing clones SA7 and SA9. In contrast, undetectable or low levels of activated Akt were observed in P-GAL3-expressing BT549 clones gal25A and gal25B. Total Akt levels among these clones were almost equal (Fig. 5a). Akt has been shown to exert its anti-apoptotic activity by phosphorylation and inactivation of several pro-apoptotic targets, including Bad and the fork head transcription factors and by increasing the intracellular level of FLIP (30, 31). We did not observe dramatic changes in C-FLIP expression or post-translational modification in fork head transcription factors between BT549 parental, control, and the GAL3-transfected clones (data not shown). Bad was phosphorylated at Ser-136 (the target of Akt) but not Ser-112 (the target of ERK) in the parental and phospho-mutant clones SA7 and SA9. In contrast, Bad was phosphorylated at Ser-112 but not Ser-136 in gal25A and gal25B cells, suggesting that Bad is most likely phosphorylated by ERK and not Akt (Fig. 5a). This is in agreement with our observation that ERK was indeed phosphorylated in gal25A and gal25B clones but not in parental or SA7 and SA9 transfectants (Fig. 5a).

To further examine the role of P-GAL3 on Akt activation and TRAIL signaling we employed RNA interference methodology. Silencing of endogenous Akt 1, 2, and 3 isoforms in BT549 parental, vehicle control and SA7 and SA9 phospho-mutant GAL3, TRAIL-resistant, clones caused a significant decrease in constitutively phosphorylated Akt and BAD, a downstream substrate of Akt (Fig. 5b) and render BT549 parental and phospho-mutant GAL3 clones SA7 and SA9 sensitive to TRAIL-induced apoptotic cell death (Fig. 5c). Control RNA (siCON), which is a scrambled nontargeting siRNA, was used to demonstrate the nonspecific effect of siRNA transfection. These results confirm the involvement of Akt in the acquisition of TRAIL resistance and implies that P-GAL3 plays an important role in Akt-dependent TRAIL-induced apoptosis.

Because PI3K activation is the major upstream signaling event leading to Akt phosphorylation (33), we examined whether two specific pharmacological inhibitors of PI3K, wortmannin and LY-294002, could inhibit constitutively active Akt and render BT549 parental and phospho-mutant GAL3 clones SA7 and SA9 sensitive to TRAIL-induced apoptosis. Exposure of cells to either LY294002 (2.5–50 μM) or wortmannin (0.25–5 μM) for 24 h in serum-free medium resulted in blockage of Akt phosphorylation in a concentration-dependent manner reaching maximum effects at a dose of 25 μM LY294002 and 2.5 μM wortmannin (supplemental Fig. S3). Treatment of BT549 parental, control, and SA7 and SA9 clones with optimal concentrations of wortmannin (2.5 μM) or LY294002 (25 μM) for 2 h completely eliminated phosphorylated/activated Akt without affecting total levels of Akt (Fig. 6a). The protein expression
of DR4, DR5, FLIP, FADD, or GAL3 was not altered by treatment with these PI3K inhibitors (data not shown).

Next, we examined the effects of these PI3K pharmacological inhibitors on BT549 sensitivity to TRAIL-induced apoptotic cell death. Pre-exposure of BT549 parental, SA7, and SA9 to LY294002 (0–50 μM) or wortmannin (0–5 μM) for 2 h prior to treatment with 100 ng/ml TRAIL (previously shown to induce a rapid and persistent apoptotic cell death of gal25A- and gal25B-sensitive clones (see Fig. 2)) sensitized the resistant clones to TRAIL-induced apoptotic cell death in a concentration-dependent manner with maximum effect observed at 25 μM for LY294002 and 2.5 μM for wortmannin (Fig. 6b), whereas LY294002 and wortmannin alone did not cause significant cell death in doses up to 50 μM and 5 μM (respectively, after 24 h). Apoptosis was confirmed by PARP cleavage (Fig. 6c). These drugs diminished Akt phosphorylation and sensitized resistant clones to TRAIL at similar concentrations and kinetics, confirming the specific mode of action of the drugs, and further suggesting that PI3K/Akt plays an important role in the acquisition of TRAIL-sensitivity mediated by P-GAL3.

Phosphorylated GAL3 Is a Novel Regulator of PTEN—The observation that phosphorylated/activated Akt levels were down-regulated in gal25A and gal25B clones prompted us to examine the expression of PTEN, a phosphatase that negatively regulates Akt activity (35). Overexpression of P-GAL3 resulted in a dramatic increase of PTEN mRNA transcripts and protein expression with a concomitant dephosphorylation of Akt in the gal25A and gal25B clones (Fig. 7, a and b). In contrast, introduction of phospho-mutant GAL3 failed to elicit a similar response. Neither up-regulation of PTEN nor dephosphorylation of Akt was observed in SA7 and SA9 clones (Fig. 7, a and b). To examine whether manipulation of P-GAL3 expressions results in par-
Galectin-3 Regulates TRAIL and PTEN Signaling

a

FIGURE 8. Coordinate expression of GAL3 and PTEN in vitro and in vivo. a, Western blot analysis of GAL3 and PTEN in total cell lysates of six breast carcinoma cell lines. α-actin is control (bottom line). b, representative tissue microarrays of human invasive breast carcinomas stained for GAL3 and PTEN. A 40× magnification of the same tumor cores in duplicate slides is shown. c, a table summarizing immunohistochemical staining of 121 breast carcinomas for GAL3 and PTEN. Positive staining is defined as described under “Experimental Procedures.” Percentages represent galectin-3-positive or -negative cases that are also PTEN-positive or -negative.

allel change in PTEN expression at the transcription level, a PTEN luciferase reporter plasmid under the control of 1344-bp PTEN promoter activity between the various clones (Fig. 7c). In addition, overexpression of GAL3 in BT549 parental cells did not augment PTEN-transcriptional activity (Fig. 7d). These results suggest that GAL3 does not effect PTEN promoter transactivation but rather regulates PTEN expression at the post-transcriptional level.

Coordinate Expression of GAL3 and PTEN in Breast Carcinomas—Five additional breast carcinoma cell lines and tissue specimens from 121 patients with invasive breast carcinoma were evaluated to determine whether GAL3 and PTEN are coordinately expressed in human breast carcinomas. The cell lines examined demonstrated coordinate expression of these proteins by Western assays (Fig. 8a). In addition, there was a highly significant correlation in expression of GAL3 and PTEN in the breast carcinoma tissues examined (p = 0.002) (Fig. 8, b and c).

Silencing of PTEN Rescues Phosphorylated GAL3-expressing BT549 Cells from TRAIL-induced Apoptosis—We next examined whether PTEN knockdown is sufficient to rescue the GAL3-expressing cells gal25A and gal25B from TRAIL-induced apoptosis. These cells were transfected with siRNA targeting PTEN and with non-silencing scrambled siRNA as a control. A dramatic decrease in PTEN protein expression with a concomitant increase in Akt phosphorylation (but not Akt protein levels) was observed 48 h after transfection. In contrast, scrambled siRNA did not affect expression of PTEN nor the status of Akt phosphorylation (Fig. 9a). Depletion of PTEN resulted in acquisition of TRAIL resistance by gal25A and gal25B clones (Fig. 9b) without changing the protein expression levels of the death receptors (DR4 and DR5), FADD, FLIP, and GAL3 (data not shown).

DISCUSSION

The data presented here demonstrate that phosphorylation of GAL3 plays a pivotal role in the acquisition of TRAIL sensitivity by human breast carcinoma cells. We identified a TRAIL-induced nonclassic caspase activation cascade mediated by P-GAL3 through induction of PTEN expression and inactivation of the PI3K/Akt survival pathway. Specifically, the death signal triggered by TRAIL activates procaspase-8 and subsequently pro-caspase-9, independently of mitochondrial activation and cytochrome c, resulting in apoptosis executed by activated caspase-3. The persistent cell death observed in BT549 transfectants expressing P-GAL3 independently of the mitochondria suggests the existence of alternative apoptotic pathways that can bypass the apoptosome and induce apoptotic death. Our results strongly suggest that the nonclassic apoptosis pathway, reported here for the first time, is a chimeric composition of the death signal-initiating phase of the extrinsic pathway (type I) and the execution phase of the intrinsic pathway uncoupled from the apoptosome (type II). The latter is consistent with previous reports by Ho et al. (23), which observed caspase-9 activation uncoupled from the apoptosome in Apaf1−/− myoblasts, and by Bitzer et al. (47), which reported uncoupling of the apoptosome in Sendai virus-infected cells in response to classic inducers of mitochondrial outer membrane permeabilization, including cisplatin, doxorubicin, and etoposide.

In the classic mitochondrial death pathway the balance of the pro-apoptotic Bad or Bax and the anti-apoptotic Bcl-2 or Bcl-XL proteins ultimately determines cell death or survival (48). In this study, we observed that expression of P-GAL3 induced phosphorylation of Bad at Ser-112 but not Ser-136, suggesting that Bad is most likely phosphorylated by ERK and not Akt. Phosphorylated Bad fails to form a complex with Bcl-2 or Bcl-XL, and the homodimer complex of Bcl-XL suppresses the activation of caspase-9 and inhibits apoptosis. This finding is consistent with the study by Fukumori et al. (49), which observed Bad phosphorylation in LNCaP prostate cancer cells overexpressing GAL3, and by our own observations and those of Lee et al. (10) that Akt is dephosphorylated and ERK is activated in BT549/GAL3 transfectants. The studies by Elad-Sfadia et al. (50) and Shalom-Feuerstein et al. (51) that showed that GAL3 triggers a Ras signal that activates ERK but not PI3K with a concomitant suppression of Akt activity in BT549/GAL3 cells further support the data presented here. A somewhat different effect of GAL3 on bladder carcinoma cells has been recently reported. Oka et al. (13) found that GAL3 overexpression in J82 cells conferred TRAIL resistance by activating Akt. These disparate findings may be due to the fact that J82 are type II cells, in which TRAIL induces a mitochondria-dependent apoptosis,
whereas in gal25A and gal25B transfectants TRAIL triggers a mitochondria-independent apoptotic pathway.

Akt is a key survival protein functionally involved in anti-apoptosis in various cancers (32). Its activity is elevated in cancers due to the loss of PTEN tumor suppressor functions and/or the activation of upstream PIK3 signaling following growth and survival factor stimulation. The anti-apoptotic role of Akt accounts for its transforming potential and for the resistance of cancer cells to the action of chemotherapeutic agents and ionizing radiation (32). Akt protects cells from apoptosis by phosphorylation of pro-apoptotic substrates that are subsequently sequestered by the chaperone 14-3-3 (52). The findings in this study, that P-GAL3 negatively regulates Akt activation, coupled with the observation that the intracellular levels of members of the DISC are not affected by reconstitution of GAL3 suggest the possibility that post-translational modifications are responsible for assembly of a nonfunctional DISC and the acquisition of TRAIL resistance by BT549 cells.

PTEN inactivation, and concurrent constitutive activation of Akt, is associated with the initiation and progression of tumors (53). PTEN expression is regulated at the transcriptional level by a set of transcription factors, including P53, and at the post-transcriptional level by protein localization, modification, and degradation (38). Recently it has been reported that stabilization and activation of p53 by proteasome inhibition leads to down-regulation of PTEN protein, and inhibition of caspases can partially rescue PTEN degradation induced by proteasome dysfunctions (45). In this study we demonstrate that GAL3 is a regulator of PTEN expression in vitro. This finding is further supported by the demonstration of coordinate expression of GAL3 and PTEN in both breast cancer cell lines and in a subset of human breast cancer specimens. P-GAL3 induced the expression of PTEN mRNA, as shown by real-time reverse transcription-PCR, without transactivating the PTEN promoter. These findings suggest that P-GAL3 regulates PTEN expression at the post-transcriptional level; however, the exact molecular mechanisms underlying GAL3-mediated PTEN expression are yet to be elucidated. One possibility, currently under investigation, is that GAL3 may interact with the RNA-binding proteins heterogeneous nuclear ribonucleoproteins to control PTEN RNA turnover. It has been previously reported that GAL3 binds directly to hnRnpA1 (54) and heterogeneous nuclear ribonucleoprotein C1/C2 (55) and that heterogeneous nuclear ribonucleoproteins form a multiprotein complex that regulates c-fos RNA degradation (56) and inhibit the mRNA editing action of Apobec (57). Binding of P-GAL3 to PTEN may also potentially alter the spatial structure of PTEN affecting its degradation.

Taken together this study demonstrates a pivotal role for P-GAL3 in promoting TRAIL sensitivity by activating a non-classic apoptosis pathway and identify P-GAL3 as a novel regulator of PTEN. It also provides the basis for innovative targeted therapeutic strategies for the activation of PTEN in cancer and affords insight into the cellular mechanisms of sensitivity and resistance to apoptosis in cancer cells.

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