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EI24/PIG8 is regulated by p53 in response to genotoxic damage and was shown to be genetically inactivated in aggressive breast cancers with frequent loss-of-heterozygosity. In addition, it was shown that ectopic expression of EI24/PIG8 resulted in apoptosis, whereas suppression of EI24/PIG8 expression resulted in increased survival after treatment with an apoptotic retinoid. We hypothesizid that genetic inactivation of EI24/PIG8 is a major contributing factor to breast cancer development and resistance to chemotherapeutic agents such as etoposide. We recently found evidence that EI24/PIG8 directly binds with Bcl-2 in the endoplasmatic reticulum (ER), and likely initiates apoptosis at this organelle by altering the activity of ER-resident Bcl-2 (purpose of Objectives 1 and 2). Our studies also showed that EI24/PIG8 likely serves to suppress tumor spreading, rather than formation of the primary tumor. Thus, the EI24/PIG8 status of breast cancers may serve as a potential novel prognostic indicator in this disease.

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INTRODUCTION

This research aims to understand the molecular action of pro-apoptotic factor and suspected breast cancer tumor suppressor EI24/PIG8 (**E**toposide-**I**nduced gene **24**/**P**53-**I**nduced gene **8**), and its putative role in breast cancer deveopment and resistance to chemotherapy in breast cancer cells.

EI24/PIG8 is regulated by p53 in response to genotoxic damage (1) and was shown to be genetically inactivated in aggressive breast cancers (2) with frequent loss-of-heterozygosity (3,4). In addition, it was shown that ectopic expression of EI24/PIG8 resulted in apoptosis (5,6), whereas suppression of EI24/PIG8 expression resulted in increased survival after treatment with an apoptotic retinoid (7). Unfortunately, otherwise there is very little known about this interesting p53-target gene. We found evidence that EI24/PIG8 directly binds with Bcl-2 in the endoplasmatic reticulum (ER), and likely initiates apoptosis at this organelle by altering the activity of ER-resident Bcl-2 (8-12; see Objectives 1 and 2). We hypothesize that point mutations in EI24/PIG8 associated with aggressive breast cancer result in functional inactivation, which we believe to be a major contributing factor to breast cancer development (Appendix 1) and resistance to chemotherapeutic agents such as etoposide (see Objectives 3 and 4). The EI24/PIG8 status may serve as a potential novel prognostic indicator in this disease, as suggested by our recently published studies (Appendix 1).

BODY

For detailed description we refer to our published report in Cancer Research 65:2125-2129, 2005 (Appendix 1).

In short, we made the following progress with regards to the original Objectives:

Objective 1 To identify domains in ei24/PIG8 responsible for apoptosis and/or Bcl-2-binding

This Objective has been accomplished, because we identified the domains in Bcl-2 and EI24/PIG8 required for binding by generating mutants and using a variety of experimental approaches that we proprosed in our original experimental plan. We decided to no longer pursue the apoptotic activity of the different mutants because we eventually found that expression of these mutants varied dramatically, as shown in our unpublished observations for fusions with eGFP in **Fig. 1**. Since all constructs are fused C-terminally with eGFP, differences in expression between the constructs must be due to the EI24/PIG8 moiety (all clones have the same Kozak-optimized translational start stite, and are cloned in the same CMV-based expression vector).

Thus, these differences are so large that no conclusions with regards to specific domain-activities can be drawn.

On the other hand, we were able to do some new experiments because we developed an antibody against EI24/PIG8 raised against residues 51-66, as mentioned in the ***Interpretation and Potential Pitfalls*** section of this Aim. This allowed us to perform:

1. immunoprecipitation assays to further confirm *in vivo* association between Bcl-2 and EI24/PIG8, as shown in our published study (Appendix 1);

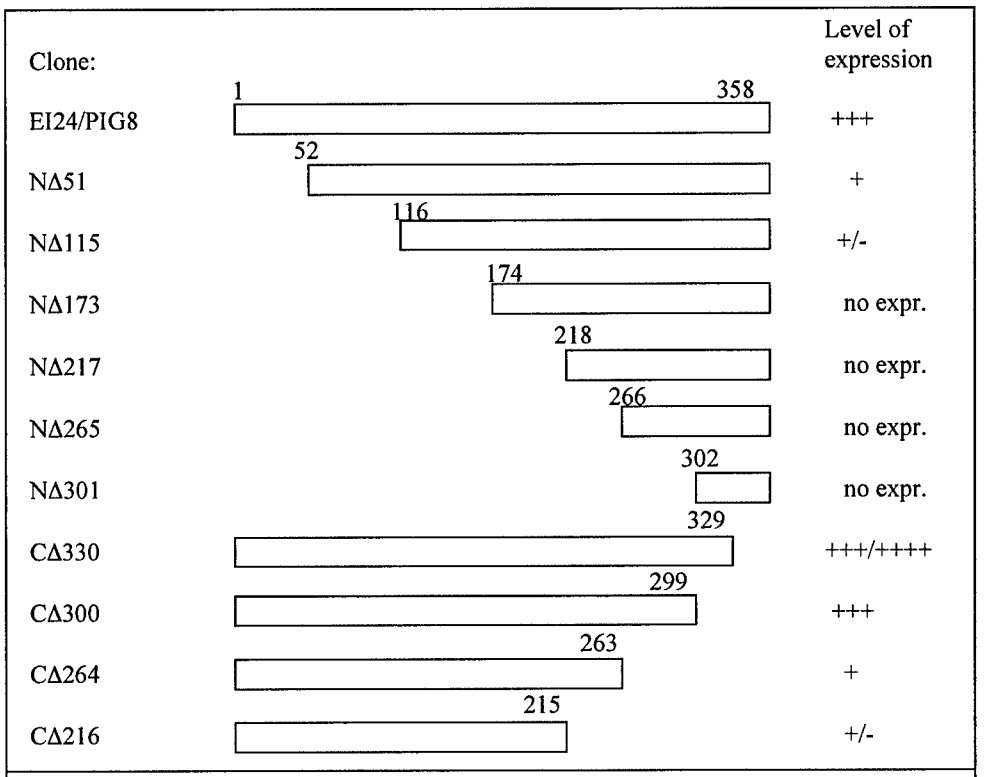

Fig. 1. Overview of expression of EI24/PIG8-eGFP truncation ("Δ") mutants. A representative selection of cells was analyzed for all mutants by fluorescence microscopy. no expr., no or low expression. EI24/PIG8-EGFP expression is arbitrarily assigned +++ "value"

2. immunohistochemistry on paraffin-embedded breast cancer development tissue arrays (obtained from the Cooperative Breast Cancer Tissue Resource, NCI; no IRB approval required), where we for the first time showed that EI24/PIG8 expression is absent from a large proportion of invasive breast cancers, but is expressed at normal levels in ductal carcinoma *in situ* and normal breast tissue. This suggests that EI24/PIG8 plays a specific role in suppression of tumor spreading (Appendix 1).

This important latter experiment was not proposed originally, but became feasible after we established that our anti-EI24/PIG8 antibody was specific, and useful for staining paraffin-embedded tissue sections.

Objective 2 To establish the mechanism of ei24/PIG8-mediated apoptosis.

Our data suggest that the interaction between Bcl-2 and EI24/PIG8 in ER directly results in apoptosis. Since ER serves to store calcium, it is conceivable that a disturbance in calcium homeostasis lies at the basis of this effect. This and Objective 4 will be pursued in the next period (see below).

Objective 3 to establish the functional activity of mutant ei24/PIG8 associated with early-onset breast cancer, and breast cancer cells.

In our published paper, we show that region 52-115 in EI24/PIG8 is the most important domain involved in binding with Bcl-2, which we believe is critical for the activity of EI24/PIG8. Interestingly, none of the reported point mutations in EI24/PIG8 correspond to this region which may indicate that these mutants still have activity ( ). Indeed, it is important to note that these mutations only represent about 22% of cases of genetically mutated EI24/PIG8 in aggressive breast cancer (the remaining 78% has much larger deletions, frame-shifts or are not expressed at all, and are clearly inactive). Even though we cannot exclude that the functional activity of the point mutants is affected in some way, we feel that we should give this Aim lower priority to optimize use of our remaining time and resources.

Although these accomplishments and progress are all very encouraging, we nevertheless lost time trying to evaluate the apoptotic activity of the EI24/PIG8 mutants, as explained above. Therefore, in the interest of time, we will lower the priority of Objective 3, for the reasons stated above, and first focus on Objective 4 (*to establish the role of wild type and mutant ei24/PIG8 in modulation of resistance to DNA damaging drugs in breast cancer cells*).

KEY RESEARCH ACCOMPLISHMENTS

See Appendix 1.

REPORTABLE OUTCOMES

See Appendix 1 (publication in Cancer Research, 65:2125-2129, 2005) and Appendix 2 (submitted abstract for ERA of HOPE Meeting, June 8-11, 2005 in Philadelphia, PA).

CONCLUSIONS

We have made good progress in the sense that we have successfully completed Objective 1, resulting in one publication and one presentation. We also have a good lead to Objective 2, because changes in calcium flux due to its interactions with ER-resident Bcl-2 most likely lie at the basis of mechanism of the apoptotic effects of EI24/PIG8. On the other hand, progress was slowed due to our failed attempts to evaluate the apoptotic activity of the EI24/PIG8 truncation mutants. We have strong indications that Objective 3 will not be very informative, which is why we have lowered its priority but if time permits, we will still assess the activity of the mutants. Instead, we will now focus on Objective 4. We feel that that this is the most sensible approach for the next year, because it maximizes our chances of success in the most time and cost-effective manner.

At this early stage in the proposal it is too difficult to tell whether our findings will result in a practical application, but our breast cancer tissue array data are promising with respect to use of EI24/PIG8 as a prognostic tool. However, this issue can be better addressed after successfull completion of Objective 4 in particular.

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Apoptosis Factor EI24/PIG8 Is a Novel Endoplasmic Reticulum–Localized Bcl-2–Binding Protein which Is Associated with Suppression of Breast Cancer Invasiveness

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Abstract

p53 is a critical tumor suppressor which removes cells with DNA damage by regulating expression and activity of a select group of *p53-i*nduced *g*enes (*PIG*) that subsequently induce apoptosis. *PIG8* was also identified as a gene induced by etoposide and named *etoposide-induced gene 24* (*EI24*). Later experiments established EI24/PIG8 as a proapoptotic factor and suggested that it may function as a tumor suppressor. Indeed, *EI24/PIG8* is relatively highly mutated in aggressive breast cancers and is located in a region which expresses frequent loss of heterozygosity. However, despite these important observations, the activity and role of EI24/PIG8 remain largely unknown. We used (immmuno)fluorescence microscopy and subcellular fractionation techniques to show that EI24/PIG8 is localized in the endoplasmic reticulum (ER). Pull-down experiments showed that it specifically binds with Bcl-2, a death regulator known to reside in mitochondria, ER, and the nuclear envelope. EI24/PIG8-Bcl-2 binding was corroborated by coimmunoprecipitation and other *in vitro* and *in vivo* protein-protein binding assays. Further analysis showed that EI24/PIG8 uses its N-terminal region to bind the BH3 domain in Bcl-2. Finally, we used immunohistochemical techniques to analyze expression of EI24/PIG8 in breast cancer tissue progression arrays and showed that loss of EI24/PIG8 is associated with tumor invasiveness but not with the development of the primary tumor. These results suggest that EI24/PIG8 is a novel, ER-localized Bcl-2–binding protein which may contribute to apoptosis by modulating the activity and/or function of Bcl-2 in this organelle. EI24/PIG8 may serve to prevent tumor spreading, consistent with its suspected role as a tumor suppressor. (Cancer Res 2005; 65(6): 2125-9)

Introduction

The role of p53 as the "gatekeeper" in the cellular defense against genotoxic damage and its function as a critical tumor suppressor, protecting the organism from potentially harmful cells which incurred DNA damage, are amply documented (1, 2). *TP53* is the most commonly deleted or mutated gene in human cancers (3), and patients with Li-Fraumeni syndrome and *TP53* knockout mice have clearly shown that loss of p53 results in a cancer-prone phenotype (4, 5).

Much effort has therefore been dedicated to the characterization of p53 target genes because they are the effectors of the apoptotic p53 response. Using adenoviral-mediated transfection of p53 into p53-deficient human colorectal cancer cells, which then undergo apoptosis, 14 *p53-i*nduced *g*enes (*PIG*) were identified which were strongly induced (6). One of these genes, *PIG8*, was also identified in an unrelated screen for genes that are induced by the chemotherapeutic drug etoposide and named *etoposide-induced gene 24* (*EI24*; ref. 7). Subsequent studies identified the p53 response element in the murine *EI24/PIG8* gene although the human promoter has not been characterized yet (8). Indeed, the p53-dependent response of *EI24/PIG8* is so robust that it is used as a biomarker for p53 activation (9, 10).

Ectopic expression of EI24/PIG8 was shown to be necessary and sufficient for induction of apoptosis (8), and our studies confirmed a major role for EI24/PIG8 in apoptosis induced by the DNA adduct-inducing synthetic retinoid CD437/AHPN in melanoma cells (11). These results suggested that EI24/PIG8 is an important apoptotic effector of p53 with a potential physiologic role as a tumor suppressor. This idea was further strengthened by a number of clinico-genetic studies. *EI24/PIG8* was mapped to human chromosome 11q23-q25 (12), a region which is often deleted in solid tumors (13). Genetic analysis of 41 early-onset breast cancers showed that *EI24/PIG8* has the highest mutation rate of four candidate tumor suppressor genes located in this area, with many of the mutations predicted to result in frameshifts or complete loss of translation (13). Moreover, this region expresses frequent loss of heterozygosity, suggesting that *EI24/PIG8* is perhaps the most important tumor suppressor gene at this locus (14).

However, despite these intriguing and promising findings, little progress has been made on the characterization of EI24/PIG8 and its activity and function in normal and cancer cells remain largely unknown. We show here that EI24/PIG8 is a novel, endoplasmic reticulum (ER)–localized Bcl-2–binding protein. Furthermore, immunohistochemical analysis showed that loss of EI24/PIG8 is associated with breast tumor invasiveness. These results suggest that induction of EI24/PIG8 results in modulation of the activity and/or function of Bcl-2 in the ER and that this event contributes to the apoptotic process. EI24/PIG8 may serve to prevent tumor spreading in breast cancer, consistent with its suspected role as a tumor suppressor.

Materials and Methods

Cell Culture and Transient Transfections. NIH 3T3 fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM with 10% (v/v) donor calf serum at 37°C in 5% CO_2 in humidified air. pMV-Bcl-2-transfected Balb cells also received 400 μg/mL neomycin. Cells were transfected with Effectene Transfection Reagent according to the

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instructions of the manufacturer (Qiagen, Valencia, CA). Etoposide was obtained from Sigma (St. Louis, MO).

Plasmid Construction. pEI24/PIG8-eGFP was constructed by cloning the murine cDNA from prKMei-flag (8) with a Kozak translational start site into the *Hin*dIII-*Sal*I sites of pEGFP-N3 (Clontech, Palo Alto, CA). For mammalian two-hybrid studies, EI24/PIG8, Bcl-2, and PCR-generated mutant cDNAs were cloned into the *Sal*I-*Not*I sites of pBIND and pACT (Promega, Madison, WI). The same cDNAs were cloned into *Eco*RI-*Not*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA) for *in vitro* translation. To generate bacterially expressed proteins, EI24/PIG8 cDNA was cloned into the *Nde*I-*Bam*HI sites of pET-16b (Novagen, Madison, WI) and Bcl-2 cDNA was cloned into pGEX4-T (Amersham Biosciences, Piscataway, NJ). pRed2ER was obtained from Clontech.

Subcellular Fractionation. Cells were collected by trypsin-EDTA digestion. Whole cell lysates were obtained by lysing cells in radioimmunoprecipitation assay buffer. To obtain the ER-enriched subcellular fraction, we essentially used the discontinuous sucrose gradient method as described before (15, 16) except that smooth and rough ER fractions were combined. Protein concentrations were determined by Pierce Protein Assay reagent (Rockford, IL).

Protein-Protein Binding Assays. Generation of recombinant His-EI24/PIG8 and GST-Bcl-2 protein and affinity columns and binding reactions were done as described (17). *In vitro* translation of EI24/PIG8 and Bcl-2 cDNAs was done in T7 TnT rabbit reticulocyte system with [^{35}S]methionine according to the protocol of the manufacturer (Promega). Whole 3T3 cell extract was prepared using E-PER Mammalian Protein Extraction reagent (Pierce). For mammalian two-hybrid assays, cells were seeded in six-well plates and transfected with 0.2 μg pACT, pBIND, and pG5Luc reporter (Promega). Total amount of plasmid was kept constant by adding empty pACT or pBIND vector as required. After 24 hours, luciferase assays were done and normalized as described (17).

Coimmunoprecipitation. pMV12-Bcl-2 Balb cells (18) were transfected with prKMei-FLAG or pEGFP-N3. Twenty-four hours later, cells were lysed in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% NP40, 50 mmol/L NaF, 0.2 mmol/L phenylmethylsulfonyl fluoride on ice for 30 minutes. Supernatants were collected and subjected to immunoprecipitation with anti-FLAG antibody (Sigma) overnight at 4°C. Complexes were precipitated by incubation with Protein A-agarose beads analyzed by Western blot as described (17). Quantitation of bands was done by densitometry (Labworks4.0, UVP, Upland, CA).

(Immuno)Fluorescence and Confocal Microscopy. For confocal imaging, 3T3 cells were plated in Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL) at 40% confluence after transfection. After 24 hours, cells were fixed with paraformaldehyde and mounted with ProLong Antifade (Molecular Probes, Eugene, OR). Nuclei were stained with 0.2 μg/mL 4′, 6-diamidino-2-phenylindole where indicated. Mitochondria in transfected cells were stained with MitoTracker Red before mounting according to the protocol of the manufacturer (Molecular Probes). Enhanced green fluorescent protein (eGFP) fluorescence was detected using a FITC filter; red fluorescent protein fluorescence and MitoTracker Red were detected with a Rhodamine filter. For immunofluorescence, cells were grown in chamber slides and transfected where indicated. Cells were fixed in methanol/acetone (1:1), incubated with anti-EI24/PIG8 antibody (1:100) for 2 hours, followed by incubation with fluorescent-conjugated anti-rabbit secondary antibody (1:1,000, Santa Cruz, Santa Cruz, CA) for 45 minutes, and examined by fluorescence microscopy.

Immunohistochemical Analysis. Breast cancer progression tissue arrays were obtained from the Cooperative Breast Cancer Tissue Resource (National Cancer Institute). Other investigators may have received slides from these same array blocks. Anti-EI24/PIG8 antibody was raised by immunization of rabbits with a conserved EI24/PIG8 peptide encoded in the N terminus (Qbiogene, Carlsbad, CA). Serum was affinity-purified against peptide (Arista Biologicals, Allentown, PA). Antibody specificity was confirmed by positive staining of EI24/PIG8 expression vector–transfected cells, compared with negative staining of control-transfected 3T3 cells (data not shown), and detection of endogenous EI24/PIG8 in etoposide-treated S91 and 3T3 cells only (see Fig. 1). Immunohistochemical staining was done according to the instructions of the manufacturer (Vector Laboratories, Burlingame, CA). Briefly, tissue sections were deparaffinized with xylene and hydrated in graded alcohol series and then incubated with anti-EI24/PIG8 antibody at 1:50 overnight at 4°C. Detection was done by incubation with biotinylated anti-rabbit antibody and staining with ABC Reagent Kit. Negative control (rabbit serum) did not give staining (data not shown). Two observers scored staining of tissue samples as follows: 0% to 25%, 26% to 50%, 51% to 75%, and 76% to 100% positive. Only undamaged samples that received identical scores by both observers were included in the final analysis. Furthermore, positive and negative staining of four sets of control tissue and cell culture samples located in different areas showed that the entire array surface was evenly stained and that the observers scored staining in a consistent manner.

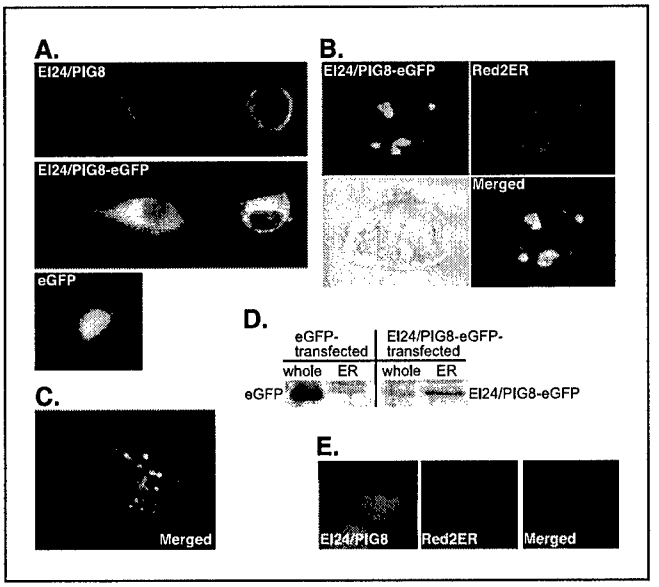

Figure 1. EI24/PIG8 specifically targets the ER. *A*, fluorescence microscopy images showing that both endogenous EI24/PIG8 (*top*, 10 μg/mL etoposide–treated 3T3 cells) and transfected EI24/PIG8-eGFP (*middle*) are expressed in the cytoplasm whereas eGFP is diffusely expressed in both cytoplasm and nucleus (*bottom*). Note appearance of apoptotic cells in top two panels (*right*). Magnification, 20×. *B* and *C*, confocal microscopy images showing that EI24/PIG8 colocalizes with ER marker Red2ER (*B*) but not with a red fluorescent mitochondrial stain (merged picture in *C*). Magnification, 40×. *D*, EI24/PIG8-eGFP, but not control eGFP, is enriched in subcellular ER fraction (*ER*) relative to whole cell lysate (*whole*). Western blot analysis (50 μg protein/lane) probed with anti-GFP antibody. *E*, fluorescence microscopy images showing that endogenous EI24/PIG8 also colocalizes with transfected ER marker Red2ER in 10 μg/mL etoposide–treated cells. Nuclei are stained with 4′,6-diamidino-2-phenylindole. Magnification, ×20.

Results and Discussion

EI24/PIG8 Targets the Endoplasmic Reticulum. We first wanted to establish the target organelle(s) of EI24/PIG8 because this provides information about the site where the death signal is likely first generated. Basal EI24/PIG8 expression levels in 3T3 fibroblasts were extremely low (data not shown). Therefore, to enable detection by immunofluorescence microscopy, cells were treated with sublethal levels of etoposide to increase p53-dependent EI24/PIG8 expression (7). As shown in Fig. 1*A*, EI24/PIG8 is a cytoplasmic protein which seems to be associated with particular intracellular structures. To identify this subcellular compartment(s), we used EI24/PIG8-eGFP expression vectors because this allows detection in both live and fixed cells and does not require additional etoposide treatment. Transfected

EI24/PIG8-eGFP displayed a similar pattern as endogenous EI24/PIG8 (Fig. 1*A*). Appearance of cells with a smaller and apoptotic morphology was also observed. In contrast, eGFP was evenly distributed over cytoplasm and nucleus without affecting cell viability. These effects, therefore, could only have been contributed by the EI24/PIG8 moiety of the fusion protein.

EI24/PIG8 could conceivably be located in the ER and/or mitochondria, organelles known to be involved in apoptotic processes (19, 20). To test these possibilities, EI24/PIG8-eGFP expression vector was cotransfected with a vector which expresses a red fluorescent protein specifically targeted to the ER (Red2ER). Our results show that EI24/PIG8-eGFP expression colocalized with the ER marker but not with a mitochondria-specific stain (Fig. 1*B* and *C*, respectively). To further corroborate ER targeting of EI24/PIG8, cells were again transfected with EI24/PIG8-eGFP or eGFP expression vectors, and whole cell lysates and ER-enriched subcellular fractions were obtained. Equal amounts of protein were then loaded and separated by SDS-PAGE, followed by immunoblotting with anti-eGFP antibody. Consistent with our microscopy data, EI24/PIG8-eGFP, but not control eGFP, was enriched in the ER fraction relative to whole cell lysate (Fig. 1*D*). Finally, we transfected etoposide-treated cells with Red2ER vector followed by immunofluorescent staining with anti-EI24/PIG8 antibody to detect endogenous protein. As shown in Fig. 1*E*, localization of EI24/PIG8 again completely overlapped with the transfected ER marker. These results establish EI24/PIG8 as an ER-localized protein, suggesting that the apoptotic signal is triggered at this organelle when levels of EI24/PIG8 increase after induction by p53.

EI24/PIG8 Is a Novel Bcl-2–Binding Protein. Many death regulators are present in the ER where they have important although poorly understood functions (19–24). For instance, Bcl-2, best known for its antiapoptotic role in mitochondria, also interferes with apoptosis induction by Bax, ionizing radiation, serum withdrawal, and c-Myc expression in the ER (20). As many of these proteins can form concentration-dependent complexes that have different activities, we speculated that EI24/PIG8 may also bind with a cell death factor at this organelle. To test this hypothesis, we first generated a His-tagged EI24/PIG8-affinity column which was incubated with cell lysate from 3T3 cells. Bound proteins were separated by SDS-PAGE and detected by immunoblotting with various antibodies, as indicated in Fig. 2*A*. Interestingly, Bcl-2, but virtually no Bcl-X_L or Bax, was specifically bound by EI24/PIG8. Bad and Bcl-X_S were undetectable (data not shown). To validate and expand on this observation, we did a coimmunoprecipitation assay to determine whether endogenous EI24/PIG8 and Bcl-2 also bind *in vivo*. S91 melanoma cells, which express high levels of Bcl-2, were treated with etoposide to induce EI24/PIG8 (7, 11). Next, cell lysates were incubated with anti-EI24/PIG8 antibody or immunoglobulin G control, and coimmunoprecipitated complexes were separated by SDS-PAGE followed by immunoblotting with anti-Bcl-2 antibody. As shown in Fig. 2*B*, endogenous Bcl-2 was specifically associated with endogenous EI24/PIG8 (note that two different Bcl-2 products can be detected in S91 cells, as described in ref. 25, 26). We also wanted to confirm this result for exogenous EI24/PIG8 and Bcl-2. FLAG-EI24/PIG8 expression vector was transfected into fibroblasts that stably overexpress Bcl-2 (12). Whole cell lysates were incubated with anti-FLAG antibody or immunoglobulin G control, and coimmunoprecipitation and immunoblotting with anti-Bcl-2 antibody was done as before. As shown in Fig. 2*C*, Bcl-2 was specifically associated with FLAG-EI24/PIG8 because no Bcl-2 was detected with immunoglobulin G control and Bcl-2 did not associate with control eGFP expressed from transfected eGFP expression vector. Thus, EI24/PIG8 is a novel Bcl-2–binding protein.

Finally, we wanted to know if the amount of etoposide-induced EI24/PIG8 would be sufficient or insufficient to bind with all ER-resident Bcl-2, which in lymphoma cells was estimated to reach 20% of total Bcl-2 (25) although lower levels seem to be present in other cell types (26). S91 cells were again treated with etoposide, and Bcl-2 expression was determined in control lysate and lysate depleted of EI24/PIG8 and associated Bcl-2 by coimmunoprecipitation with anti-EI24/PIG8 antibody. Fig. 2*D* shows that depletion of EI24/PIG8 removes 33% of Bcl-2, suggesting that the amount of induced EI24/PIG8 is sufficient to bind all available ER-resident Bcl-2.

Mapping of EI24/PIG8-Bcl-2 Binding Domains. To characterize the interaction between EI24/PIG8 and Bcl-2 in more detail, we assessed binding of ^{35}S-labeled Bcl-2 and Bcl-2 mutants to a His-EI24/PIG8 affinity column. As shown in Fig. 3*A* (*left*), deletion of residues 78 to 91, 6 to 82 (which includes the BH4 domain),

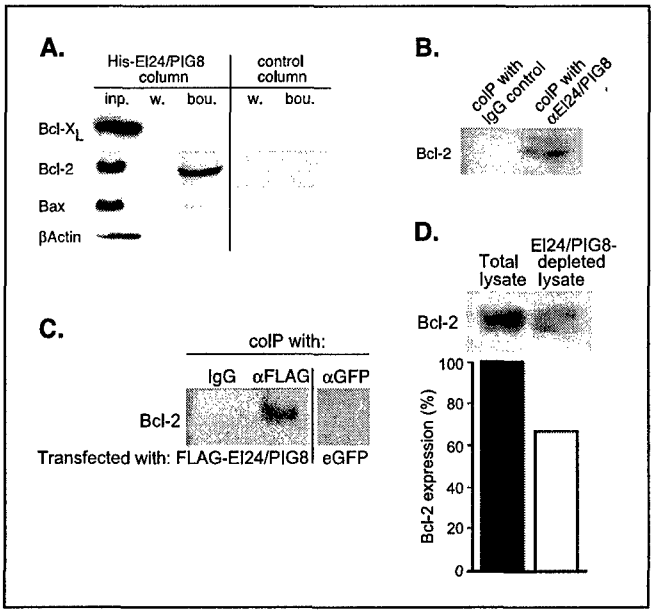

Figure 2. EI24/PIG8 is a novel Bcl-2–binding protein. *A*, 3T3 cell lysate (1 mg protein) was incubated with His-EI24/PIG8 or control column, and bound protein was analyzed by immunoblotting with the indicated antibodies. Only Bcl-2 is specifically retained. β-Actin serves as negative control. *inp.*, lysate input (10%); *w.*, final wash; *bou.*, bound protein. *B* and *C*, coimmunoprecipitation (*coIP*) experiments showing that endogenous (*B*) and exogenous (*C*) EI24/PIG8 is associated with Bcl-2. *B*, S91 cells were treated for 24 hours with 10 μg/mL etoposide to induce EI24/PIG8 expression. Cell lysates were subjected to coimmunoprecipitation with anti-EI24/PIG8 antibody or immunoglobulin G control. Protein complexes were then separated by 12% SDS-PAGE followed by immunoblotting with anti-Bcl-2 antibody. *C*, Bcl-2-overexpressing fibroblasts were transfected with FLAG-EI24/PIG8 expression vector and subjected to coimmunoprecipitation with anti-FLAG antibody or immunoglobulin G control, followed by immunoblotting of complexes with anti-Bcl-2 antibody. *Right*, Bcl-2 is not associated with overexpressed eGFP after transfection with eGFP vector, followed by coimmunoprecipitation with anti-GFP antibody. *D*, depletion of EI24/PIG8 from etoposide-treated S91 cell lysate results in 33% loss of Bcl-2, which exceeds the amount of ER-resident Bcl-2 (25, 26). Cells were etoposide-treated as above, and Bcl-2 expression in control cell lysate and lysate depleted of EI24/PIG8 and associated Bcl-2 was determined by coimmunoprecipitation with anti-EI24/PIG8 antibody and immunoblotting with anti-Bcl-2 antibody (60 μg/lane). *Bottom*, quantitation of bands after densitometry.

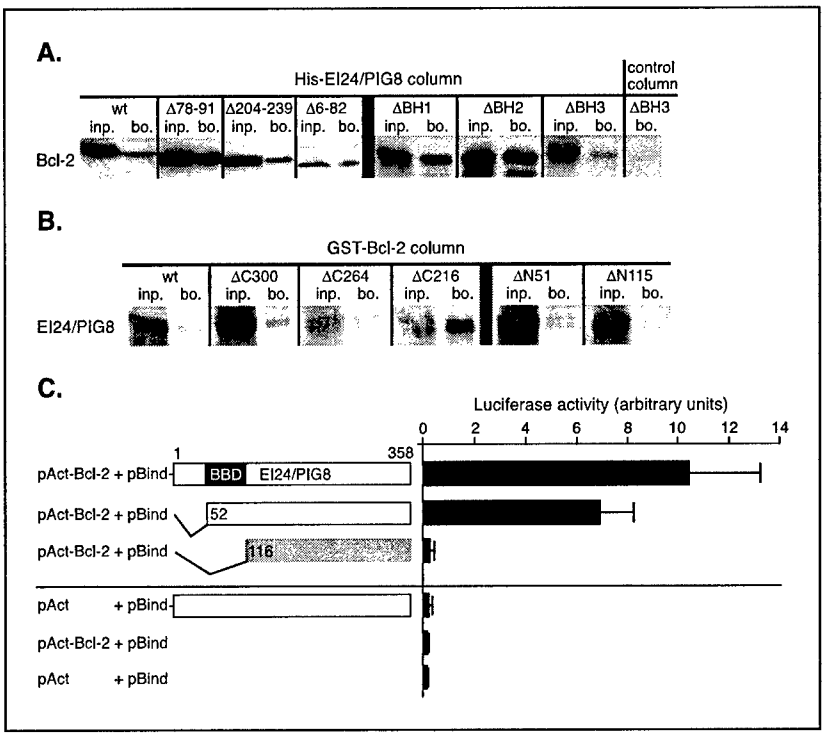

Figure 3. *In vitro* and *in vivo* domain-mapping analysis of EI24/PIG8 and Bcl-2. *A*, *in vitro* generated ^{35}S-labeled wild-type (wt) and mutant Bcl-2 were incubated with His-EI24/PIG8 column, and bound protein was analyzed by autoradiography. Only Bcl-2 mutant ΔBH3 lost all His-EI24/PIG8–binding capability, equal to binding to control column loaded with bacterial extract without EI24/PIG8 (*far right*). *B*, reciprocal experiment showing that deletion of N-terminal residues 1 to 115, but not 1 to 51, of EI24/PIG8 abrogates binding with GST-Bcl-2 , suggesting that region 52 to 115 contains the Bcl-2–binding domain (*BBD*). *inp.*, lysate input (20%); *bo.*, bound protein. *C*, mammalian two-hybrid assay showing that Bcl-2 (cloned in pACT vector) and EI24/PIG8 (cloned in pBIND vector) functionally interact and likely directly bind (*top*). Deletion of the identified Bcl-2-binding domain abrogates the interaction. Negative controls are shown below the divider.

and 204 to 239 (which contains the membrane insertion domain) was dispensable for binding with EI24/PIG8, suggesting that the critical binding domain resided between residues 92 and 203. This region contains BH domains 1 to 3 (21), which were then individually deleted, and binding of BH mutants to EI24/PIG8 was again tested. As shown in Fig. 3*A* (*right*), Bcl-2 mutants missing BH1 (residues 136-155) or BH2 (residues 187-202) were not affected. The BH3 mutant, which misses residues 93 to 107, no longer bound at levels equal to that of a control column (*far right*). In the reciprocal experiment we assessed binding of ^{35}S-labeled EI24/PIG8 and a series of truncation mutants to a GST-Bcl-2 affinity column. As shown in Fig. 3*B*, all C-terminal deletions and an N-terminal mutant missing residues 1 to 51 still bound. However, further N-terminal deletion of residues 1 to 115 abrogated all binding. These results suggest that the Bcl-2–binding domain in EI24/PIG8 is located between residues 52 and 115 and that this region binds with the BH3 domain of Bcl-2. This latter domain constitutes the heterodimerization interface in the BH3 domain–containing protein families, and thus EI24/PIG8 also seems to use this surface (21, 22).

Finally, we used a mammalian two-hybrid system to test the functional interactions between Bcl-2 and EI24/PIG8 *in vivo*. Bcl-2 cDNA was fused to the Gal4 DNA-binding domain in vector pACT, whereas EI24/PIG8 cDNA was cloned in frame with the VP16 transcriptional activation domain in vector pBIND. Cells were cotransfected with these and/or empty control vectors together with a luciferase reporter plasmid, and normalized luciferase activity (expressed as arbitrary units) was determined. As shown in Fig. 3*C*, significant luciferase activity was detected when Bcl-2 and EI24/PIG8 were coexpressed but not when they were transfected as single vectors. The N-terminal mutant missing residues 1 to 51 still interacted with Bcl-2, but further deletion of the Bcl-2-binding domain resulted to loss of luciferase activity. These results are consistent with our *in vitro* protein-protein binding studies and provide strong evidence that Bcl-2 and EI24/PIG8 can bind in the ER.

Loss of EI24/PIG8 Is Associated with Breast Tumor Invasiveness. In the final series of experiments, we wanted to obtain information about the potential role that loss of EI24/PIG8

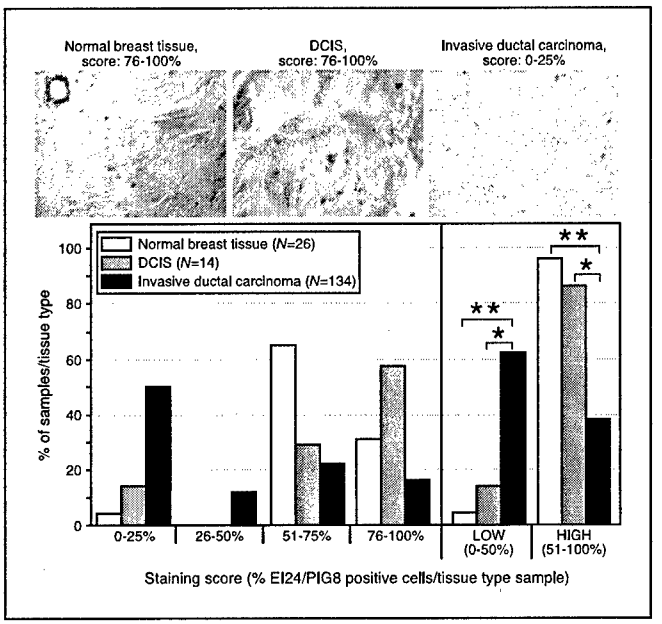

Figure 4. Immunohistochemical analysis showing that loss of EI24/PIG8 expression is associated with invasive ductal carcinoma but not normal breast tissue and ductal carcinoma *in situ*. Anti-EI24/PIG8 antibody was used for staining of breast cancer tissue progression arrays. Representative samples of all three immunostained tissue types are shown for illustration purposes (magnification, ×10). Staining scoring ranges are indicated. Results are summarized (*right*) comparing low (0-50%) versus high (51-100%) EI24/PIG8 expression. *, **: $P < 0.001$ (χ^2 analysis).

plays in breast cancer development, as suggested by earlier chromosomal studies (8, 9). We analyzed expression of EI24/PIG8 in normal breast tissue, ductal carcinoma *in situ*, and invasive ductal adenocarcinoma on breast cancer tissue progression arrays. Each sample was scored for the fraction of EI24/PIG8-positive cells as illustrated in representative samples of each tissue type in Fig. 4. The results are shown for each category and are summarized as low (0-50%) or high (51-100%) EI24/PIG8 expression. In normal tissue and ductal carcinoma *in situ*, 96% and 86% of specimens have high EI24/PIG8 expression, respectively. However, in invasive cancers this fraction has dropped to only 38%. Conversely, only 4% of normal breast tissue and 14% of ductal carcinoma *in situ* samples are classified as low in EI24/PIG8 expression whereas this number increases to 62% in invasive cancers. This statistically significant reduction of EI24/PIG8 expression in invasive cancers relative to normal breast tissue and its slightly less reduction in ductal carcinoma *in situ* suggest a role for EI24/PIG8 in suppressing tumor invasiveness. No correlation between loss of EI24/PIG8 and estrogen or progesterone receptor status or grade of the tumors was observed (data not shown).

A Model for the Action and Function of EI24/PIG8. Bcl-2 is not only present in mitochondria but is also found in the nuclear envelope and ER (25, 26). In the latter organelle, Bcl-2 is involved in regulating calcium fluxes between ER, cytoplasm, and mitochondria, which can greatly affect downstream enzyme activities and cell viability (24). It was recently shown that the ratio of Bcl-2:Bax/Bak, but not the absolute amount of Bcl-2, is a major factor in this process presumably because shifts in concentration control which death factor complexes are formed, with dramatic consequences (27). Our data suggest that binding of EI24/PIG8 with Bcl-2, which occurs after p53-dependent induction of EI24/PIG8 increases its concentration to relatively high levels compared with the amount of resident Bcl-2, leads to perturbations in the activity of Bcl-2 which may affect the entire physiology of the ER. It is conceivable that this event contributes to the apoptotic EI24/PIG8-dependent signaling cascade which ultimately may involve multiple organelles (28). In accordance with this interpretation, it was shown that overexpression of Bcl-X_L, which we showed to be incapable of directly binding with EI24/PIG8, can inhibit EI24/PIG8-dependent apoptosis in transfected fibroblasts, suggesting that Bcl-X_L can functionally substitute for EI24/PIG8-bound Bcl-2 (8).

Our immunohistochemical analysis suggests that EI24/PIG8 prevents tumor spreading rather than contributes to the development of the primary tumor, which is consistent with the suspected tumor suppressor activity of *EI24/PIG8* in aggressive breast cancer. This indicates that EI24/PIG8 may interfere with the activity of late-stage metastasis genes during mammary carcinogenesis. Answers to these important new questions, as well as further assessment of loss of EI24/PIG8 as a prognostic biomarker for disease progression and perhaps resistance to etoposide and/or other chemotherapeutics, may result in new insights into the biology and treatment of this disease.

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Apoptosis factor EI24/PIG8 is a novel endoplasmic reticulum-localized Bcl-2-binding protein which is associated with suppression of breast cancer invasiveness

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ABSTRACT

P53 is a critical tumor suppressor which removes cells with DNA damage by regulating expression and activity of a select group of p53-Induced Genes *(PIG*s) that subsequently induce apoptosis. *PIG8* was also identified as a gene induced by etoposide, and named *Etoposide-Induced gene 24 (EI24)*. Later experiments established EI24/PIG8 as a pro-apoptotic factor and suggested that it may function as a tumor suppressor. Indeed, *EI24/PIG8* is relatively highly mutated in aggressive breast cancers and is located in a region which expresses frequent loss of heterozygosity. However, despite these important observations, the activity and role of EI24/PIG8 remain largely unknown. We utilized (immmuno)fluorescence microscopy and subcellular fractionation techniques to show that EI24/PIG8 is localized in the endoplasmic reticulum (ER). Pull-down experiments showed that it specifically binds with Bcl-2, a death regulator known to reside in mitochondria, ER and the nuclear envelope. EI24/PIG8-Bcl-2-binding was corroborated by co-immunoprecipitation and other *in vitro* and *in vivo* protein-protein binding assays. Further analysis showed that EI24/PIG8 utilizes its N-terminal region to bind the BH3 domain in Bcl-2. Finally, we used immunohistochemical techniques to analyze expression of EI24/PIG8 in breast cancer tissue progression arrays, and showed that loss of EI24/PIG8 is associated with tumor invasiveness, but not development of the primary tumor. These results suggest that EI24/PIG8 is a novel, ER-localized Bcl-2-binding protein which may contribute to apoptosis by modulating the activity and/or function of Bcl-2 in this organelle. EI24/PIG8 may serve to prevent tumor spreading, consistent with its suspected role as a tumor suppressor.