**Proteomic Identification of Glycosylphosphatidylinositol Anchor-Dependent Membrane Proteins Elevated in Breast Carcinoma**

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Running Title: GPI anchor-dependent membrane proteins from breast cancer

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**Keywords:** Breast Cancer, Glycobiology, Mass Spectrometry, Tissue, Serum  

**Background:** GPI anchored proteins are elevated in breast carcinoma.

**Results:** We have utilized mass spectrometry and molecular biology techniques to capture and identify GPI anchored proteins from breast carcinoma.

**Conclusions:** Increased GPI anchor addition contributes to the dedifferentiation of malignant breast epithelial cells.

**Significance:** We have identified new potential diagnostic and therapeutic targets for breast carcinoma.

**SUMMARY**

The glycosylphosphatidylinositol (GPI) anchor is a lipid and glycan modification added to the C-terminus of certain proteins in the endoplasmic reticulum (ER) by the activity of a multiple subunit enzyme complex known as the GPI transamidase (GPIT). Several subunits of GPIT have increased expression levels in breast carcinoma. In an effort to identify GPI anchored proteins and understand the possible role of these proteins in breast cancer progression, we employed a combination of strategies. First, alpha toxin from *Clostridium septicum* was used to capture GPI anchored proteins from human breast cancer tissues, cells, and serum for proteomic analysis. We also expressed short interfering RNAs targeting the expression of the GPAA1 and PIGT subunits of GPIT in breast cancer cell lines to identify proteins whose membrane localization is dependent on GPI anchor addition. Comparative membrane proteomics using nano ESI-RPLC-MS/MS led to the discovery of several new potential diagnostic and therapeutic targets for breast cancer. Furthermore, we provide evidence that increased GPI anchor addition in malignant breast epithelial cells promotes the dedifferentiation of malignant breast...
epithelial cells in part by increasing the levels of cell surface markers associated with mesenchymal stem cells.

Posttranslational addition of a glycosylphosphatidylinositol anchor (GPI), is performed in eukaryotic cells via the activity of the GPI transamidase (GPIT) (1). GPIT is a multisubunit enzyme complex required for the expression of GPI anchored proteins on the cell surface. GPI anchored proteins are predicted to comprise approximately 1-2% of translated proteins in mammals (2). Several GPI anchored proteins identified to date are tumor antigens such as carcinoembryonic antigen (3), mesothelin (4), prostate specific stem cell antigen (5), and urokinase plasminogen activator receptor (6), suggesting possible roles for this class of proteins in promoting tumorigenesis.

The predictive annotation of GPI anchoring in mammalian protein databases is difficult as there are no common consensus sequences that clearly indicate that a protein will receive a GPI anchor. There are several amino acid features that have been characterized in the C-terminus of proteins that receive the GPI anchor (7). The discovery of these common characteristics led to the development of algorithms to predict the probability of GPI anchor addition such as FragAnchor (8), GPI SOM (9), and Big-PI (10). Furthermore, the experimental isolation and identification of GPI anchored proteins from mammalian cells is often hampered due to the lower expression levels of GPI anchored proteins in many cell types coupled with difficulty in extracting these proteins due to the presence of both lipid and glycan structures (Fig. 1A, core GPI structure). GPI anchored proteins can be released into a soluble form using the bacterial enzyme GPI-specific phospholipase C (PI-PLC) (11). However, certain GPI anchored proteins may be phospholipase C insensitive due to acylation within the GPI anchor (12). In an effort to overcome these obstacles, we are employing the use of the bacterial toxin known as alpha toxin (AT), isolated from Clostridium septicum, to capture and enrich GPI anchored proteins from breast carcinoma for identification by mass spectrometry (See the fractionation scheme in Fig. 1B). AT is a member of the aerolysin-like pore forming toxins that bind with GPI anchored proteins (13). The diversity of GPI anchored proteins that AT can bind with suggests that the binding occurs via the GPI anchor without peptide requirements.

Human breast carcinomas express elevated levels of several GPIT subunits such as GPAA1 (GPI Anchor Attachment Protein 1) and PIGT (GPI Class T) due to gain of chromosome copy number (14). Increased expression of these subunits has been shown to induce tumorigenicity in vitro and in vivo (14). In our study, we document that increased expression of GPIT subunits results in increased levels of GPI anchored proteins in breast cancer epithelial cells evidenced by binding of AT from C. septicum. We isolate and identify proteins binding to AT using nano ESI-RPLC-MS/MS analysis. Our data indicate that the membrane abundance of several cell surface receptors that are also found in mesenchymal stem cell populations are dependent on the expression of GPAA1 and PIGT. We report that increased expression of GPAA1 and PIGT positively regulates the expression of the
embryonic Forkhead/Fox transcription factor FOXC2. Elevated expression of GPI anchored proteins also increases the expression of several mitochondrial membrane proteins that may promote the growth and survival of breast cancer. We also provide evidence that AT binds with GPI anchored proteins released into serum allowing the capture and detection of potential markers for the detection of breast cancer. Overall, these results indicate that GPI anchored proteins are abundant in breast cancer cells with functions that promote tumor growth and spread, making these proteins ideal diagnostic and therapeutic targets.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Reagents*  
The following reagents were purchased from Sigma (St Louis, MO): Puromycin, Polybrene, DTT, Iodoacetamide, Immidazole, Urea, and anti-kindlin-3 rabbit polyclonal. All secondary antibodies and the following primary antibodies were purchased from Santa Cruz Biotechnology: anti-ERK, anti-FoxC2, anti-filamin A.

*Specimens and Cell line Information*  
Tissue specimens, matched adjacent normal and tumor, from patients with histologically proven invasive ductal breast carcinoma were collected in accordance with approved institutional review board Human Subject guidelines at GHSU. Blood from non-diseased and patients with ductal invasive breast carcinoma was collected pre-operatively and in accordance with approved institutional review board Human Subject guidelines at GHSU. Serum fractions were stored at -70°C. The MDAMB231, MCF10A, and 293 cell lines were obtained from the ATCC and cultured as recommended.

*Immunohistochemistry Staining*  
Paraffin-embedded tissue sections from patient matched tissue sets of normal and invasive ductal breast carcinoma were de-waxed and re-hydrated. Tissues were blocked with 0.5% hydrogen peroxide for 30 minutes. Tissues were rinsed in PBS with 0.2% tween 20 prior to blocking in 2.5% blocking serum (Vector Labs) for 20 minutes. Biotin labeled AT (2 μg/ml) diluted in PBS/0.2% tween 20 was added for 2 hours at room temperature. Following washes in PBS/0.2% tween 20 the tissues were incubated with the ABC reagent (Vector Labs) for 30 minutes. Positive staining was detected using DAB substrate followed by hematoxylin counterstain.

*AT Purification and Labeling*  
The plasmid pBRS10 encoding native AT expressing a histidine-tag (15) was transformed into BL21(DE3) E. coli. Bacteria were grown in 2XYT media supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenical at 37°C overnight. The culture was diluted 20 fold, at 1.0 OD protein expression was induced for 4 hours at room temperature using 0.2 mM IPTG. Bacterial pellets were resuspended in 0.5X PBS with protease inhibitors and lysed using a French Press. AT was purified using Talon cobalt resin (Clontech). Bound toxin was eluted using step immidazole gradients in 25 mM MES pH 6.5 buffer supplemented with 150 mM NaCl. AT
fractions were pooled and dialyzed to remove imidazole prior to SP cation-exchange chromatography. AT fractions were concentrated and buffer exchanged into 25 mM MES pH 6.5, 150 mM NaCl before storage at -80°C. AT was dialyzed into 1X PBS pH 9.0 prior to biotin labeling using Sulfo-NHS-LC-Biotin (Pierce) as recommended followed by buffer exchange using a 10,000 MWCO membrane.

Membrane protein extraction/AT binding/peptide preparation. Tissue (100 mg) or cell pellet (100 mg) was resuspended in 10 mM Hepes pH 7.5 plus protease inhibitors using a polytron (1 ml volume). The slurry was placed in a glass dounce homogenizer and cells lysed using 10 strokes each of the large and fine pestle. The solution was incubated on ice for 1 hour. Nuclei were removed by transferring to a microcentrifuge tube and centrifuging at 3,000 rpm for 5 minutes. The supernatant was removed to a fresh tube and the centrifugation was repeated twice. The final supernatant was placed in a Beckman ultracentrifuge tube and centrifuged at 100,000 x g for 1 hour at 4°C. The pellet containing a total membrane preparation was rinsed in 40 mM ammonium bicarbonate. The pellet was resuspended by sonication in 300 μl of 40 mM ammonium bicarbonate/10 mM DTT and rotated at room temperature for 2 hours to reduce proteins. An equal volume of iodoacetamide (10 mg/ml in 40 mM ammonium bicarbonate) was added and the tubes were vortexed before incubating in the dark for 45 minutes at room temperature. The protein solution was dialyzed overnight at 4°C into 10 mM ammonium bicarbonate using 4000 MWCO tube-O-dialyzer (G-Biosciences). Proteins were dried in the speed vacuum for long term storage at -80°C or used directly. Membrane proteins were also extracted from the cells using Triton X-114 (16). The detergent fractions were treated with 10 units of PI-PLC (Invitrogen) for 1 hour at 37°C. The aqueous fraction containing the GPI anchored proteins was precipitated with cold acetone. Proteins were resuspended in 1X PBS for binding to alpha toxin as described below for the total membrane extractions. Digest containing 60 μg of membrane proteins was prepared with 5 μg of sequencing grade modified trypsin (Promega) for sample analysis. Dried proteins or proteins PI-PLC released from Triton X-114 extractions were resuspended in 1X PBS by sonication. A protein assay was performed and 600 μg were incubated with 10 μg of biotinylated AT overnight at 4°C. For the analysis of serum samples, 10 μL of patient serum was incubated with 10 μg of biotinylated AT overnight at 4°C in a 300 microliter volume of 1X PBS.

Bound toxin reactive proteins were captured using 100 μl paramagnetic streptavidin particles (Promega) at 4°C for 2 hours. After washing in 1X PBS, captured proteins were eluted with 200 μl of 4M Urea/4 mM DTT/40 mM ammonium bicarbonate at 52°C for 1 hour. The eluted fraction was separated from the paramagnetic streptavidin particles using a magnetic stand. Alpha toxin bound proteins from Triton X-114 extractions were separated on 4-12 % polyacrylamide gels and each sample lane was cut into 6 gel slices. Proteins were reduced, carboxyamidomethylated, and released using standard in-gel trypsin digest protocols. Proteins eluted from streptavidin beads from the total membrane isolation protocol were digested with 5 μg of sequencing grade
Trypsin at 37°C overnight. Tryptic peptides were acidified with 200 μl of 1% trifluoroacetic acid and desalting was performed using C18 spin columns (Vydac Silica C18, The Nest Group, Inc.). Peptides were dried in the speed vac and resuspended in 19.5 μl buffer A (0.1 % formic acid) and 0.5 μl of buffer B (80 % acetonitrile/0.1 % formic acid) and filtered through a 0.2 μm filter (nanosep, PALL). Samples were loaded off-line onto a nanospray column/emitter (75μm x 13.5 cm, New Objective) self-packed with C18 reverse-phase resin in a nitrogen pressure bomb for 10 minutes. Peptides were eluted via a 160-minute linear gradient of increasing B at a flow rate of approximately 250 nl/min. directly into a linear ion trap mass spectrometer (LTQ XL ETD, Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray ion source). The top eight ions from the full MS (300-2000 m/z) were selected for MS/MS in CID at 34 % normalized collision energy with a dynamic exclusion of 2 repeat counts at 30 seconds duration.

Proteomic Data Analysis  The raw peptide data was converted to mzXML using ReAdW, a software written at the Institute for Systems Biology in Seattle, WA (http://www.systemsbiology.org). MS/MS spectra were searched against the International Protein Index (IPI) human sequence database (IPI.HUMAN.v.3.71) using MyriMatch (17). The MyriMatch search criteria included only tryptic peptides, all cysteines were presumed carboxyamidomethylated, and methionines were allowed to be oxidized. MyriMatch searches allowed a precursor error of up to 1.25 m/z and a fragment ion limit of within 0.5 m/z. All ambiguous identifications that matched to multiple peptide sequences were excluded. The identified proteins (2 or more peptides assigned) from each individual tumor and normal sample were filtered and grouped using IDPicker software. IDPicker software incorporates searches against a separate reverse database, probability match obtained from MyriMatch, and DeltCN scores to achieve false discovery rates of < 5%. Information about IDPicker tools can be found at http://www.mc.vanderbilt.edu/msrc/bioinformatics/.

Biological Function Annotation Proteins (defined by 2 or more peptides assigned in at least 2 biological samples) binding to AT were converted to gene symbols and uploaded to DAVID 2009 (the Database for Annotation, Visualization and Integrated Discovery) for analysis.

RNA interference Constructs RNA interference (RNAi) target sequences were chosen using the Oligoengine software. Two target sequences for each gene were tested and the following sequences were chosen based on >80 % knockdown of mRNA following infection in MDAMB231 cells. GPAA1 target : (NM_003801) 5’ TCTTCCTCTACTTGCTCCC 3’ and PIGT target : (NM_015937) 5’ GACACTGACCACTACTTTC 3’ were synthesized in oligos as recommended by the manufacturer for cloning into pSIH-H1shRNA vector (SBI System Biosciences).

Construction of GPAA1 and PIGT lentiviral expression vectors Full length GPAA1 and PIGT were PCR amplified using primers with flanking sequence matching the pCDH1-MSCV-MCS-EF1-Puro cDNA expression vector (SBI System Biosciences). PCR products (gel purified) mixed with gel purified
NotI/EcoRI cut vector were ligated and transformed using the Cold Fusion Kit (SBI System Biosciences).

**Lentivirus Production and Cell Transduction**

Lentivirus was produced by transfection of 293T cells using Lipofectamine 2000 with the following amounts of plasmids: 8 μg envelope (pMD.G), 5 μg lentiviral siRNA vector or lentiviral expression clone, and 8 μg of packaging plasmid. Approximately 5.5 ml of 293T cell suspension (1.2 x 10⁶) in growth media were seeded onto lipofectamine 2000 complexes (formed in Opti-MEM I media) in 10 cm tissue culture plates. The next day the cells were replenished with fresh media and infectious lentivirus supernatant was collected at 48 hours and 72 hours posttransfection. Polybrene was added to lentiviral supernatants at a final concentration of 8 μg/ml and the virus was placed on cells to be infected. Virus entered cells during centrifugation at 2,500 rpm for 30 minutes at 37°C. Cells were fed with normal growth media between infections. Two rounds of infection were performed prior to selection of infected cells using puromycin 1 μg/ml.

**Quantitative RT-PCR**

Samples (50 μl packed cells) were extracted using TriZol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After DNase treatment, RNA (2 μg) was reverse transcribed using Superscript III (Invitrogen) with random hexamers and Oligo (dT). Primer pairs for assay genes and control genes were designed within a single exon using conditions described (18,19). Primers were validated with respect to primer efficiency and single product detection. Primer sequences are included in supplemental Table 1. The control gene, Ribosomal Protein L4 (RPL4, NM_024212) was included on each plate to control for run variation and to normalize individual gene expression. Samples were run with negative control templates prepared without reverse transcription to ensure amplification is specific to cDNA. Triplicate Ct values for each gene were averaged and the standard deviation from the mean was calculated. Data was converted to linear values and normalized as described previously (18,19).

**Results**

**Specificity of AT for GPI Anchored Proteins** - AT has been used previously by researchers to screen for cells that carry mutations of enzymes in the GPI biosynthetic pathway (20). Despite evidence that AT binds to cells that display GPI anchored proteins, in vitro binding of proteins to AT has not been described. Therefore, to establish the specificity of AT binding for GPI anchored proteins in vitro, we created breast cancer cell lines using MDA-231 breast cancer cells that express a control short interfering RNA that does not target any human genes (control siRNA) or short interfering RNA targeting the GPI anchor attachment protein 1 (GPAA1) and the GPI Class T (PIGT) subunits of the GPIT. The addition of the GPI anchor to the C-terminus of proteins by GPIT is required for the surface expression of GPI anchored protein receptors such as PrP (21). The subunits GPAA1 and PIGT are each essential for the addition of the GPI anchor to proteins by the GPIT (22,23). The MDA-231 cells expressing short interfering RNA targeting the GPAA1 and PIGT genes reduced the level of mRNA expression for each subunit by at least 80% (Fig.
2A, 2B). We used these cell lines to establish that AT binding to proteins requires the GPI anchor. Membrane proteins from these cells were extracted and used in a binding assay with biotin labeled AT and streptavidin magnetic beads in vitro (See Scheme in Fig. 1B). The silver stained gel shown in Fig. 2C demonstrates that several membrane proteins isolated from MDAMB231 cells expressing control siRNA are bound by AT. However, in MDAMB231 cells expressing siRNA that targets the GPAA1 or PIGT gene there is a dramatic reduction in the levels of membrane proteins binding to the toxin, similar to the low levels observed for non-transformed mammary MCF10A cells. The total protein amounts present in each AT binding reaction were equivalent verified by protein assay and nano-ESI-RPLC-MS/MS analysis prior to AT binding (see Fig. 1B) and a silver stained gel representing 10% of the protein inputs are shown in Fig. 2C. Therefore, we report that AT can be used to bind with extracted membrane proteins in vitro and binding requires the presence of a GPI anchor verified by the dependence of AT binding on GPAA1 and PIGT gene expression.

Elevated Expression Levels of Enzymes in the GPI Biosynthetic Pathway Result in Increased Levels of GPI anchored Proteins in Breast Carcinoma Cells. The GPI anchor is assembled by stepwise assembly in the ER membrane prior to addition to the C-terminus of proteins by the GPIT (Fig. 1A, blue asterisk). We have compared the mRNA expression levels for each enzyme in this pathway in malignant MDAMB231 cells and non-transformed mammary MCF10A cells, using quantitative real-time PCR. Our data indicate that the enzymes involved in the last 3 steps of the GPI biosynthetic pathway, additions of phosphoethanolamine by PigO and PigG and enzymes that comprise the GPIT, are significantly increased in breast carcinoma cells relative to non-transformed mammary cells (Fig. 3A). MCF10A expression levels were set to 1 to demonstrate the fold increase of each enzyme in the MDAMB231 cells. The mRNA levels of enzymes participating in the earlier steps of this pathway were not significantly different between MCF10A and MDAMB231 (data not shown).

AT Shows High Levels of Binding to Human Breast Carcinoma Tissue Compared with Adjacent Normal Breast Tissue. Next, we wanted to determine the cell types that may be expressing GPI anchored proteins in human breast cancer tissue. Therefore, we stained human ductal breast carcinoma and adjacent normal breast tissue with biotin labeled AT. Results shown in Fig. 3B demonstrate that AT binds very weakly to the stromal cell compartment of normal breast tissue (Fig. 3B left), however, the toxin binds with high affinity to malignant breast epithelial cells in the ductal breast carcinoma tissue (Fig. 3B right). These results verify that GPI anchored proteins are increased in breast cancer. This data also reveals that GPI anchored proteins are expressed in cell types different between normal and malignant breast tissue. The stromal cells express low levels of GPI anchored proteins in normal breast; while the epithelial cells are expressing high levels of GPI anchored proteins in the tumor.

Identification of Membrane Proteins Binding AT from Breast Carcinoma Tissue, Serum, and Cells. GPI anchored proteins represent
valuable therapeutic and/or diagnostic markers due to the fact that they are localized on the surface of malignant cells and certain GPI anchored proteins may be cleaved by phospholipase activity into circulation. Therefore, to identify proteins receiving the GPI anchor in breast carcinoma, we isolated these proteins from human tissue and serum using AT. The proteins identified by nano ESI-RPLC-MS/MS from tissue and serum were compared with data obtained using the human cell lines expressing control siRNA, GPAA1 siRNA, or PIGT siRNA. Cumulatively, over 1,000 individual proteins were identified from the membrane extractions of matched breast cancer tissues (3 cases-stage III ER+/PR+, stage II ER-/PR-, stage III ER+/PR+), cell lines (MCF10A and MDAMB231), and serum analysis (3 cases non-diseased and 3 cases invasive ductal breast carcinoma). As shown in Fig 4A, total membrane isolation prior to nano ESI-RPLC-MS/MS led to membrane proteins being identified as 48% of the total proteins prior to AT enrichment. This value can be compared to 7-8% of membrane proteins being identified by MS/MS without prior membrane protein enrichment. The distribution of proteins after AT capture (proteins listed in Table 1) is mainly membrane (>80%) as shown in Fig. 4B. Furthermore, over 50% of the membrane proteins identified binding with AT are localized at the plasma membrane. Therefore, AT enriches for proteins at the cell surface that represent a large pool of potential diagnostic or therapeutic biomarkers. Table 1 lists all proteins, in alphabetical order with respect to official gene code, (2 or more peptides assigned for each protein from 2 separate biological samples) detected binding with AT from breast tissues (T), serum (S), and cell lines (CL). The percent coverage and complete peptide list for each protein in Table 1 is shown in Supplemental Table 2. Surprisingly, there is an abundance of mitochondrial membrane proteins that are enriched binding to AT from breast cancer samples. We utilized DAVID (Database for Annotation, Visualization, and Integrated Discovery) to annotate the functions of the proteins listed in Table 1. Many of the proteins binding with AT function to bind and/or transport molecules such as amino acids, ions, lipids, and nucleotides, examples include: ATP1A, AT2A2, GOT2, RAB11B, SLC1A5, SLC25A24, and SLC25A5. A large number of proteins binding the toxin function in cell signaling and cell communication such as EPHA2, F3, GNAS, GNAI2, ITGA2, ITGA3, MYH9, CAV1, FERMT3 and FLNA. Several have enzymatic activities such as ATPase (ATP5B, ABCDF2, VCP, ATP1A1, RUVBL1, and HSPA8) or oxidoreductase activity (IMPDH2, HSD17B4, MDH2, and UQCRC2). Numerous cell surface glycoproteins binding with AT have IgG domains and function in cell adhesion, protein-protein clustering, integrin activation or antigen presentation such as BCAM, BSG, F3, HM13, IGHM, ITGA2, ITGA3, ITGA6, MUCB, and VCL. Finally, AT enrichment has led to the discovery of a breast cancer-specific uncharacterized membrane protein known as TMEM165 that is annotated to be GPI anchored.

**Cluster of Differentiation Markers that Require GPAA1 and PIGT Expression for Membrane Localization are also Found in Mesenchymal Stem Cell Populations.** We compared the membrane proteome of MDAMB231 cells expressing control siRNA, GPAA1
siRNA, and PIGT siRNA to identify proteins that change abundance in the membrane in response to changes in GPAA1 and PIGT expression levels. Many of the proteins binding with AT listed in Table 1 show GPIT dependent membrane localization. A protein listed as showing “GPIT dependence” indicates that the number of peptides detected and spectral abundance for these peptides identified from MDAMB231 cells were reduced by greater than 2 fold following GPAA1 or PIGT suppression. We identified all cluster of differentiation (CD) receptors that show GPIT dependence (Table 2). Overwhelmingly, the CD markers listed in Table 2 have been reported in mesenchymal stem cell (MSC) populations (24-31). All of these proteins have tumor-specific expression in breast cancer tissue with the exception of CD36. Many of these proteins partition into the detergent fraction following triton X-114 extraction and can be released into the aqueous phase following PI-PLC treatment (mass spectrometry data). These results demonstrate that the expression of GPAA1 or PIGT in breast carcinoma contributes to the de-differentiation of breast epithelial cells leading to the cell surface expression of CD markers found in mesenchymal stem cell populations. Suppression of GPI addition by reducing the expression of GPAA1 and PIGT reduced the abundance of these CD markers in the cell membrane.

**GPAA1 and PIGT Expression Levels Regulate FOXC2 Expression.**

Most of the proteins listed in Table 1 and Table 2 are not detected by mass spectrometry in non-transformed breast epithelial cultured cells or normal breast tissue and normal serum. In supplemental Table 3, we list the AT bound proteins identified in normal MCF10A cells, normal breast tissue, or normal serum. Therefore the proteins in Table 1 and Table 2 are induced in malignant breast epithelial cells. Furthermore, due to the high prevalence of tumor-specific proteins associated with mesenchymal cell populations, we analyzed the relative expression levels of embryonic transcription factors that may induce mesenchymal gene expression using quantitative real-time PCR. We discovered that FOXC2 levels were decreased by >80% in MDAMB231 cells expressing GPAA1 or PIGT siRNA compared with control siRNA cells (Fig. 5A). This data suggests that FOXC2 expression is dependent on the expression of GPAA1 and PIGT. We wanted to further test for a relationship between the expression level of FOXC2 and GPAA1 or PIGT expression levels. Therefore, we cloned the cDNA for GPAA1 and PIGT into a lentiviral expression vector and expressed these genes in non-transformed MCF10A mammary cells. Stable cell lines express GPAA1 and PIGT mRNA at a 4-fold increase compared with vector only cells (data not shown). Lentiviral expression of either GPAA1 or PIGT in MCF10A cells results in increased levels of FOXC2 mRNA (Fig 5B). FOXC2 protein levels in these cells were also elevated for GPAA1 and PIGT expressing MCF10A cells compared with control MCF10A cells (Fig. 5C). Therefore, we find a positive correlation between FOXC2 levels and GPAA1 and PIGT levels. Suppression of GPAA1 and PIGT in breast carcinoma significantly reduces FOXC2 expression; while increased expression of GPAA1 and PIGT in non-transformed mammary cells leads to elevated FOXC2 expression.
Serum Proteins Binding to AT are Potential Biomarkers for the Detection of Breast Carcinoma. AT can be used to capture GPI anchored proteins from serum. Pooled serum from 5 non-diseased patients was compared with pooled serum from 5 patients with ductal invasive breast carcinoma following AT binding on magnetic beads. The silver stained gel shown in Fig. 6A indicates that GPI anchored proteins are captured using alpha toxin from the sera of breast cancer patients with very few proteins adhering to the toxin from normal serum. The serum proteins binding with AT were identified by nano-ESI-RPLC-MS/MS from 3 non-malignant serum samples and 3 of the serum samples from ductal breast carcinoma patients. Proteins binding with AT that were found in cancer tissue or cells as well as serum, are included in Table 1. In supplemental Table 4, we list serum proteins that were found binding AT from cancer patient serum that were never found in tissues or cells. Interestingly, we found proteins involved in the coagulation pathway (PLG, F11, and KNG1) that were not identified from tissue and cells that bind AT in breast cancer patient serum. How these proteins are connected with breast cancer is unclear; however, these proteins were never identified from non-diseased patient sera after AT pull down.

We chose 2 of the proteins listed in Table 1 for further validation based on prevalence in breast cancer tissue and serum cases by nano-ESI-RPLC-MS/MS analysis. We have analyzed by Western blot sera from 15 cases of ductal invasive breast carcinoma and 10 cases of non-malignant sera, including 8 women with benign polycystic breast disease. Selected results shown in Fig. 6B demonstrate that following AT capture, FERMT3 (kindlin 3) and FLNA (filamin A) are detected cumulatively in at least 90% of breast cancer sera analyzed. Results from our analysis of the 10 non-malignant cases, (serum from the polycystic breast disease patients shown in Fig. 6C), indicate that FERMT3 was detected in only 1 of 10 cases with no detection of FLNA in all 10 cases. Gelsolin (GSN) is detected following AT capture from both non-malignant and malignant serum and serves as a control for the input and quality of the serum. Overall, these results suggest that the detection of AT reactive FERMT3 and FLNA may be useful for the detection of breast cancer from patient serum.

DISCUSSION

Mass spectrometry-based comparative membrane proteomics can enable the identification of novel cancer biomarkers by distinguishing proteins that change membrane localization between normal and malignant tissues and cells. We have extended these capabilities by adding an additional selective enrichment using AT from C. septicum to identify proteins receiving a GPI anchor or associating with GPI anchored proteins in normal and malignant breast tissues, cells, and serum. To our knowledge this is the first study that uses AT as an in vitro capture agent and as a probe for GPI anchored proteins in tissue sections. Numerous proteins identified binding with AT (31%) listed in Table 1 were annotated to be GPI anchored proteins using the Frag Anchor, GPI SOM, or Big-PI algorithms.

The cell compartment data analysis described in Fig 4 reveals that the majority of membrane proteins bound by AT are found at the plasma
membrane or in membrane vesicles, suggesting possible involvement of the GPI anchor in facilitating protein movement from membrane organelles to the cell surface. GPI anchored proteins have previously been reported in the ER, Golgi, exocytic vesicles, endocytic vesicles, and at the cell surface in analysis of parasite GPI anchored proteins (32). The localization of GPI anchored proteins in lipid rafts has been reported to allow them to serve as platforms to mediate vesicle trafficking and signal transduction. In fact, GPI anchored proteins are probably the most mobile since they can be transferred readily between different cell surfaces (33,34). However, GPI anchored proteins have never been reported in the mitochondria. Our data showing that several mitochondrial membrane proteins isolated from tissues and cells are bound by AT represents the first suggestion of association with GPI anchored proteins or GPI anchor addition for some of these proteins. Four of the top 5 most abundant mitochondrial membrane proteins binding AT (IMMT, HADHB, SLC25A5, and GOT2) are annotated by databases to be GPI anchored. Also, ACO2 a mitochondrial enzyme binds AT in cancer patient serum, tissue, and malignant cells indicating release from the cell. Therefore, these proteins may represent novel biomarkers for breast cancer.

Our finding that CD markers associated with mesenchymal stem cell populations are decreased in the cell membrane in response to GPAA1 or PIGT suppression is novel. This is the first report of a link between GPAA1 and PIGT expression and the expression of mesenchymal stem cell markers. The CD44 antigen present on cancer stem cells (CSCs) (CD44^{high}/CD24^{low}) does not change abundance in the membrane in response to GPAA1 or PIGT suppression (data not shown). Moreover, the transcription factors Snail and Twist that often change expression levels in CSC populations are not changing in response to changes in GPAA1 and PIGT expression levels (data not shown). These data suggest that breast cancer cells expressing high levels of GPI anchored proteins may have a distinct mesenchymal stem cell niche, and warrant further investigations.

We also report for the first time that the levels of the embryonic transcription factor Forkhead/Fox FOXC2 are correlated with changes in the expression levels of GPAA1 and PIGT. These results demonstrate that increased GPAA1 and PIGT expression may influence cell signaling pathways that activate FOXC2 expression. The FOXC2 transcription factor has been reported to be elevated in basal-like breast cancers (35). However, a recent study analyzed FOXC2 levels using the T-MTA-6A tissue array and found this transcription factor overexpressed in the majority of breast cancers and colon cancers suggesting a role in tumor progression (36). Ectopic expression of FOXC2 in normal adipose tissue induces mitochondrial biogenesis and increases the metabolic capacity of the cells (37). Our proteomic data indicating an enrichment of mitochondrial membrane proteins supports a possible hypothesis that the induction of FOXC2 in malignant breast epithelial cells due to increased GPIT expression leads to increased mitochondriogenesis and promotes the growth and expansion of dedifferentiated epithelial cells.

Mass spectrometry-based proteomic analysis of serum proteins is
often an arduous task requiring removal of abundant proteins and multiple steps of protein fractionation. We report that AT can be used to bind and isolate GPI anchored proteins released into serum, thereby simplifying the enrichment and proteomic analysis of potential markers from human serum. Using this method, we have verified that AT bound FERMT3 and FLNA from human serum are potential markers useful for the detection of breast carcinoma. Our analysis of a small set of sera from breast cancer patients and controls indicate that these markers can be bound by AT in 90% of the cancer cases with very low AT binding detected in non-malignant patients. A common obstacle to establishing a serum-based detection assay is the formation of protein complexes in serum and the inability of antibodies to detect certain proteins that may be found in these complexes. We have shown that AT binds very well to proteins from serum in vitro (Fig. 6). Therefore, AT can be used to isolate protein markers before detection using established platforms such as ELISA. 

In conclusion, we have developed AT as a reagent useful for the detection and capture of GPI anchored proteins. We have shown that GPI anchored protein expression is elevated in malignant breast epithelial cells. Our mass spectrometry-based comparative membrane proteomic results suggest new roles for GPI anchored proteins in breast cancer progression such as epithelial dedifferentiation and increased mitochondrial protein expression. In addition, we showed that FOXC2 expression is regulated by GPAA1 and PIGT expression levels. These findings indicate that molecular therapeutics targeting GPI biosynthetic machinery or specific GPI anchored cell surface receptors may be useful to control the de-differentiation of breast epithelial cells in malignant disease. Our discovery that AT can be used to capture GPI anchored proteins from serum has led to the discovery of new potential detection markers for breast cancer such as FERMT3 and FLNA. The discovery of AT binding to these proteins in both malignant tissue and cancer patient serum suggests that these proteins may be important in tumor progression and may be useful for the detection and prognostic monitoring of breast cancer using patient serum.

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REFERENCES
FIGURE LEGENDS

Figure 1. (A) Schematic of the GPI biosynthetic pathway. The core GPI anchor structure is assembled stepwise in the ER and added en bloc to the C-terminus of proteins that display a C-terminal signal sequence by the multisubunit enzyme complex GPIT (blue asterisk). Enzymes increased in breast carcinoma are labeled. (B) Schematic of the sample processing and isolation of GPI anchored proteins. Patient matched tissue samples and cells from the indicated cell lines were used for extraction of total membrane proteins. Membrane extractions were analyzed by nano-ESI-MS/MS to verify equivalent protein levels and quality. Biotinylated AT was added to membrane extractions and serum obtained from women with ductal invasive breast carcinoma or women with benign breast disease. GPI anchored proteins with bound AT were captured with streptavidin magnetic beads and analyzed by nano-ESI-MS/MS.

Figure 2. AT capture of membrane proteins requires the expression of GPAA1 and PIGT and therefore, the presence of a GPI anchor. (A) Real-time PCR measurement of GPAA1 mRNA levels in human breast cancer MDA MB 231 cells stably expressing control siRNA or GPAA1 siRNA. GPAA1 levels were normalized to RPL4 and the control level set to 1.0 for comparison. Error bars represent the standard error of the mean (SEM) for triplicate measurements. (B) Real-time PCR measurement of PIGT mRNA levels standardized and measured as described for GPAA1. (C) Silver stained gel of AT bound proteins obtained using equivalent inputs of membrane proteins isolated from MDA MB 231 stable cell lines and MCF10A cells.

Figure 3. GPI biosynthetic enzyme transcript levels and GPI anchored proteins are elevated in human breast cancer. (A) Real-time PCR analysis of enzymes in the GPI biosynthetic pathway using RNA isolated from MDA MB 231 cells and non-transformed human mammary MCF10A cells. Transcript levels are normalized to RPL4 and the MCF10A levels are set to 1.0 for comparison. Error bars represent the SEM for triplicate measurements. (B) Immunohistochemistry analysis of AT binding to GPI anchored proteins in ductal invasive breast carcinoma tissue and patient matched adjacent normal tissue, magnification 40X.

Figure 4. Alpha toxin capture enriches for membrane proteins. (A) Cell compartment analysis of proteins identified from nano-ESI-RPLC-MS/MS analysis of total membrane proteins from human breast cancer before AT capture. (B) Cell compartment analysis of proteins identified from nano-ESI-RPLC-MS/MS analysis of human breast cancer after AT capture. The percentage of proteins in the membrane compartment increases to 82%, and from this 82%, >50 % of the proteins are found in the plasma membrane.

Figure 5. GPAA1 and PIGT expression levels regulate FOXC2 transcript levels. (A) Real-time PCR analysis of FOXC2 transcript levels in MDA MB 231 cells stably expressing the indicated siRNAs. Error bars represent the SEM of data from 2 separate experiments with 5 replicates each normalized to RPL4. (B) Real-time PCR analysis of FOXC2 transcript levels in MCF10A cells stably expressing GPAA1 or PIGT cDNA. Error bars represent data from 2 separate experiments with 5 replicates each normalized to RPL4. (C) Representative Western blot analysis of FOXC2 protein levels in MCF10A cells for control (vector), GPAA1, or PIGT. Densitometry graph represents band density normalized to ERK levels.
Figure 6. GPI anchored proteins from serum bind AT. (A) Silver stained gel of AT bound proteins from pooled serum (n=5) obtained from non-diseased patients (Normal) and pooled serum (n=5) from patients with ductal invasive breast carcinoma (Breast Cancer).  (B) Western blot analysis of serum from patients with ductal invasive breast carcinoma following AT capture. The blot was probed using the indicated antibodies. (C) Western blot analysis of serum from women with benign polycystic breast disease following AT capture. Blots were probed using the indicated antibodies. Gelsolin binds with AT in serum obtained from patients with breast carcinoma and non-malignant serum samples and serves as a control for serum quality and input.
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(a) Two independent peptide matches required in two separate samples.
(b) International Protein Database.
(c) Proteins listed in italics have tryptic peptides identified from the N-terminal end of the protein and are not likely to be GPI-anchored.
(d) Source: S-12 serum, T = invasive ductal breast carcinoma tissue, CL = cell line MDAMB231.
(g) PI-PLC sensitive indicates release of the protein into the aqueous phase following treatment of triton X-114 detergent phase with the GPI-specific PI-PLC enzyme from B. Cereus.
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(a) Two independent peptide matches required in two separate samples, spectral counts and number of peptides reduced by ≥ 2 fold following suppression of GPAA1 or PICT (b) International Protein Database (c) GPI annotation by the GPI-D8 containing FragAnchor and GPI-SOM predicted proteins (http://navat.iichawaii.edu/~fraganchol/cgi-bin/18ddb.pl) or Big-PI http://mendel.imp.ac.cn/gp/gpi_server.html
Fig. 1

A. GPI Anchor Biosynthesis

B. Sera from women with ductal invasive breast carcinoma or benign breast disease

Patient Matched Breast Tissues

Extract Membrane Proteins

Pre-Toxin nano-ESI-MS/MS

Add Biotinylated Purified Recombinant Alpha Toxin

Capture Proteins with GPI anchor using Streptavidin-Magnetic Beads

Toxin Bound Fraction nano-ESI-MS/MS

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Proteomic identification of glycosylphosphatidylinositol anchor-dependent membrane proteins elevated in breast carcinoma

Peng Zhao, Alison V. Nairn, Shanterian Hester, Kelley W. Moremen, Ruth M. O'Regan, Gabriella Oprea, Lance Wells, Michael Pierce and Karen L. Abbott

J. Biol. Chem. published online May 31, 2012

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