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Proteomic profiling of lipid rafts in a human breast cancer model of tumorigenic progression

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Abstract

Tumor biomarkers assist in the early detection of cancer, act as therapeutic targets for intervention, and function as diagnostic indicators for the evaluation of therapeutic responses. To identify novel human breast cancer biomarkers, we have analyzed the protein content of lipid rafts isolated from a series of human mammary epithelial cell lines with increasing tumorigenic potential. Since lipid rafts function as platforms for protein interaction critical to several biological processes, we hypothesized that the abundance of proteins associated with proliferation, invasion and metastasis would be dysregulated in highly transformed cells. For this purpose, the MCF10A epithelial lineage, which include benign MCF10A cells, premalignant AT and TG3B cells, and malignant CA1a tumor cells, was utilized. Detergent-resistant membranes were isolated from each line and proteins were identified and relatively quantitated using iTRAQ™ reagents and tandem mass spectrometry. 57 proteins were identified, and 1667 peptide identifications, mapping to 49 proteins, contained sufficient information for semi-quantitative analysis. When comparing malignant to benign cells, we observed consistent alterations in groups of proteins, such as a 5.7-fold average decrease in G protein content (n=5), 2.7-fold decrease glycosylphosphatidylinositol-linked proteins (n=7) and 3.3-fold increase in intermediate filaments (n=9). Several of the identified proteins, including caveolin-1, filamin A, keratins 5,6 & 17, and vimentin, are bona fide or candidate biomarkers in clinical studies, underscoring the usefulness of the MCF10A series as a model to better understand the biological mechanisms underlying cancer progression.

Keywords

lipid rafts; biomarkers; breast cancer; caveolin-1; filamin A; iTRAQ; keratin; mass spectrometry; MCF10A; vimentin

Introduction

The plasma membrane is comprised of numerous of species of glycerophospholipids, sphingolipids, triglyceride variants, fatty acid and sterol-based structures, as well as integral proteins. Order arises from this highly complex environment in the form of demixing into two observed 'phases', liquid disordered (Ld) and liquid ordered (Lo) [1,2]. A high degree of lateral diffusion occurs within the liquid disordered phase. The liquid ordered domains, referred to as rafts, result from alignment of the rigid planar sterol rings of cholesterol with the long and highly saturated hydrocarbon chains of sphingolipids. Rafts are highly dynamic structures that may coalesce or disperse depending on specific lipid-lipid, lipid-protein, or

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protein-protein interactions. Furthermore, rafts are heterogeneous with respect to the types of proteins they harbor. Caveolae, a subclass of rafts, contain at least 15 caveolin molecules which form flask-like invaginations of the plasma membrane. Other rafts are specialized for apoptotic signaling by sequestering FAS, FASL and members of the DISC complex. Proteins carrying mostly saturated unbranched lipid modifications, such as acyl or glycosylphosphatidylinositol (GPI) groups, readily partition into the liquid ordered domain.

Lipid rafts participate in a wide variety of cellular activities, including receptor signaling, molecular transport across the membrane, and actin cytoskeleton rearrangement. They provide a platform for coordinating the interaction of groups of proteins, including receptors and effectors involved in signal transduction [3,4]. Numerous transmembrane G protein-coupled receptors including adrenergic, muscarinic, opioid, angiotensin and bradykinin receptors as well as receptor tyrosine kinases such as EGFR, IR, PDGFR and VEGFR have been identified in rafts. Signaling molecules that are typically associated with the inner layer of the plasma membrane such as G-proteins, adenylyl cyclases, PKA, PKC, MAPKs, SRC family members and eNOS have also been localized to lipid rafts. The close association of so many proteins involved in signal transduction within the lipid raft compartment indicates that rafts are important functional structures within cells. By extension, disease processes that result in aberrant signaling would be expected to result in observable changes in the protein composition of rafts. Mass spectrometry-based proteomic identification and quantitation can provide a snapshot of the lipid raft microenvironment and insight into disease processes.

As a tumor cell population evolves, signaling networks that regulate normal cell growth, development and apoptosis become altered in favor of increased clonal expansion. Although this evolution is unique from patient to patient, morphologically changes often occur sequentially. For human breast carcinomas these changes entail progression from normal epithelium to benign hyperplasia to atypical hyperplasia to carcinoma *in situ* and eventually to fully malignant invasive tumors with high metastatic potential. MCF10A cells have been used as a progenitor line from which a series of cell lines have been developed that partially replicate these classifications [5-8]. MCF10A is a spontaneously immortalized non-transformed mammary epithelial cell line derived from a woman with benign fibrocystic disease [5]. When xenotransplanted into nude mice, MCF10AT cells form nonproliferative xenograft lesions that appear benign, but sporadically progress to tumors [6], and MCF10ATG3B frequently progress to atypical hyperplasia and ductal carcinoma *in situ* (DCIS) [7]. Fully malignant MCF10CA1a cells give rise to rapidly growing tumors with 100% efficiency and form lung metastases following intravenous injection [8]. Although there are limitations in any model system, the use of the MCF10A series has the advantage of the ability to perform replicate proteomic analyses on isogenic cells.

In this study, we investigated changes in the lipid raft proteomes isolated from four cell lines in the MCF10A lineage that model breast cancer progression. Our hypothesis was that cells with different degrees of tumorigenicity should present unique lipid raft protein profiles. To this end, detergent-resistant membranes were isolated from each cell line, and proteins were digested and labeled with one of four unique isobaric tags. The iTRAQ-labeled peptides were combined and then proteins were identified and quantitated using tandem mass spectrometry. 49 proteins were identified with enough information for semi-quantitative analysis. Proteomic results were verified on a subset of these proteins using immunoblotting and immunocytochemistry. For some of the proteins identified in lipid rafts, the change in expression level of the protein in the whole cell was similar to the change in abundance observed in the lipid rafts. This was not the case for other proteins indicating that there are biological processes specific to the cell lineage that are modulating the composition of the lipid raft proteome. These data suggest that the dynamic processes regulating protein

association with lipid rafts may have an important role in mammary tumorigenesis, and that subcellular localization of proteins needs to be considered when evaluating cancer biomarkers.

Methods

Cell lines and culture conditions

Cells in the MCF10A cell lineage were obtained from Dr. Fred Miller at the Karmanos Cancer Institute (Detroit, MI, USA). MCF10A cells (10A) are spontaneously immortalized breast epithelial cells obtained from a woman with fibrocystic breast disease [5]. MCF10AT cells (AT) were generated by stably transfecting a mutated T₂₄ Ha-ras gene into MCF10A cells [6]. When implanted subcutaneously into nude mice, about 25% of the animals develop carcinomas. MCF10ATG3B cells (TG3B) were generated by transplantation of AT cells into nude/beige mice and re-establishment in culture three times [7]. These premalignant cells progress to highly proliferative lesions (atypical hyperplasia, DCIS, invasive carcinoma) in greater than 50% of test animals. The MCF10CA1a cells (CA1a), also derived from AT cells, were generated by serially growing a trocar transplantation [8]. This fully malignant cell line gives rise to rapidly growing tumors with 100% efficacy.

Cells were cultured in Dulbecco's Modified Eagle Medium/F-12 medium (DMEM/F-12, Invitrogen) supplemented with 10 µg/mL of human insulin (Invitrogen), 20 ng/mL of epidermal growth factor (Invitrogen), 0.5 µg/mL of hydrocortisone (Sigma), 5% horse serum (Invitrogen), 100 U/mL of penicillin (Invitrogen), and 100 µg/mL of streptomycin (Invitrogen). Cells were maintained in a humidified environment of 5% CO₂/95% air at 37°C.

Lipid Raft Isolation

The method for isolating lipid rafts was modified from a previously published detergent-based protocol [9]. For each cell line, cells were plated in fifteen 100 mm dishes. On day 3 (~80-90% confluency), cells were washed 3X with cold PBS. The remainder of the procedure was performed on ice or at 4°C. The remaining liquid in the plate was removed and replaced with 90 µl of MES-buffered saline (MBS; 25 mM MES, 150 mM NaCl, pH 6.0) with 1% Triton X-100. Extracts were scraped from the plate, pooled, and incubated for 20-30 min. Extracts were homogenized with 20 strokes of a Dounce (glass on glass) tissue grinder. 2 ml of the homogenate was mixed with 2 ml of a 90% sucrose solution and placed on the bottom of a ultracentrifuge tube. This was layered with 4 ml of 35% sucrose and 4 ml of 5% sucrose. The sucrose solutions were diluted with MBS/1% Triton X-100. Tubes were centrifuged at 200,000g for 16 h. The layer containing the visible detergent-insoluble membranes at the 35%-5% sucrose interface was carefully removed, combined with MBS, and recentrifuged at the same speed for 2 h. The pellet was washed twice under the same conditions. Liquid was carefully removed from the tubes, which were then sealed and frozen at -80°C for future use. A set of lipid raft isolations from each of the 4 cell lines was prepared in 3 separate experiments.

Protein reduction and alkylation

Detergent-insoluble membranes were resuspended in 5 µl of 2% sodium dodecyl sulfate (SDS). After a 5 min incubation at room temperature, 45 µl of 100 mM ammonium bicarbonate was added, and the solution transferred to a 1.5 ml tube. The ultracentrifuge tube was washed with an addition 25 µl of ammonium bicarbonate, which was combined with the previous step. Protein was quantitated by the bicinchoninic acid method. The remainder of the extract was reduced with 5 mM dithiothreitol for 30 min at 60°C, followed

by alkylation with 15 mM iodoacetamide for 45 min at room temperature. Aliquots were frozen at -80°C .

Peptide labeling and purification

1.5 μg of protein was diluted to 20 μl with iTRAQ Dissolution Buffer (Applied Biosystems). Proteins were digested with 0.1 μg of sequencing-grade trypsin (Promega) overnight at 37°C . Peptides were labeled with one-third the contents of an iTRAQ 4-plex reagent tube (Applied Biosystems) in a final concentration of 90% acetonitrile (ACN) at room temperature. Lipid rafts from each cell line were labeled with a unique iTRAQ tag. Samples were combined and purified by off-line hydrophilic interaction chromatography (HILIC) as follows. Sample pH was adjusted to 3.0 with the addition of 0.5% (final concentration) trifluoroacetic acid (TFA). A polysulfoethyl aspartamide column (SCX CapTrap, Michrom Bioresources) was equilibrated with 90% ACN. Samples were passed through the column and washed with 90% ACN. Peptides were sequentially eluted with 50 μl volumes of 85%, 70% and 5% ACN. Equilibration, wash and elution solutions contained 0.025% TFA for a final pH of 3.0. Samples were dried and stored at -80°C .

Mass spectrometry

Samples were resuspended in 5% ACN, 0.1% formic acid and 0.005% TFA and loaded onto a 3 μ 200 $\text{\AA}$ Magic C18AQ (0.1 $\times$ 150 mm) column (Michrom). Peptides were separated over a 7-35% ACN gradient with 0.1% formic acid using a Paradigm MS4 HPLC instrument (Michrom), ionized with the ADVANCE source (Michrom), and introduced into a LTQ-XL linear ion trap mass spectrometer (Thermo Scientific). The top 4 MS1 ions above a 5×10^3 threshold were selected for fragmentation. Dynamic exclusion was enabled (repeat count=2; repeat duration=10s; exclusion duration=30s). Peptides were fragmented in pulsed-Q dissociation (PQD) mode (normalized collision energy=32; activation Q=0.75; activation time=0.1ms). The automatic gain control (AGC) target was set at 5×10^4 for MSⁿ, and 2 μscans were averaged with a maximum injection time of 200 ms.

Protein identification

MS2 spectra were searched against a human IPI protein database (ver 3.69) using ProteomeDiscoverer (ver 1.1, Thermo) and Mascot (ver 2.3.01, Matrix Sciences) software. Mascot settings included 1 missed tryptic cleavage; assigned charges of +2 and +3; decoy database search (strict target FDR=0.1, relaxed target FDR=0.2); precursor and fragment mass tolerances of 2.2 and 0.8 Da, respectively; and dynamic modifications of carbamidomethyl C, oxidation of M, iTRAQ on K, and iTRAQ on peptide N-termini. A MudPIT strategy was employed, in that all 9 LC/MS/MS runs (3 elutions from each of 3 biological replicates) were combined into a single search. Proteins were considered to be positively identified if at least 2 unique peptides scored above the set FDR threshold. iTRAQ labeling efficiency of identified peptides was 93.4%.

Relative quantitation and normalization

Griffin has reported that summing, rather than averaging, of reporter ion intensities to calculate an overall ratio for each protein was the best method for iTRAQ quantitation with a LTQ-XL mass spectrometer [10,11]. To verify this finding, we used a set of iTRAQ-labeled standard proteins in known amounts and relative abundance (e.g. ratios of 1:1, 1:10, 5:1) and then compared various methods of ratio calculation. Similar to Griffin's work, we observed a great amount of variability in reporter ion ratios between peptides that mapped to a protein. Furthermore, we found that summation of peptide reporter ion intensities significantly increased accuracy when compared to the known ion ratios (data not shown). Accuracy improved with increased total reporter ion intensity and with the number of

peptides that mapped to a protein. Therefore, to achieve more accurate quantitative information on the less abundant proteins in our preparations, data analysis was prioritized to acquire the greatest amount of total reporter ion intensity per protein. To this end, MS2 data for the three biological replicates in this study were combined into a single search result, and the reporter ion intensities were summed.

Peak intensities were scored within a ± 0.3 Da window of the reported iTRAQ tag sizes. Only peptides with at least 3 iTRAQ labels were utilized, and missing labels were assigned an intensity of zero. Relative quantitation was performed on proteins with at least 2 unique peptides with sufficient iTRAQ peak intensity information. To normalize for protein measurement differences between lipid raft preparations, the relative ratios were adjusted such that the column averages in Table 1 would equal 1 for each cell line.

Western blotting

Four proteins identified in the lipid raft preparations as differentially abundant in the cell lines and considered to be representative of the entire data set were selected for verification by western blot and immunocytochemistry. Protein blotting and immuno-detection were performed using ECL Plus reagents (GE Healthcare) by methods described by the manufacturer. Whole cell protein extracts were prepared as described previously [12]. 20 μ g of whole cell or 1 μ g of lipid raft protein were loaded per lane. Instead of selecting a single protein as a loading control western blots were stained with Ponceau reagent and visually inspected to ensure consistent protein transfer and equivalent loading across the samples. Two independent samples were analyzed for each cell line to further reduce the possibility of a spurious confirmation of the MS results, and optical intensities were averaged for replicates in each experiment. Primary antibodies for caveolin-1 and -2 were purchased from BD Biosciences and used at 1:5000 dilution; Filamin-1, prohibitin-2 and G_{i-2} antibodies were from Santa Cruz Biotechnology (1:2000); and HRP-linked mouse and rabbit secondary IgG were from GE Healthcare (1:30000).

Immunocytochemistry

Procedures for growing cells on coverslips, fixation, staining and fluorescence microscopy have been described previously [13], with the exception that nuclei were stained with SlowFade Gold with DAPI (Invitrogen). The same primary antibodies against filamin A and prohibitin as described above was used for immunocytochemistry, except at lower dilution (1:500). Alexa Fluor 488 goat anti-mouse and goat anti-rabbit IgGs were purchased from Invitrogen and applied with a 1:10000 dilution. In some experiments, cell surface labeling of filamin A and/or lipid rafts (Vybrant Lipid Raft Labeling Assay kit; Invitrogen) were performed in live cells. Briefly, cells cultured on cover slips were washed with cold PBS and incubated at 4°C in complete media for 10 min with primary antibody, washed, and then 15 min with secondary antibody. Cells were washed and fixed with 4% paraformaldehyde for 20 min at 4°C. Images were captured using an Axioplan 2 Imaging Microscope equipped with an ApoTome optical sectioning device, and a Plan-Neofluar 100X/1.30 Oil objective (Carl Zeiss, Cologne, Germany).

Results

The principal aim of this study was to quantitatively compare proteomic profiles for lipid rafts isolated from a series of human mammary epithelial cell lines with increasing tumorigenic potential. Lipid rafts were separated by their characteristic insolubility in a detergent solution, and by their mobility in a sucrose gradient after ultracentrifugation. Proteins remaining with the lipid raft component were reduced, alkylated, and digested with trypsin. Subsequently, peptides from each cell line were labeled with a unique iTRAQ tag,

combined into one sample, purified, and analyzed by LC/MS/MS (Figure 1). 57 proteins were identified in the detergent-resistant membranes with at least 2 unique peptides above the FDR threshold (Tables 1 and S1). 1667 peptide identifications, mapping to 49 proteins, contained sufficient information for semi-quantitative analysis.

To determine whether the lipid rafts from tumorigenic cell lines were different in composition compared to parental cells, identified proteins were grouped according to function and location (Figure 2). These groupings were based on literature searches of all cell types, and are not mutually exclusive. As might be expected, AT groupings were not statistically different from the parental cell line. In contrast, there were significant changes within specific groups from tumor xenograft-derived TG3B and CA1a lines. Signaling proteins were only a third as abundant in the lipid rafts of CA1a cells (Figure 2A). A subset of this group, the heterotrimeric G proteins, had an average 5.7-fold decrease in CA1a cells relative to 10A. Similarly, adhesion proteins were significantly decreased only in CA1a cells, to about half of parental levels. Signaling scaffold proteins, which have an essential role in intracellular signal transduction by providing a platform for signaling molecules to interact, were also decreased 2-fold in CA1a cells, but this finding was not statistically significant ($p=0.074$).

Structural proteins were 2-fold more abundant in the lipid rafts from the TG3B and CA1a cell lines relative to the other cell lines. However, a disparity emerges when this group is separated into intermediate filaments (IFs) and non-IFs. The IF group, including vimentin and keratins, is increased over 3-fold in CA1a cells (Figure 2A), whereas the non-IF group, which includes α -actin, α -tubulin, and filamins A & B, is decreased to about a third of parental levels.

Differences were also found when proteins were grouped according to subcellular location (Figure 2B). CA1a outer plasma membrane proteins, including those with GPI linkages, were decreased by ~62% compared to 10A levels, and inner membrane proteins were decreased by ~54%. Integral membrane and cytosolic proteins were not significantly different in the CA1a lipid rafts. Nuclear proteins were significantly decreased in TG3B and CA1a lipid rafts. Most of these proteins, such as Hsc71 and Ubiquitin C, are found in multiple cellular compartments. Only two proteins, histones H1c and H4, are thought to be exclusively nuclear, and are possibly present in these samples as an artifact of lipid raft preparation.

From this approach it is impossible to ascertain whether differences in lipid raft protein profiles derive from changes in global protein expression or by other mechanisms such as alterations in the biochemistry of the lipid rafts themselves. To gain insight into this question, and to verify the mass spectroscopic analysis for a subset of the proteins, antibody-based expression and localization studies were conducted. Caveolins are a well characterized family of proteins known to reside within a subpopulation of lipid rafts. Only caveolin-1 was detected by LC/MS/MS analyses of the four cell lines, and its expression profile in CA1a lipid rafts was 43% of parental levels (Table 1). Immunoblot analysis of caveolin-1 in whole cell extracts showed a similar decrease for that protein in CA1a cells (Figure 3A). Caveolin-2 was not identified by LC/MS/MS in this study, but its expression pattern appears to be unrelated to caveolin-1 in whole cell extracts as it is virtually unchanged across the 4 cell lines. Caveolin-3 was not detected by immunoblot (data not shown).

Although it has diverse functions, the main role of filamin A is to act as a scaffold for actin cytoskeleton reorganization [15]. In whole cell extracts, filamin A was found to be expressed as a full-length (280 kDa) and as a smaller form, possibly a 180 kDa calpain

cleavage product [16] (Figure 3B). Similar to caveolin-1, filamin A protein content was decreased in CA1a cells relative to parental 10A cells. The decreased cellular expression of these two proteins likely contributes to the lower abundance of caveolin-1 and filamin A we observe in lipid rafts.

To better understand filamin A localization, two immunocytochemical methods were employed. In the first, cells were fixed, permeabilized and stained with primary antibodies against filamin A followed by a fluorescent-tagged secondary antibodies (Figure 4A). The intensity of filamin A staining was severely diminished in CA1a cells compared to the other cell lines. Furthermore, filamin A staining was most prevalent in the periphery of CA1a cells. To determine whether filamin A co-localized with lipid rafts on the outer cell surface, live cells at 4°C were co-stained with antibodies against filamin A and plasma membrane ganglioside G_{M1}, which has been reported to partition in lipid rafts [17]. Cells were then incubated with secondary antibodies, fixed, and imaged by optical sectioning as before. Filamin A staining could not be readily detected by this method (data not shown), suggesting that filamin A is most likely associated with lipid rafts via the inner membrane surface. Representative images of lipid raft staining are shown in Figure 4B. Taken together, these studies indicate that there is less overall filamin A protein expression in CA1a relative to the other cell lines, but the filamin A that is present in these cells appears to be linked to lipid rafts from the inner membrane surface.

The function of prohibitins is poorly understood, but they have a role in cell cycle progression, differentiation and gene transcription [18]. Prohibitins are primarily thought of as mitochondrial proteins but have previously been detected in lipid raft preparations from various cell types [19,20, 21]. Furthermore, it has recently been shown that palmitoylation of prohibitin facilitates its translocation to the plasma membrane where it interacts with the lipid raft protein EHD2 [22]. Our immunocytochemical data confirms that the majority of prohibitin-2 appears to be associated with intracellular structures that are likely to be mitochondria (Figure S1, compare to [14]). However, the identification of prohibitin-2, but no other mitochondrial proteins, in isolated lipid rafts by mass spectrometry is most consistent with prohibitin-2 also being a component of lipid rafts in the MCF10A lineage. In contrast with results obtained with caveolin-1 and filamin A, quantitative LC/MS/MS results did not appear similar to densitometric analyses on duplicate whole cell extracts (Figure 5B), but more closely resembled the immunoblot expression pattern of lipid raft preparations (Figure 5A).

There were five G-proteins identified in lipid rafts that were under-represented in CA1a cells compared to the parental line. Guanine nucleotide-binding protein G(i) alpha-2 subunit (GNAI2) was examined by immunoblot analysis to determine whether this variation resulted from global down-regulation of protein expression or cell type-dependent differences in protein localization. In whole cell extracts, expression of GNAI2 remained relatively constant across the 4 cell lines, and even increased slightly in CA1a cells (Figure 5D). Similar to prohibitin-2, the pattern of GNAI2 abundance in lipid raft protein isolates is discordant with whole cell lysates, and more closely aligned with LC/MS/MS results.

Discussion

Identification of molecular markers of tumor progression is crucial for both disease diagnosis and as a therapeutic target for intervention [23,24]. For this study, protein abundance in detergent-resistant lipid rafts was compared in a series of human breast cell lines with increasing characteristics of malignancy. Lipid rafts are of particular importance as a subcellular structure in cells with aberrant regulation of growth because they support the formation of proteins networks known to be important in regulating growth and cell

signaling. Accordingly, proteins associated with cell signaling comprised about at third of those that were identified in this study. Our hypothesis was that cells with different degrees of tumorigenicity should present unique lipid raft protein profiles, which may assist in the identification of novel cancer biomarkers or critical signaling networks.

Mariotti and co-workers compared lipid raft protein profiles in a melanoma model of tumor progression [25]. Similar to the data presented here, AHNAK was the most prominent lipid raft protein as determined by spectral counting. Their findings that caveolin-1, galectin-1 and 5'-nucleotidase were less abundant in the lipid rafts of metastatic lines are also in agreement with our results. In contrast to the MCF10A breast cancer model, however, levels of seven G proteins in lipid raft preparations from the melanoma model did not change with progression. The exception within this class of proteins was alpha 13 which was found to be enriched in metastatic melanoma cells [25]. We identified five G proteins in the MCF10A cell lines. As a group, the G proteins have a greater than 5-fold decrease in abundance in the metastatic CA1a line compared to the parental cells. The MCF10A series is based on transformation with a constitutively active G protein, Ha-ras [6], but G protein content in the stably transfected AT line did not vary significantly compared to the parental line. The decrease in abundance of G proteins in the lipid rafts appears to be counter-intuitive for the process of cancer progression in which cell signaling networks are constitutively activated and result in enhanced proliferation. However, the decrease in lipid raft G protein abundance that we have documented may represent the end result of hyperactivation of G protein-coupled receptors since agonist stimulation can promote exchange of GTP for GDP and redistribution of the subunits from the membrane to the cytoplasm [26].

GPI-linked proteins are another group of proteins that are under-represented in the lipid rafts of metastatic cells. Taken as a group, the seven GPI-linked proteins were about half as abundant in CA1a versus 10A cells. The glycosylphosphatidylinositol post-translational modification of proteins at their C-termini allows them to be anchored in the outer plasma membrane and to be preferentially located within lipid rafts [27]. These results suggest that a phospholipase which cleaves the GPI anchor may have been activated in the malignant cells. This has been proposed as a method of signal regulation, in which membrane-bound proteins are released to alter local function or to act upon distant cells. For example, a 3-fold increase in intracellular GPI-specific phospholipase D (GPI-PLD) was shown to drastically decrease the level of GPI-linked protein levels [28]. Furthermore, it has been shown that GPI-PLD mRNA expression increased with tumor progression in human skin epithelial cells as well as Ha-rastransfected murine bladder carcinoma cells [29].

Our results show that intermediate filament (IF) proteins are enriched in lipid rafts during tumorigenic progression of the MCF10A lineage. Levels of vimentin and eight keratins were cumulatively over 3-fold higher in CA1a cells compared to the parental line. In addition, two IF-associated proteins, plectin-1 and desmoglein-3, were also more abundant. IF proteins are best known for their cell type specificity and their structural role as components of the cell cytoskeleton. The role of IFs in dynamic cellular functions and mammary tumorigenic progression is more poorly understood. Keratins are typically used in breast cancer studies as immunohistochemical biomarkers for cell identification. The epithelial mesenchymal transition (EMT) has long been associated with breast cancer cell invasiveness. Enhanced protein expression of vimentin and keratins have been associated with EMT, a phenomenon that has been correlated with metastatic potential [30,31], and poor prognosis [32,33]. Studies have shown that increased expression of keratins 5, 6 and 17 are associated with invasive carcinomas [34,35] as well as adverse clinical outcome [36]. These three IFs had the highest expression levels in metastatic CA1a cells of all proteins identified in this study (Table 1). Some progress has been made in understanding the role of vimentin in tumor progression. Cells that stably overexpress vimentin displayed increased

rates of proliferation, invasive potential, clonagenicity and tumorigenicity compared to control cells [37], and down-regulation of vimentin inhibited migration and adhesion of cancer cell lines [38]. In a study examining changes in the global proteome for the MCF10A lineage model, vimentin expression was found to be about 4-fold higher in CA1a cells compared to 10A [40]. It is not known if the changes in vimentin and keratin abundance within CA1a lipid rafts are a general phenomenon and would be detected in tumor-derived lipid rafts.

Immunoblot data on a subset of identified proteins was obtained to confirm the iTRAQ results. Although this data is limited, it clearly validates the large differences in caveolin-1, filamin A, prohibitin-2 and GNAI2 abundance that were detected as a function of cell line for the MCF10A series. These data indicate that some lipid raft protein content differences can be accounted for by global changes in protein expression, whereas for other proteins there is a marked difference between whole cell protein expression and lipid raft protein abundance. For example, in CA1a cells caveolin-1 and filamin A content decreased in whole cell lysates and in the lipid raft compartment. For prohibitin-2 and GNAI2, however, whole cell lysate immunoblot patterns of expression did not align with the abundance of those proteins in the lipid rafts. It is not known whether the difference between global and raft proteome profiles is the result of protein-specific events, such as G protein activation or GPI phospholipase activity, or if changes in the lipid components of lipid rafts during tumorigenic progression leads to changes in the composition of imbedded proteins. To address this question, Mariotti quantitated levels of cholesterol and gangliosides GD2, GD3 and GM1 in lipid rafts, and found higher levels of GD3 in metastatic melanoma cells [25]. More comprehensive lipidomic studies are planned to determine whether lipid raft composition is altered in the MCF10A series.

Caveolin-1 is the main structural component of caveolae, a subset of lipid rafts characterized by flask-shaped invaginations of the cell membrane that have a role in compartmentalizing important signaling molecules. A growing body of literature indicates that loss of caveolin-1 is associated with human breast cancer progression. Several human breast cancer cell lines and cells transformed with Ha-ras, v-Abl or Bcr-Abl have reduced intracellular caveolin-1 levels relative to untransformed cells, and re-expression of caveolin-1 caused significant reduction in cell proliferation and/or anchorage-independent growth and invasion capabilities [40-45]. In clinical specimens, caveolin-1 is rarely expressed in invasive lobular carcinomas [46,47] and the absence or decreased abundance of stromal caveolin-1 is predictive of poor outcome in breast cancer [48-50]. However, the role of caveolin-1 as a tumor suppressor in breast cancer is not clear since it is preferentially expressed in invasive ductal carcinomas of basal-like phenotype and metaplastic carcinomas [51-54]. Our finding that caveolin-1 expression is diminished only in metastatic CA1a cells underscores the usefulness of the MCF10A series as a model to further understand the role of caveolin-1 in breast cancer progression.

Filamin acts as a scaffold for actin organization and plays a role in signal transduction and cell migration [55]. Expression of filamin A has been reported in the cytosol and nucleus, but it also accumulates in lipid rafts where it regulates cytoskeletal rearrangement [56,57]. Moreover, Liscovitch has shown that filamin A becomes hyper-phosphorylated by Akt when caveolin-1 is stably expressed in MCF-7 human breast cancer cells, and that these 3 proteins co-immunoprecipitate [58]. Similar to our results with caveolin-1, filamin A expression is greatly decreased in whole cell lysates and lipid rafts in CA1a cells compared to the other 3 cell lines by iTRAQ quantitation and western blot analyses. Immunocytochemical staining also suggests that filamin A expression is decreased in CA1a cells, and that filamin A is primarily localized in punctate domains in the vicinity of the inner membrane of CA1a cells, whereas 10A, AT and TG3B cells have a broader distribution. These results are in

agreement with a recent report which showed that filamin A levels were significantly decreased in breast tumor samples when comparing invasive breast cancer versus benign disease, and in lymph node-positive versus lymph node-negative breast cancer [59]. Furthermore, down-regulation of filamin A expression led to enhanced cancer cell migration, invasion and metastasis in breast cancer cells cultured *in vitro* or after transplantation into the mammary fat pad of SCID mice [59].

In conclusion, we have identified and quantified proteins in detergent-insoluble membranes from an *in vitro* model of human breast cancer progression. We observed consistent changes in the abundance of groups of proteins with the progression toward malignancy, such as decreased content of G proteins and GPI-linked proteins, and an increase in intermediate filaments. Differential protein abundance in the lipid rafts could be explained in some instances by alterations in the global expression changes, whereas for other proteins there was an inverse correlation between whole cell and lipid raft protein contents. These findings highlight the importance of using caution when interpreting microarray and proteomic experiments on whole cell extracts since they may not accurately reflect the changes in protein abundance in critical subcellular regions. Finally, many of the proteins identified here, including caveolin-1, filamin A, keratins and vimentin, are bona fide or potential biomarkers for clinical studies, and therefore the MCF10A model can be used to better understand the biological mechanisms underlying their alteration during cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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abbreviations

DCIS	ductal carcinoma <i>in situ</i>
EMT	epithelial mesenchymal transition
FDR	false discovery rate
G protein	guanine nucleotide-binding proteins
GPI	glycosylphosphatidylinositol
IF	intermediate filament
MudPIT	multidimensional protein identification technology

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Fig. 1. Schematic outline of experimental method. HILIC, hydrophilic interaction chromatography; ICC, immunocytochemistry; W.blot, Western blot.

Fig. 2.

Relative proteomic expression profiles of proteins by group. Proteins were grouped according to function (A) or subcellular localization (B). Protein group composition information can be found in Table 1. Error bars represent $\pm$ standard error of the mean. Statistics were performed with a one sample t test against a hypothetical mean of 1. *, $p < 0.005$; #, $p < 0.05$.

Fig. 3. Global expression patterns of caveolins 1 and 2 and filamin A in the MCF10A series of human breast cell lines. Whole cell lysates were separated by SDS-PAGE and stained by immunoblotting with primary antibodies against (A) caveolin-1 (CAV1) and caveolin-2 (CAV2), and (B, C) filamin A (FLNA). Panel C is a long term autoradiographic exposure of the same blot as panel B to demonstrate the presence of filamin A in CA1a cell extracts. Each cell line was run as two independent biological replicates. Numbers below and above pictures represent the average relative densitometric signal intensities. This experiment was repeated with similar results. N.D., not detected.

Fig. 4. Subcellular localization of filamin A in the MCF10A series of human breast cell lines. Cells were fixed and stained according to procedures described in Materials and Methods. Two independent photographs are shown per cell line. Contrast/brightness adjustments remained constant throughout each panel. **(A)** Filamin A expression is shown in green, and nuclei are stained blue. Imaging exposure times are shown in the bottom right corner. **(B)** For comparison, a subset of cell surface lipid rafts were localized by staining ganglioside G_{M1} (red). Cells were then fixed and the nuclei stained with DAPI (blue). This experiment was repeated with similar results.

Fig. 5.

Protein expression patterns of prohibitin-2 (PHB2) and guanine nucleotide-binding protein G(i) alpha-2 subunit (GNAI2) in the MCF10A series of human breast cell lines. Lipid raft preparations (**A,C**) and whole cell lysates (**B,D**) were separated by SDS-PAGE and stained by immunoblotting with primary antibodies against PHB2 (**A,B**) and GNAI2 (**C,D**). Each cell line was run as two independent biological replicates. Numbers below and above pictures represent the average relative densitometric signal intensities. N.D., not detected.

Table 1

Relative quantitation of lipid raft proteins in the MCF10A human mammary epithelial series.

#	Accession	Protein Name (alternative or gene name)	AT ^a	TG3B ^a	CA1a ^a	Function; Location ^b
1	IPI00009456.1	5'-Nucleotidase (CD73; NT5E)	1.15	2.95	0.36	Out(G); Oth
2	IPI00478896.3	60S Ribosomal protein L7a (RPL7A)	0.45	0.80	0.50	Cyt; Oth
3	IPI00894365.2	Actin, beta (ACTB)	0.85	0.67	0.60	Cyt; Str
4	IPI00221224.6	Aminopeptidase N (CD13; ANPEP)	1.08	1.57	0.10	Intgl; Sig
5	IPI00455315.4	Annexin A2 (ANXA2)	0.89	0.42	0.20	Out(G); Scaf
6	IPI00009236.5	Caveolin-1 (CAV1)	0.71	1.47	0.43	Int; Trans,Scaf
7	IPI00788676.1	CD109	1.69	2.14	0.28	Out(G); Sig
8	IPI00011302.1	CD59	1.29	2.35	0.55	Out(G); Sig
9	IPI00028931.2	Desmoglein 2 (DSG2)	0.70	0.74	0.20	Intgl; Adh
10	IPI00031547.2	Desmoglein 3 (DSG3)	1.02	0.41	1.25	Intgl; Adh
11	IPI00936931.1	ER lipid raft associated 1 (ERLIN1)	0.57	1.27	0.84	Int; Oth
12	IPI00553169.5	Filamin A (filamin-1; FLNA)	0.82	0.70	0.14	In; Str,Scaf
13	IPI00945610.1	Filamin B (256 kDa protein; FLNB)	1.16	0.66	0.45	In; Str,Scaf
14	IPI00748145.2	G protein, alpha inhibiting activity polypeptide 2 (GNAI2)	1.50	1.40	0.29	In; Sig(G)
15	IPI00220578.3	G protein, alpha inhibiting activity polypeptide 3 (GNAI3)	1.29	1.14	0.08	In; Sig(G)
16	IPI00026268.3	G protein, beta polypeptide 1 (GNB1)	1.14	1.20	0.09	In; Sig(G)
17	IPI00003348.3	G protein, beta polypeptide 2 (GNB2)	0.93	1.00	0.30	In; Sig(G)
18	IPI00221232.9	G protein, gamma 12 (GNG12)	0.83	1.01	0.12	In; Sig(G)
19	IPI00022624.1	G protein-coupled receptor, family C, group 5, member A (GPRC5A)	1.83	1.62	1.02	Intgl; Sig
20	IPI00219219.3	Galectin-1 (LGALS1)	0.66	0.38	0.10	Cyt,Nuc,Out; Adh,Sig
21	IPI00015688.1	Glypican-1 (GPC1)	0.81	0.51	0.20	Out(G); Sig
22	IPI00939595.1	Heat shock cognate 71 kDa protein (HSC71; HSPA8)	0.58	0.13	0.22	Cyt,Nuc,In,Out; Oth
23	IPI00217465.5	Histone H1.2 (HIST1H1C)	0.64	0.81	0.66	Nuc; Oth
24	IPI00453473.6	Histone H4 (HIST1H4A)	0.57	0.60	0.37	Nuc; Oth
25	IPI00554711.3	Junction plakoglobin (gamma-catenin; JUP)	1.07	1.05	0.52	In; Adh,Sig
26	IPI00009865.4	Keratin, type I cytoskeletal 10 (KRT10)	0.76	0.45	1.31	Cyt; Str(IF)
27	IPI00450768.7	Keratin, type I cytoskeletal 17 (KRT17)	0.90	0.70	7.43	Cyt; Str(IF)
28	IPI00554788.5	Keratin, type I cytoskeletal 18 (KRT18)	0.68	0.52	2.51	Cyt; Str(IF)
29	IPI00019359.4	Keratin, type I cytoskeletal 9 (KRT9)	1.00	0.83	2.71	Cyt; Str(IF)
30	IPI00220327.4	Keratin, type II cytoskeletal 1 (KRT1)	1.55	1.00	3.11	Cyt; Str(IF)
31	IPI00021304.1	Keratin, type II cytoskeletal 2 epidermal (KRT2)	1.31	0.75	1.34	Cyt; Str(IF)
32	IPI00009867.3	Keratin, type II cytoskeletal 5 (KRT5)	0.93	0.95	6.23	Cyt; Str(IF)
33	IPI00293665.9	Keratin, type II cytoskeletal 6B (KRT6B)	1.11	0.90	3.81	Cyt; Str(IF)
34	IPI00382733.2	Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1)	0.92	0.76	0.47	Cyt,Nuc; Oth
35	IPI00643788.1	Ly6/PLAUR domain containing protein 3 (LYPD3)	2.12	1.49	0.66	Out(G); Adh
36	IPI00298690.2	Mesothelin (43 kDa protein, MLSN)	0.51	0.49	0.35	Out(G); Adh

#	Accession	Protein Name (alternative or gene name)	AT ^a	TG3B ^a	CA1a ^a	Function; Location ^b
37	IPI00021812.2	Neuroblast differentiation-associated protein AHNAK (AHNAK)	0.99	0.63	0.39	Cyt,Nuc; Scaf
38	IPI00398776.3	Plectin-1 (PLEC)	0.82	0.85	1.55	Cyt,In; Scaf
39	IPI00017334.1	Prohibitin (PHB)	0.58	0.83	0.37	Cyt,Nuc; Sig
40	IPI00027252.6	Prohibitin-2 (PHB2)	0.70	1.11	0.51	Cyt,Nuc; Sig
41	IPI00013981.4	Proto-oncogene tyrosine-protein kinase Yes (YES1)	0.54	0.86	0.23	Cyt,In; Sig
42	IPI00016670.3	RhoA activator C11orf59 (C11orf59)	2.01	2.26	1.08	In; Sig
43	IPI00010214.1	S100 calcium binding protein A14 (S100-A14)	1.83	0.41	0.40	Cyt,Nuc,In; Oth
44	IPI00062120.1	S100 calcium binding protein A16 (S100-A16)	1.68	0.57	0.63	Cyt,Nuc; Oth
45	IPI00909762.1	Tubulin, alpha 1a (TUBA1A)	0.53	0.48	0.17	Cyt; Str
46	IPI00789823.1	Ubiquitin C (16 kDa protein; UBC)	1.09	1.10	0.93	Cyt,Nuc,In,Out; Oth
47	IPI00552689.1	Vimentin (VIM)	0.60	1.47	1.59	Cyt; Str(IF)
48	IPI00743576.1	V-type proton ATPase 116 kDa subunit a isoform 1 (ATP6VOA1)	0.65	0.86	0.98	Intgl; Trans
49	IPI00299719.2	V-type proton ATPase 116 kDa subunit a isoform 3 (TCIRG1)	0.95	1.72	0.37	Intgl; Trans

^aNormalized quantitative value relative to MCF10A cells (1.00).

^bOut, Outer membrane; Out(G), subset of Outer membrane proteins with reported GPI linkages; Intgl, Integral membrane protein; In, Inner membrane protein; Cyt, Cytoplasmic protein; Nuc, Nuclear protein; Sig, Signaling protein; Sig(G), G protein subset of signaling proteins; Scaf, Signaling scaffold protein; Str, structural protein; Str(IF), intermediate filament subset of structural proteins; Adh, Adhesion protein; Trans, Transport protein; Oth, Other.