THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 8, Issue of February 21, pp. 6482–6489, 2003

Comprehensive Proteomic Analysis of Breast Cancer Cell Membranes Reveals Unique Proteins with Potential Roles in Clinical Cancer*[S]

Received for publication, October 4, 2002, and in revised form, November 25, 2002
Published, JBC Papers in Press, December 10, 2002, DOI 10.1074/jbc.M210184200

Paul J. Adam‡§, Robert Boyd‡§, Kerry L. Tyson‡, Graham C. Fletcher‡, Alasdair Stamps‡, Lindsey Hudson‡, Helen R. Poyser‡, Nick Redpath‡, Matthew Griffiths‡, Graham Steers¶, Adrian L. Harris¶, Sonal Patel‡, Joanne Berry‡, Julie A. Loader‡, R. Reid Townsend‡, Laurent Daviet‖, Pierre Legrain‖, Raj Parekh‡, and Jonathan A. Terrett‡**

From ‡Oxford Glycosciences, The Forum, 86 Milton Park, Abingdon, Oxford OX14 4RY, United Kingdom, the ¶Cancer Research United Kingdom Molecular Oncology Laboratories, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom, and ‖Hybrigenics, 3-5 Impasse Reille, Paris 75014, France

Proteins associated with cancer cell plasma membranes are rich in known drug and antibody targets as well as other proteins known to play key roles in the abnormal signal transduction processes required for carcinogenesis. We describe here a proteomics process that comprehensively annotates the protein content of breast tumor cell membranes and defines the clinical relevance of such proteins. Tumor-derived cell lines were used to ensure an enrichment for cancer cell-specific plasma membrane proteins because it is difficult to purify cancer cells and then obtain good membrane preparations from clinical material. Multiple cell lines with different molecular pathologies were used to represent the clinical heterogeneity of breast cancer. Peptide tandem mass spectra were searched against a comprehensive data base containing known and conceptual proteins derived from many public data bases including the draft human genome sequences. This plasma membrane-enriched proteome analysis created a data base of more than 500 breast cancer cell line proteins, 27% of which were of unknown function. The value of our approach is demonstrated by further detailed analyses of three previously uncharacterized proteins whose clinical relevance has been defined by their unique cancer expression profiles and the identification of protein-binding partners that elucidate potential functionality in cancer.

Breast cancer is the most frequently diagnosed cancer in women and accounts for 30% of all cancers diagnosed in the United States (1). The implementation of screening programs for the early detection of breast cancer and the advent of anticancer treatments such as chemotherapy, radiotherapy, and anti-estrogen therapies to augment surgical resection have improved the survival of breast cancer patients. However, some breast tumors become refractory to such treatments as the cancer cells develop resistance to chemotherapy drugs or lose their hormone sensitivity, leading to recurrent or metastatic disease that is often incurable. Cancer membrane-associated proteins form the basis of a number of new drug and antibody cancer therapeutics such as Gleevec (abl-kinase) (2), herceptin (her2neu) (3), Panorex (Ep-CAM) (4), and IRESSA (EGF[1] receptor) (5). Furthermore many other membrane-associated proteins such as small GTPases, kinases, and catenins are implicated in carcinogenesis. Thus a comprehensive definition of cancer cell membrane-associated proteins can reveal further proteins involved in cancer biology which may themselves represent new therapeutic targets.

Tumor-specific proteins have been identified for a number of cancer types using techniques such as differential screening of cDNAs (6) and the purification of cell surface proteins that are recognized by tumor-specific antibodies (7). More recently, DNA "chips" containing up to 12,000 expressed sequence elements have been used to characterize tumor cell gene expression (8). However, there are several reasons why the numerous and extensive previous transcriptomic analyses of breast cancer may not have revealed all tumor-associated proteins. These include (i) a lack of correlation between transcript and disease-associated protein levels; (ii) translocation of a protein in the disease state rather than simply differential levels of the transcript; (iii) novel/uncharacterized genes that are not highly represented within the "closed system" of a cDNA array where there are restrictions on the number of expressed sequence elements per chip and the knowledge and availability of DNA clones.

There have been previous attempts to use plasma membrane-enriched preparations from cancer cell lines to discover new proteins associated with tumor biology (9). In this study we have improved the proportion and total number of membrane proteins obtained from around 30% in previous studies (9) to more than 50%, with 31% being trans-plasma membrane or plasma membrane-associated. Furthermore, 27% of the pro-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Table I.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY069977 (BCMP11).

The nucleotide sequences reported in this paper have been submitted to the Swiss Protein Database under Swiss-Prot accession number(s) Q8TD06 (BCMP11).

§ Both authors contributed equally to this work.

** To whom correspondence should be addressed. Tel.: 1235-207-687; Fax: 1235-207-670; E-mail: jon.terrett@ogs.co.uk.

[1] The abbreviations used are: EGF, epidermal growth factor; AFP, autofluorescent protein; BCMP, breast cancer membrane protein; EST, expressed sequence tag; IM, integral membrane-associated; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry; ORF, open reading frame; PA, plasma membrane-associated; PBS, phosphate-buffered saline; RT, reverse transcription; T, trans-plasma membrane; TOF, time of flight.

teins identified are of unknown function. Indeed, the three uncharacterized proteins described in this paper (named breast cancer membrane protein BCMP11, BCMP84, and BCMP101) all show cancer cell membrane association as well as protein expression levels and localizations that are unique to cancer cells over normal breast epithelial cells.

MATERIALS AND METHODS

Preparation of Membrane Fractions and Protein Separation—The human breast carcinoma cell lines MDA-MB-468 (ATCC:HB-132), T-47D (ATCC:HB-133), BT-474 (ATCC:HTB-20), and MCF-7 (ATCC:HTB-22) were cultured in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Adherent cells (2×10^8) were washed three times with PBS and scraped using a plastic cell lifter. Cells were centrifuged at $1,000 \times g$ for 5 min at 4 °C, and the cell pellet was resuspended in homogenization buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM vanadate, and 0.02% azide protease inhibitors). Cells were fractionated using a ball bearing homogenizer (8.002-mm ball, HGM Laboratory Equipment) until approximately 95% of the cells were broken. Membranes were fractionated using the method described by Pasquali *et al.* (10). The fractionated cells were centrifuged at $3,000 \times g$ for 10 min at 4 °C, and the postnuclear supernatant was layered onto a 60% sucrose cushion and centrifuged at $100,000 \times g$ for 45 min. The membranes were collected using a Pasteur pipette, layered on a preformed 15–60% sucrose gradient, and spun at $100,000 \times g$ for 17 h. Proteins from the fractionated sucrose gradient were run on a 4–20% one-dimensional gel (Novex) and subjected to Western blotting. Those fractions containing alkaline phosphatase and transferrin immunoreactivity but not oxidoreductase II or calnexin immunoreactivity were pooled and represented the plasma membrane fraction.

Proteolysis and MALDI-TOF Mass Spectrometry—Proteins excised from the one-dimensional gels were analyzed by MALDI-TOF mass spectrometry (Voyager STR, Applied Biosystems, Framingham, MA) using a 337-nm wavelength laser for desorption and the reflectron mode of analysis. Selected masses for BCMP11 ((M+H) = 1,226.604, 1,258.674, and 922.455), BCMP84 ((M+H) = 1,648.697), and BCMP101 ((M+H) = 2,099, 1,649 and 1,170) were characterized further by MS/MS using a quadrupole TOF mass spectrometer equipped with a nanospray ion source (Micromass U. K. Ltd., Manchester). Prior to MALDI analysis the samples were desalted and concentrated using C18 Zip Tips™ (Millipore, Bedford, MA). Samples for MS/MS were purified using a nano LC system (LC Packings, Amsterdam, The Netherlands) incorporating C18 SPE material. Using the SEQUEST search program (11), uninterpreted tandem mass spectra of tryptic digest peptides were searched against a FASTA data base of public domain proteins constructed of protein entries in the nonredundant data base held by the National Center for Biotechnology Information (NCBI) and Swiss-Prot which are accessible at www.ncbi.nlm.nih.gov/and www.expasy.com. Peptide matches identified by SEQUEST were filtered according to their cross-correlation score, normalized difference correlation score, compatibility with trypsin digestion, and number of observations of proteins and peptides. Peptides were only used for protein identification where the cross-correlation score was ≥ 1.2 and the normalized difference correlation score ≥ 0.2. For conservative identification of abundant proteins two peptides with these scores were required, but for some of the novel lower abundance proteins and low molecular weight proteins identified we accepted a single tandem peptide with these scores as long as there were mass matches on the same protein sequence from the same gel slice.

Cloning, AFP Tagging, and Transient Transfection Analysis—Total RNA was prepared from cultured MDA-MB-468 and T-47D cells using Trizol reagent (Invitrogen) and resuspended in RNase-free water at a concentration of 1 $\mu g/\mu l$. 5 μg of total RNA was used for oligo(dT) primed first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). The predicted full-length BCMP11, BCMP84, and BCMP101 open reading frames (ORFs) were amplified by PCR from T-47D and MDA-MB-468 cDNAs using the following primers: BCMP11F, 5′-ggccaagtcagcttcttctg-3′; BCMP11R, 5′-gtatttgtcaatgtgccagagg-3′; BCMP84F, 5′-ataggacaacagaactctcacc-3′; BCMP84R, 5′-gcttcaacggaactttgcagag-3′; BCMP101F, 5′-tgtgcaaatgaccctggagttg-3′; BCMP101R, 5′-ggctgctactgcaaacagttcc-3′. Reactions contained 10 ng of cDNA and reagents for PCR (Qiagen) and used 40 cycles at 94 °C for 30 s and 60 °C for 30 s. PCR products were column purified (Qiagen), cloned into T:A vector (Invitrogen) and the sequence verified. For fluorescent tagging, the BCMP11, BCMP84, and BCMP101 full-length ORFs were PCR cloned into the pQBI25/50-fN1 vector (Qbiogene) resulting in an in-frame addition of the SuperGlo™ autofluorescent protein (AFP) to the C terminus of each expressed protein. Transient transfection of SuperGlo™ AFP-tagged BCMP11, BCMP84, and BCMP101 cDNAs into MDA-MB-468, T-47D, and normal human mammary epithelial (Clonetics, BioWhittaker, Inc. International) cell lines was achieved using Superfect™ transfection reagent (Qiagen).

Immunohistochemistry and Immunocytochemistry—Immunohistochemical analysis was carried out on formalin-fixed paraffin-embedded tissue microarrays containing 1-mm sections of breast carcinoma tissue from 50 donors as well as 200 sections of various normal tissues (Clinomics Laboratories Inc., Pittsfield, MA). A variety of other ethically approved carcinoma tissues sections were provided by the University of Oxford, U. K. Formalin-fixed sections were deparaffinized by two 5-min washes in xylene, then rehydrated through successive graded ethanol solutions and washed for 5 min in PBS. Antigen retrieval was achieved in 0.01 M citrate buffer (pH 6) and microwaving for 10 min at full power (950 W). In addition, detection with the BCMP84 antibody required that the tissue be treated with 1 mg/ml pepsin for 1.5 min at room temperature. Thawed frozen sections were fixed in acetone for 10 min at room temperature then washed twice in PBS. Endogenous hydrogen peroxidase activity was quenched by treating the slides in 3% hydrogen peroxidase and PBS for 10 min. The tissue was blocked in 10% donkey serum and PBS for 1 h before the addition of 2 μg/ml primary polyclonal antibody (in 2.5% donkey serum). The BCMP11, BCMP84, and BCMP101 polyclonal antibodies were raised in rabbits immunized with two specific peptides (ABCAM Ltd., Cambridge, U. K.). The BCMP11 peptides used were CAQNEEIQEMAQNKFIMLNLMHET and CTYEPRDLPLLIENMKKALRLIQSEL, the BCMP84 peptides used were CEGGKETLTPSELRDLV and CEAAKSVKLERPVRGH, and the BCMP101 peptides used were SYKEVPTADPTGVDR and LTDASQGRRGRVVND. Western blot analysis of T-47D, MDA-MB-468, and other negative cell line lysates was used to confirm that each antibody cross-reacted with a single band of the predicted size in the correct samples. After three washes in PBS the tissue sections were incubated with biotin-conjugated secondary antibodies (Biotin-SP-conjugated AffiniPure donkey anti-rabbit, Jackson ImmunoResearch) diluted at 1:200 (2.5 μg/ml in 2.5% donkey serum and PBS) for 1 h. Slides were washed three times in PBS and the tissue incubated with streptavidin-horseradish peroxidase (Jackson ImmunoResearch) diluted 1:100 (5 μg/ml in 2.5% donkey serum and PBS). Immunostaining was detected using DAB substrate solution (Dako Ltd.) according to the manufacturer's instructions. Immunocytochemical analysis was carried out on MDA-MB-468 cells fixed in 4% paraformaldehyde and PBS and permeabilized on 0.3% Triton X-100. After a 1-h incubation in blocking buffer (10% donkey serum and PBS) BCMP84 antibodies (1:50 dilution in blocking buffer) were incubated on the cells for a further 1 h. Cells were washed three times in PBS and incubated for 1 h in biotinylated anti-rabbit secondary antibody (Jackson Laboratories) followed by the addition of streptavidin-Alexa 488 (Molecular Probes).

In Situ RT-PCR—Direct *in situ* RT-PCR detection of BCMP101 mRNA expression was examined in formalin-fixed, paraffin-embedded breast cancer tissues (provided by Human Research Tissue Bank, Department of Cellular Pathology, Peterborough District Hospital, Peterborough PE3 6DA, U. K.). Briefly, dewaxed and rehydrated tissue was permeabilized in 0.01% Triton X-100 for 3 min followed by treatment with proteinase K for 30 min at 37 °C. Direct *in situ* RT-PCR was carried out in a GeneAmp in Situ PCR System 1000 (PerkinElmer Life Sciences) using a GeneAmp Thermostable rTth RT-PCR kit (PerkinElmer Life Sciences). The primers used to amplify BCMP101 were: sense, 5′-ttcacctctccgcgggtagcct-3′, antisense, 5′-ggaagttacccacatatacggc -3′. The thermal cycling parameters were 1 cycle of 94 °C for 2.5 min followed by 20 cycles of 94 °C for 40 s, 60 °C for 50 s, and 72 °C for 30 s. Amplified product was detectable through the direct incorporation of alkali-stable digoxigenin-11-dUTP (Roche Molecular Biosciences), which was added to the reaction mix. After washing in PBS an anti-digoxigenin-gold antibody (Roche) was incubated on the tissue section for 30 min at room temperature. This was followed by a silver enhancement step (Roche silver enhancement reagents) during which time the amplified expression product became visible by light microscopy. The tissue was counterstained with hematoxylin (Dako Ltd.).

Yeast Two-hybrid Cloning Analysis—Baits were PCR amplified (*Pfu*, Stratagene) and then cloned in the pB6 plasmid derived from the original pAS2ΔΔ (12). Random and oligo(dT)-primed cDNA libraries from human placenta poly(A)$^+$ RNA and pooled breast cancer-derived cell line (T47-D, MDA-MB-468, MCF-7, BT-20) poly(A)$^+$ RNA were constructed into the pP6 plasmid derived from the original pACT2

plasmid (13) and transformed in *Escherichia coli* (DH10B; Invitrogen). The complexity of the primary libraries was more than 50 million clones. The libraries were then transformed into yeast, and 10 million independent yeast colonies were collected, pooled, and stored at −80 °C as equivalent aliquot fractions of the same library.

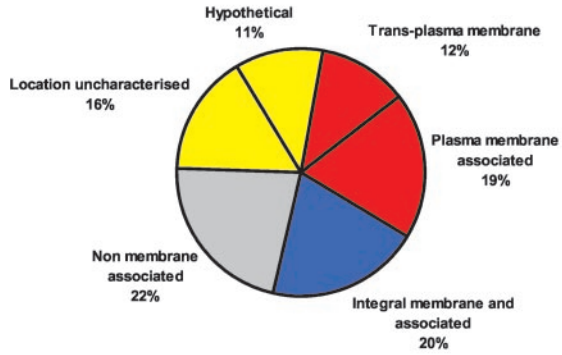
The mating protocol has been described elsewhere (12). Briefly, the screening conditions were adapted for each bait (test screen) before performing the full-size screening. The selectivity of the *HIS3* reporter gene was modulated with 3-aminotriazole to obtain a maximum of 384 histidine-positive clones. For all selected clones, LacZ activity was measured in a semiquantitative 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside overlay assay. The interacting "prey" fragments of the positive clones were amplified by PCR, analyzed on an agarose gel, and sequenced at their 5′- and 3′-junctions on a PE3700 sequencer. The resulting sequences were then used to identify the corresponding gene in the GenBank data base (NCBI).

Selection of Protein Candidates for Further Analysis—To discover proteins with novel relevance to breast cancer, bioinformatic filters were applied to exclude those expressed ubiquitously or at high levels in vital organs (liver, lung, heart, central nervous system, digestive system, kidney). Proteomic data bases were queried for frequency, and tissue and subcellular distribution, of proteins identified in the present study. Accumulated cDNA data were also queried for absolute abundance in various tissue types. Proteins were identified for further analysis on the basis of the highest expression in breast cancer, coupled with low or absent expression in vital organs. A further exclusion step was applied to remove proteins already known to be involved in breast cancer or those obviously not associated with plasma membranes. The resulting class of breast cancer-specific or transcriptionally low level proteins were examined for further evidence relevant to tumorigenic processes, such as homology to protein families known to be associated with breast cancer or involved in cancer biology. Other proteins in this class which were previously supported only by cDNA evidence were also included as potential novel breast cancer-specific membrane proteins. Of the 22 proteins selected using the above methods, the three described here were particularly interesting with respect to mRNA distribution, breast cancer-specific changes, and relevant interacting proteins.

RESULTS

MS/MS Analysis of Membrane-associated Proteins in Breast Cancer-derived Cell Lines—Purified cell membrane protein preparations were isolated from the MDA-MB-468, T-47D, BT-474, and MCF-7 breast cancer cell lines. The T-47D/MCF-7 estrogen receptor-positive and MDA-MB-468/BT-474 EGF receptor-positive cell lines were pooled, and each pool was independently separated by one-dimensional-PAGE. Sequential 0.5-mm gel slices containing the proteins were subjected to trypsinolysis, and the resulting peptide fragments analyzed by MALDI-TOF and MS/MS. Proteins were identified by mass- and fragment-based data base searching (11, 14). Selection of peptides for MS/MS analysis was deliberately biased away from those masses contained in highly abundant proteins so as to increase the discovery potential and coverage of lower abundance proteins. In total, 501 distinct proteins were identified from both cell membrane pools (see Table I in the supplemental material). This table provides an accession to each protein sequence obtained by MS/MS and indicates in which cell line pool each protein was identified. The tandem mass peptides used to identify each protein have also been included in supplemental Table I because accession numbers can frequently change, particularly for the more recently identified hypothetical/functionally uncharacterized proteins. The proteins are categorized into five locational groups based on Swiss-Prot annotations, literature citations, and homology searches. However, it is noteworthy that many of the proteins identified can exist in multiple cell locations, but where there is good literature evidence of membrane association we have reflected this in the categorizations in supplemental Table I, which determines the contribution of each locational group to Fig. 1*A*. The proportion of proteins in each category is illustrated in Fig. 1*A*. Categories T, PA, and IM, which represented 51% of the total,

A



B

ACCESSION	DESCRIPTION	CLASSIFICATION	CELL-LINE POOL
O00688	Epidermal Growth Factor receptor	Trans-plasma membrane	MDA 468/BT474
P09758	GA733-2 (EpCAM)	Trans-plasma membrane	BOTH
Q14876	MUC-1	Trans-plasma membrane	T47D/MCF-7
P04626	erbB2/HER2-neu	Trans-plasma membrane	MDA 468/BT474
P02786	Transferrin receptor (CD71)	Trans-plasma membrane	BOTH
Q93020	G-Protein, G(I) alpha-2	PM associated	BOTH
P24407	RAB-8 (oncogene C-MEL)	PM associated	T47D/MCF-7
O00161	SNAP-23	PM associated	T47D/MCF-7
Q13277	Syntaxin 3	PM associated	T47D/MCF-7
P07947	Tyrosine kinase YES (P61-YES)	PM associated	MDA 468/BT474
Q14108	Lysosomal membrane protein II	Integral membrane	T47D/MCF-7
P51149	RAB-7	Integral membrane	BOTH
Q13190	Syntaxin 5	Integral membrane	T47D/MCF-7
O15258	RER-1	Integral membrane	T47D/MCF-7
P49257	ERGIC-53	Integral membrane	BOTH

FIG. 1. **Classification of proteins identified from breast cancer cell membrane preparations.** *A*, proteins were classified into the five groups illustrated using the data in supplemental Table I. The *numbers* indicate the percentage fraction of identified proteins represented by each group. *B*, examples of proteins identified in each of the membrane categories shown in *A*. The breast cancer cell line pools in which each protein was identified are indicated (MDA 468/BT474, T47D/MCF-7, or both).

contained known trans-plasma membrane (T), plasma membrane-associated (PA), and integral membrane-associated (IM) proteins, respectively (Fig. 1*A*). Examples of proteins in each of these categories are shown in Fig. 1*B* and include known breast cancer membrane antigens such as erbB2/her2neu tyrosine kinase receptor (accession P04626) and EGF receptor (O00688). Intracellular signaling proteins, such as Ras-related proteins and nonreceptor tyrosine kinases, which bind to plasma membrane-associated proteins, were observed in category PA (Fig. 1*B* and supplemental Table I), demonstrating that multiple components of cancer signal transduction pathways can be extracted and identified by these proteomic methods.

Of the proteins identified, 27% were either hypothetical (proteins predicted from the transcriptome) or of unknown function and cell localization (Fig. 1*A*). Based on the number of known membrane-associated proteins found, it is likely that ~50% of these hypothetical/uncharacterized proteins represent trans-plasma membrane or generally membrane-associated proteins. The two breast cancer cell line pools, one representing estrogen receptor-positive cell lines and the other EGF receptor-positive cell lines, were deliberately chosen to reflect some of the different molecular pathologies known in breast cancer. Although failure to identify a protein is not evidence of its absence in a membrane preparation, many proteins were apparently restricted to, or denoted in, either the EGF receptor pool or the estrogen receptor-positive pool (supplemental Table I). From

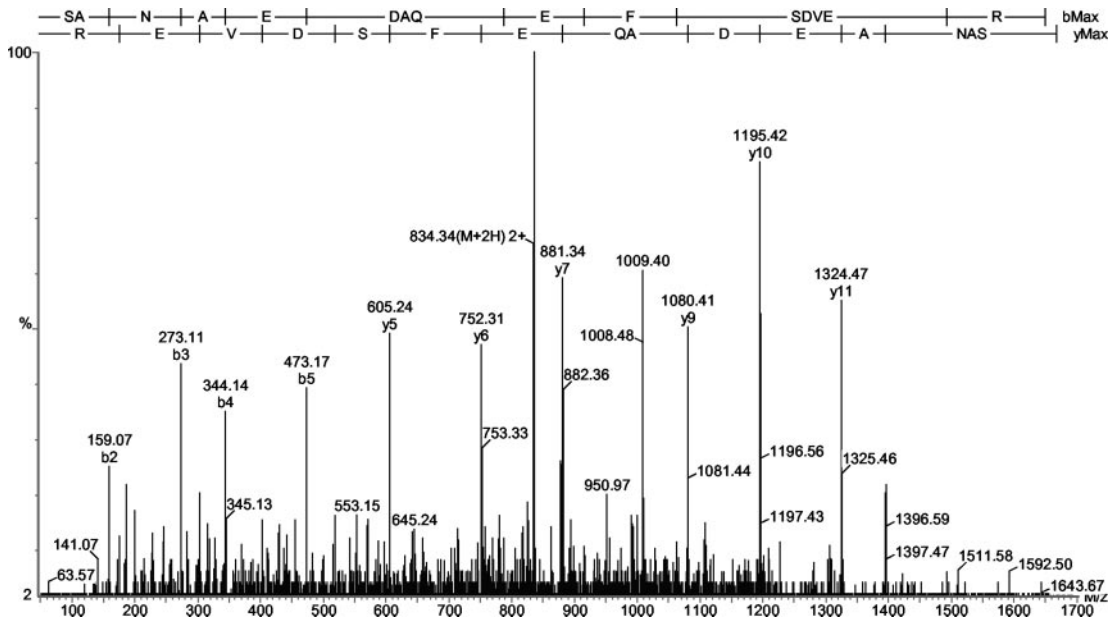


FIG. 2. **MS/MS showing the *m/z* 1,648.697 ion used to identify a tandem peptide (SANAEDAQEFSDVER) representing BCMP84.** The y and b ion trails are shown.

the pool of hypothetical/uncharacterized proteins we describe more detailed analyses on three, BCMP11, BCMP84, and BCMP101. A representative MS/MS result used to identify protein BCMP84 is shown in Fig. 2. All three unique proteins were represented by a low number of expressed sequence tags (ESTs) that did not represent a complete expression profile. In addition, BCMP11 was chosen because of its similarity to the *Xenopus laevis* XAG embryonic differentiation proteins. BCMP84 was an uncharacterized member of the S100 protein family, several of which are associated with tumor biology. BCMP101 was chosen because, with no known functionally characterized homologs, it represented a completely unique breast cancer membrane-associated protein.

Identification and Cloning of BCMP11, BCMP84, and BCMP101—Four spectra from protein BCMP11, isolated from T-47D/MCF-7 cell membranes, were found to match a translation of an EST from a human lung carcinoma library (accession AI458391) (supplemental Table I) defining an ORF of 166 amino acids (Fig. 3*A*). A BLAST search of a human EST data base (www.ncbi.nlm.nih.gov/blast) identified several overlapping ESTs with matches to the sequence of AI458391, providing additional 5′- and 3′-untranslated region sequences. Primers designated to the 5′- and 3′-untranslated region sequences were used to amplify a full-length clone from T-47D cDNA by PCR (Fig. 3*A*). The predicted BCMP11 protein is highly homologous to hAG-2, a novel human protein encoded by a cDNA cloned from the MCF-7 breast cancer cell line (15) and localized to chromosomal band 7p21.3 (16). hAG-2 is the human homolog of XAG-2, a secreted *X. laevis* protein that is thought to be involved in the regulation of dorsoanterior ectodermal cell fate during cement gland differentiation (17). BCMP11, like hAG-2, is predicted to be an extracellular protein with an N-terminal signal sequence (psort.nibb.ac.jp). In view of the high degree of sequence identity between the two proteins, we have named BCMP11 "hAG-3" (GenBank AY069977; Swiss-Prot Q8TD06).

Protein BCMP84 was isolated from the MDA-MB-468/BT-474 cell membrane pool. Amino acid sequences from a single MS/MS were found to match a translation of an EST from a human colon carcinoma cell line (accession number AA315020) (Fig. 3*B*). Overlapping ESTs were identified which established a complete ORF of 104 amino acids. A full-length clone was amplified by PCR from MDA-MB-468 cDNA (Fig. 3*B*). The predicted BCMP84 protein shows similarity to the S100 family of calcium-binding proteins and the predicted protein product of a recently identified cDNA (AY007220), which is identical to BCMP84. The protein encoded by AY007220 has been named S100A14 and annotated as a novel member of the S100 family of calcium binding proteins (Swiss-Prot Q9HCY8). The BCMP84 gene lies at chromosomal position 1q21 within the S100 calcium binding protein gene cluster (18).

Three spectra from protein BCMP101, isolated from MDA-MB-468/BT-474 cell membranes, were found to match a translation of an EST from a human pooled library of testis, fetal lung, and B-cell cDNA (GenBank accession AI827549; dbEST identification 2915502). Additional overlapping ESTs were identified, and alignment with genomic clone AC021396.2 established a complete ORF of 310 amino acids. Primers designed to the 5′- and 3′-untranslated region sequences were used to amplify a full-length clone from MDA-MB-468 cDNA by PCR (Fig. 3*C*). The predicted protein product of a recently identified, uncharacterized cDNA clone, NSE-2 (GenBank AJ417849; Swiss-Prot Q96KN1) is identical to BCMP101. The BCMP101 gene lies on chromosome 8 at position q24.21, and the ORF is encoded by a single exon. The predicted BCMP101 protein has a homolog on chromosome 2 (GenBank CAD10038); however, the functions of both proteins are unknown, and analysis of the BCMP101 protein sequence identified no motifs that might suggest a particular function or cellular location.

Cellular Localization and Proteins Interacting with BCMP11, BCMP84, and BCMP101—C-terminal tagging with green SuperGlo™-AFP and immunocytochemistry were used to determine the cellular localization of BCMP11, BCMP84, and BCMP101 proteins in MDA-MB-468 and T-47D cell lines. In addition, yeast two-hybrid cloning analysis was used to identify tumor cell line-derived proteins interacting with full-length BCMP84 and BCMP101 proteins and mature BCMP11 protein (signal sequence deleted).

Transient transfection analysis of AFP-tagged BCMP11 cDNA construct demonstrated that the translated AFP fusion protein was localized in secretory or endosome-like organelles in both T-47D and MDA-MB-468 cells (Fig. 4*A*). Indeed, BCMP11 has a putative signal sequence and is predicted to be a secreted extracellular protein. In addition, yeast two-hybrid cloning identified the human homolog of a rat glycosylphos-

A

```
GGCCAAGTCAGCTTCTTCTGAGAGAGTCTCTAGAAGACATGATGCTACACTCAGCTTTGG
                                          M  M  L  H  S  A  L  G
GTCTCTGCCTCTTACTCGTCACAGTTTCTTCCAACCTTGCCATTGCAATAAAAAAGGAAA
  L  C  L  L  L  V  T  V  S  S  N  L  A  I  A  I  K  K  E  K
AGAGGCCTCCTCAGACACTCTCAAGAGGATGGGGAGATGACATCACTTGGGTACAAACTT
  R  P  P  Q  T  L  S  R  G  W  G  D  D  I  T  W  V  Q  T  Y
ATGAAGAAGGTCTCTTTTATGCTCAAAAAAGTAAGAAGCCATTAATGGTTATTCATCACC
  E  E  G  L  F  Y  A  Q  K  S  K  K  P  L  M  V  I  H  H  L
TGGAGGATTGTCAATACTCTCAAGCACTAAAGAAAGTATTTGCCCAAAATGAAGAAATAC
  E  D  C  Q  Y  S  Q  A  L  K  K  V  F  A  Q  N  E  E  I  Q
AAGAAATGGCTCAGAATAAGTTCATCATGCTAAACCTTATGCATGAAACCACTGATAAGA
  E  M  A  Q  N  K  F  I  M  L  N  L  M  H  E  T  T  D  K  N
ATTTATCACCTGATGGGCAATATGTGCCTAGAATCATGTTTGTAGACCCTTCTTTAACAG
  L  S  P  D  G  Q  Y  V  P  R  I  M  F  V  D  P  S  L  T  V
TTAGAGCTGACATAGCTGGAAGATACTCTAACAGATTGTACACATATGAGCCTCGGGATT
  R  A  D  I  A  G  R  Y  S  N  R  L  Y  T  Y  E  P  R  D  L
TACCCCTATTGATAGAAAACATGAAGAAAGCATTAAGACTTATTCAGTCAGAGCTATAAG
  P  L  L  I  E  N  M  K  K  A  L  R  L  I  Q  S  E  L  *
AGATGATAGAAAAAAGCCTTCACTTCAAAGAAGTCAAATTTCATGAAGAAAACCTCTGGC
ACATTGACAAATAC
```

B

```
ATAGGACAACAGAACTCTCACCAAAGGACCAGACACAGTGAGCACCATGGGACAGTGTCG
                                                M  G  Q  C  R
GTCAGCCAACGCAGAGGATGCTCAGGAATTCAGTGATGTGGAGAGGGCCATTGAGACCCT
  S  A  N  A  E  D  A  Q  E  F  S  D  V  E  R  A  I  E  T  L
CATCAAGAACTTTCACCAGTACTCCGTGGAGGGTGGGAAGGAGACGCTGACCCCTTCTGA
  I  K  N  F  H  Q  Y  S  V  E  G  G  K  E  T  L  T  P  S  E
GCTACGGGACCTGGTCACCCAGCAGCTGCCCCATCTCATGCCGAGCAACTGTGGCCTGGA
  L  R  D  L  V  T  Q  Q  L  P  H  L  M  P  S  N  C  G  L  E
AGAGAAAATTGCCAACCTGGGCAGCTGCAATAACTCTAAACTGGAGTTCGGGAGTTTCTG
  E  K  I  A  N  L  G  S  C  N  N  S  K  L  E  F  G  S  F  W
GGAGCTGATTGGAGAAGCGGCCAAGAGTGTGAAGCTGGAGAGGCCTGTCCGGGGGCACTG
  E  L  I  G  E  A  A  K  S  V  K  L  E  R  P  V  R  G  H  *
AGAACTCCCTCTGGAATTCTTGGGGGGTGTTGGGGAGAGACTGTGGGCCTGGAGATAAAA
CTTGTCTCCTCTACCACCACCCTGTACCCTAGCCTGCACCTGTCCTCATCTCTGCAAAGT
TCCGTTGAAGC
```

C

```
TGTGCAAATGACCCTGGAGTTGGTTTCGCTTTCTCCCCTTGCGGCGGTGTGAACGTGTG
CCGCAGCGTGATGGGCAACCAGGTGGAGAAATTGACCCACCTAAGTTACAAGGAAGTTCC
            M  G  N  Q  V  E  K  L  T  H  L  S  Y  K  E  V  P
CACGGCCGACCCGACTGGCGTGGACCGGGACGACGGGCCCCGCATTGGGGTCTCCTACAT
  T  A  D  P  T  G  V  D  R  D  D  G  P  R  I  G  V  S  Y  I
TTTCTCCAATGACGATGAGGACGTGGAGCCGCAGCCGCCGCCTCAGGGGCCAGATGGCGG
  F  S  N  D  D  E  D  V  E  P  Q  P  P  P  Q  G  P  D  G  G
CGGCTTGCCCGACGGTGGGGACGGGCCGCCGCCCCAGCCGCAGCCCTACGATCCGCG
  G  L  P  D  G  G  D  G  P  P  P  P  Q  P  Q  P  Y  D  P  R
GCTGCACGAGGTGGAATGCTCCGTGTTCTACCGGGACGAATGCATCTACCAGAAGAGCTT
  L  H  E  V  E  C  S  V  F  Y  R  D  E  C  I  Y  Q  K  S  F
CGCGCCGGGCTCGGCGGCGCTGAGTACCTACACGCCCGAGAACCTGCTCAACAAGTGCAA
  A  P  G  S  A  A  L  S  T  Y  T  P  E  N  L  L  N  K  C  K
GCCGGGCGATCTGGTGGAGTTCGTGTCGCAGGCTCAGTACCCGCACTGGGCCGTATATGT
  P  G  D  L  V  E  F  V  S  Q  A  Q  Y  P  H  W  A  V  Y  V
GGGTAACTTCCAGGTGGTGCACCTGCACCGGCTGAGGTGATTAACAGCTTCCTGACTGA
  G  N  F  Q  V  V  H  L  H  R  L  E  V  I  N  S  F  L  T  D
CGCCAGCCAGGGCCGTCGCGGCCGCGTGGTCAACGATCTGTACCGCTACAAGCCGCTAAG
  A  S  Q  G  R  R  G  R  V  V  N  D  L  Y  R  Y  K  P  L  S
CTCCAGCGCCGTGGTGCGCAACGCGCTGGCGCACGTGGGTGCCAAGGAGCGCGAGCTGAG
  S  S  A  V  V  R  N  A  L  A  H  V  G  A  K  E  R  E  L  S
CTGGCGCAACTCGGAGAGTTTCGCCGCCTGGTGCCGCTACGGCAAGCGCGAGTTCAAGAT
  W  R  N  S  E  S  F  A  A  W  C  R  Y  G  K  R  E  F  K  I
CGGCGGCGAGCTGCGCATCGGCAAGCAGCCCTACCGGCTGCAGATTCAGCTGTCGGCGCA
  G  G  E  L  R  I  G  K  Q  P  Y  R  L  Q  I  Q  L  S  A  Q
GCGCAGCCACACGCTCGAGTTCCAGAGTCTAGAGGACCTGATCATGGAGAAGCGACGCAA
  R  S  H  T  L  E  F  Q  S  L  E  D  L  I  M  E  K  R  R  N
CGACCAGATCGGGCGCGCGGCCGTGCTGCAGGAGCTCGCCACGCACCTGCACCCGGCGGA
  D  Q  I  G  R  A  A  V  L  Q  E  L  A  T  H  L  H  P  A  E
GCCGGAGGAGGGCGACAGCAACGTGGCGCGGACTACGCCGCCTCCCGGGCGCCCCCCTGC
  P  E  E  G  D  S  N  V  A  R  T  T  P  P  P  G  R  P  P  A
GCCCAGCTCCGAGGAGGAGGACGGAGAGGCAGTGGCACACTGATGGGCGAGCTGAGCGCA
  P  S  S  E  E  E  D  G  E  A  V  A  H  *
GAGCTGCGAAGGGGAACTGTTTGCAGTAGCAGCC
```

FIG. 3. **DNA and protein sequence of BCMP11 (*A*), BCMP84 (*B*), and BCMP101 (*C*).** Peptides assigned to BCMP11, BCMP84, and BCMP101 are in *bold* and *underlined*; tandem spectra are in *bold* and *italicized*.

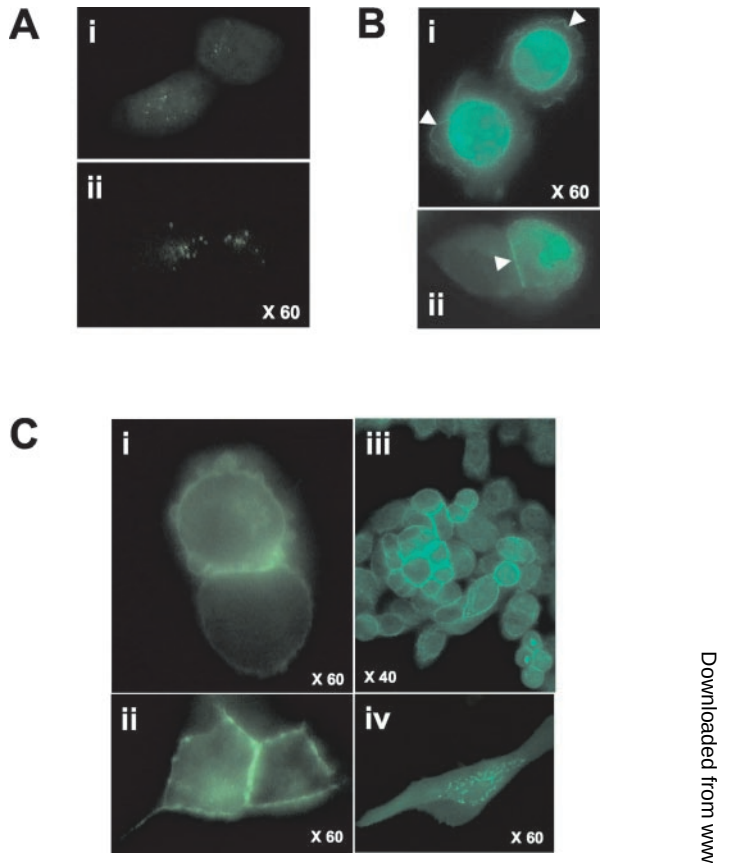


FIG. 4. **Cellular localization of BCMP11, BCMP84, and BCMP101 breast cancer cell lines.** *A*, fluorescence microscopy showing apparent endosomal expression of transiently transfected AFP-tagged BCMP11 in MDA-MB-468 (*i*) and T-47D (*ii*) cell lines. *B*, fluorescence microscopy showing expression of AFP-tagged BCMP101 in MDA-MB-468 (*i*) and T-47D (*ii*) cell lines. Membrane localization is indicated by *white arrowheads* (magnification, ×60 using oil immersion objective). *C*, fluorescence microscopy showing plasma membrane-associated expression of BCMP84 with both transiently transfected AFP-tagged BCMP84 and immunofluorescence using a BCMP84-specific antibody. AFP-tagged BCMP84 expression in MDA-MB-468 (*i*) and T-47D (*ii*) cell lines is shown (magnification, ×60 using oil immersion objective). *iii*, immunofluorescence microscopy of BCMP84 in the MDA-MB-468 cell line (magnification, ×40). *iv*, AFP-tagged BCMP84 expression in human mammary epithelial cells (magnification, ×60) showing solely cytosolic localization.

phatidylinositol-anchored metastasis-associated protein, C4.4A, (GenBank NM_014400) (19) and the extracellular domain of dystroglycan as BCMP11-interacting proteins (20). An interaction between BCMP11 and C4.4A is particularly interesting given that both proteins are expressed in carcinoma tissues prone to metastasis.

Analysis of the cellular location of an AFP-tagged BCMP101 demonstrated widespread intracellular localization but also significant expression associated with the plasma membrane in both MDA-MB-468 and T-47D cell lines (Fig. 4*B*). Indeed, we observed particularly high levels of BCMP101 plasma membrane localization in areas of cell-cell contact (Fig. 4*B*, *ii*). Consistent with this, BCMP101 was found to interact specifically with α1-catenin (Swiss-Prot accession P35221) by yeast two-hybrid analysis. α1-Catenin protein was also identified in our breast cancer cell membrane preparations (see supplemental Table I; plasma membrane-associated accession P35221).

Transient transfection analysis of an AFP-tagged BCMP84 cDNA construct demonstrated that the translated AFP fusion protein was localized in the plasma membrane in both MDA-MB-468 and T-47D cancer cells (Fig. 4*C*, *i–ii*). Because both our breast cancer cell line membrane proteome data (supplemental Table I) and Western blot analysis with an anti-BCMP84 antibody demonstrated the absence of BCMP84 in the T-47D cell line, these data indicate that T-47D cancer cells artificially expressing BCMP84 also target the protein to the plasma membrane. In addition, immunocytochemical analysis with an anti-BCMP84 antibody also showed clear plasma membrane localization of BCMP84 protein in MDA-MB-468 cells (Fig. 4*C*, *iii*).

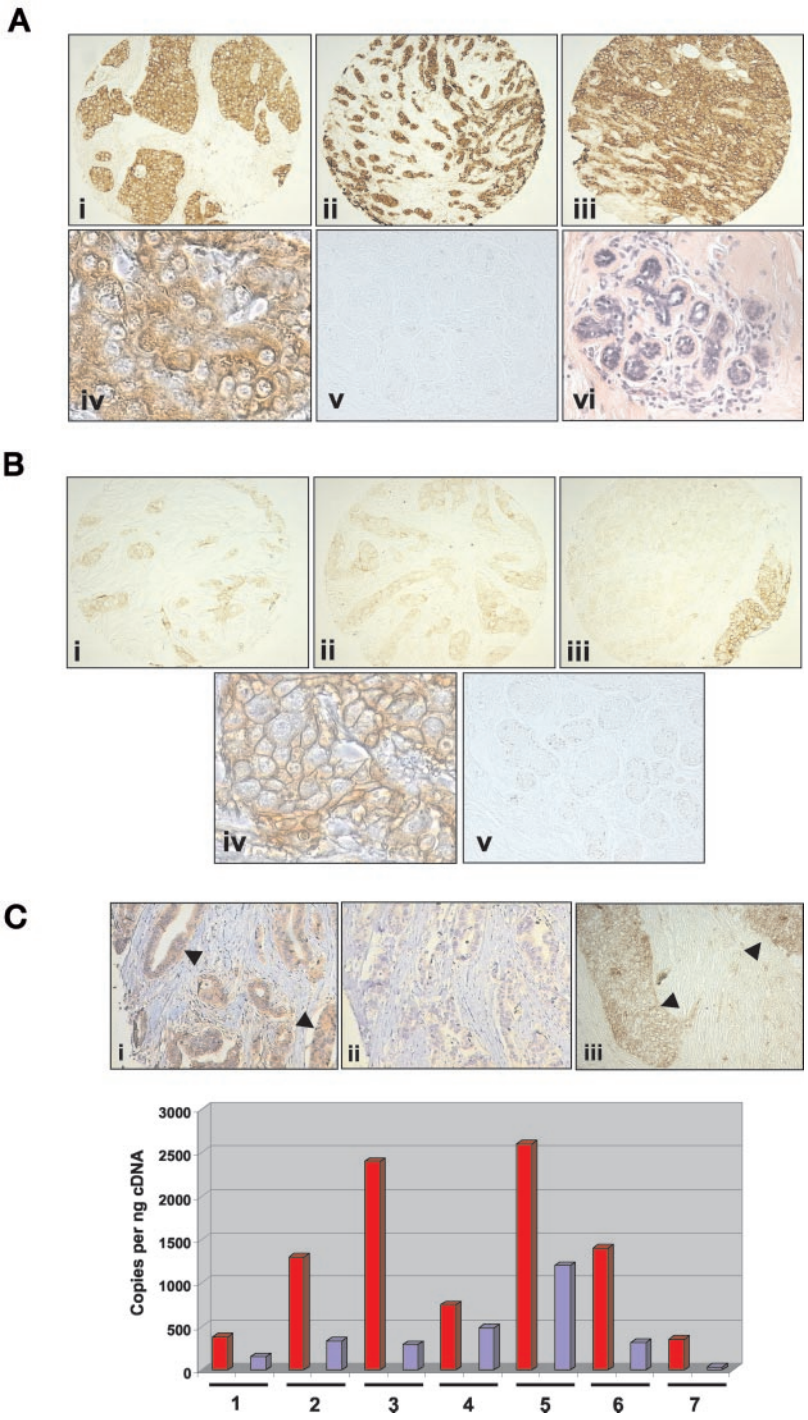


FIG. 5. **Expression of BCMP11, BCMP84, and BCMP101 in breast cancer tissue sections.** Immunohistochemical analysis of BCMP11 (*A*) and BCMP84 (*B*) protein in three separate 1-mm core sections of breast invasive ductal carcinoma sections (*i–iii*) is shown. *Panels* labeled *iv* show high power microscopy, demonstrating cytoplasmic staining of BCMP11 and plasma membrane-associated staining of BCMP84 in ductal carcinoma epithelial cells. *Panels* labeled *v* show expression in normal breast ductal epithelial cells (a representative hematoxylin and eosin stained section of this tissue is shown in *vi*). *C*, *in situ* RT-PCR (*i*) and immunohistochemical analysis (*iii*) of BCMP101 in breast cancer tissue sections are shown. A nonspecific primer *in situ* control RT-PCR is shown on a consecutive section to indicate levels of background amplification (*ii*). Expression of BCMP101 mRNA and protein in carcinoma cells is indicated by *arrowheads*. Real time RT-PCR quantification of BCMP101 mRNA levels in breast tumor and adjacent normal tissues from seven donors is also shown (tumor tissue is represented by *red bars*, normal tissue is represented by *blue bars*).

In contrast to this tumor cell plasma membrane localization, a primary culture of non-tumor-derived human mammary epithelial cells transfected with the AFP-tagged BCMP84 construct showed only cytosolic BCMP84 expression (Fig. 4*C*, *iv*). Yeast two-hybrid cloning identified nucleobindin (CALNUC) protein as a BCMP84-interacting protein. Nucleobindin is found in both cytosolic and membrane fractions (see supplemental Table I, plasma membrane-associated accession Q02818) where it is thought to act as a major calcium-binding protein (21).

BCMP11, BCMP84, and BMCP101 Proteins Are Expressed in Clinical Breast Cancer Tissues—Immunohistochemical analysis was used to determine the expression of BCMP11, BCMP84, and BCMP101 proteins in sections of normal and breast cancer tissues. BCMP11 showed a restricted expression profile in normal tissues, with only the epithelial lining of the colonic mucosa showing significant levels of staining with a specific antibody raised against BCMP11, entirely consistent with the mRNA distribution of BCMP11 (data not shown). In contrast, very high levels of BCMP11 staining were seen in 43 of 58 (74%) breast cancer donor tissues but not normal breast ductal epithelial cells (Fig. 5*A*). In each case the BCMP11 staining was restricted to the cancerous epithelial cells of the tissue and was localized in the cytoplasm of these cells (Fig. 5*A*, *iv*).

Real time quantitative RT-PCR, *in situ* RT-PCR, and immunohistochemical analysis of BCMP101 expression demonstrated very low levels in multiple normal tissues (data not shown). In contrast, high levels of BCMP101 mRNA and protein were detected in the carcinoma cells of breast cancer

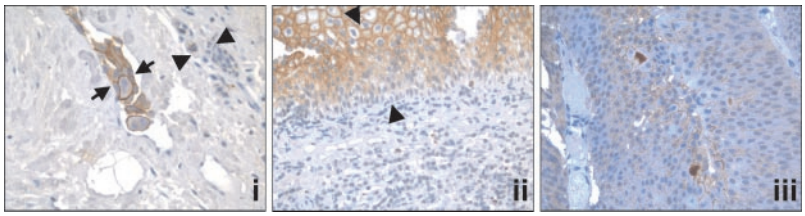


FIG. 6. **Immunohistochemical analysis demonstrating subcellular translocation of BCMP84 protein in tumor tissues.** *i*, BCMP84 immunostaining in breast ductal carcinoma (grade 3) tissue (*arrows*) and adjacent normal tissue (*arrowheads*). Compare the strong plasma membrane-associated staining in the tumor cells with the granular cytoplasmic staining in the normal tissue. BCMP84 immunostaining in squamous cell tonsil carcinoma (*ii*) and bladder papillary transitional cell carcinoma (*iii*) is shown. Note the predominantly cytoplasmic staining in the less well differentiated carcinoma cells, which becomes distinctly plasma membrane-associated as the cells become more differentiated (*arrows* in *ii*). All tissues were counterstained with hematoxylin. Magnification, ×40.

tissues (Fig. 5*C*). Real time RT-PCR analysis also demonstrated that BCMP101 mRNA was up-regulated more than 2-fold in six of seven (85%) breast cancer donor tissues relative to donor-matched adjacent normal breast tissue (Fig. 5*C*).

The distribution of BCMP84 protein in normal tissues was restricted to the stratified squamous epithelium of the skin, cervix, and tonsil (data not shown). Strong immunoreactivity to the BCMP84 antibody was observed in 10 of 58 (17%) breast cancer donor tissues with weak staining seen in normal breast ductal tissue (Fig. 5*B*, *v*). In each case BCMP84 tumor staining was restricted to the cancerous epithelial cells of the tissue and, as with the immunocytochemical analysis in the MDA-MB-468 cell line (Fig. 4*C*, *iii*), showed a clear localization in the plasma membrane (Fig. 5*B*, *iv*). In contrast, we consistently observed weaker cytoplasmic staining in adjacent normal ductal breast epithelial tissue (Fig. 6, *i*). This is consistent with the cytosolic expression of AFP-tagged BCMP84 in primary normal human mammary epithelial cells (Fig. 4*C*, *iv*). Subsequent analysis of multiple other tumor tissues demonstrated elevated BCMP84 protein expression in squamous tonsil and bladder papillary transitional cell carcinoma tissues (Fig. 6, *ii–iii*). For both of these tumor tissues we observed that the less differentiated basal cancer cells showed cytoplasmic BCMP84 staining that became distinctly plasma membrane localized as the cells became more differentiated (Fig. 6).

DISCUSSION

Identification of tumor cell-specific plasma membrane-associated proteins is an important first step in the development of antibody and small molecule cancer therapies. In this paper we describe and list, to our knowledge, the largest example of a tumor cell-derived membrane proteome.

The standard approach for resolving proteins involves two-dimensional gel electrophoresis; however, two-dimensional gels are particularly poor at separating relatively insoluble hydrophobic membrane proteins. For this reason we chose to resolve our membrane preparations on one-dimensional gels, which has allowed us to identify a spectrum of membrane and associated proteins ranging from small membrane-associated Ras signaling proteins to large multitransmembrane proteins (see supplemental Table I). This comprehensive annotation of breast cancer cell line proteins has been achieved by avoiding repetitive sequencing of high abundance proteins and subjecting more than 16,000 peptides of low relative intensity (<10% from MALDI) to MS/MS. Thus we observed a wide dynamic range of breast cancer proteins, from the highly abundant and amplified her2neu to rare unique proteins such as BCMP101 which have never been sequenced at the protein level before and in this case show only four matching ESTs from breast tissues. In addition to one-dimensional gel electrophoresis, other exploratory peptide separation technologies such as multidimensional protein identification technology (22) or isotope-coded affinity tagging (23) as well as more complex protein fractionation methods and new MS technologies such as anchor targets (www.brukerdaltonics.com) coupled to TOF-TOF instruments (www.appliedbiosystems.com) could enable the direct identification of even lower abundance proteins. To ensure a representative and plasma membrane-enriched breast cancer cell membrane preparation we have used breast cancer-derived cell lines. The membrane preparations that can be obtained from clinical breast tissue are far less pure and in many instances are "contaminated" with non-tumor cell membranes.[2] The four breast cancer-derived cell lines used in this study, two representing estrogen receptor-positive cell lines and the other two EGF receptor-positive cell lines, were deliberately chosen to reflect some of the different molecular pathologies known in breast cancer. Indeed, demonstrating immunohistochemically that the three unique proteins we characterized further are significantly expressed in clinical breast tumor tissues has validated our use of tumor-derived cell lines in proteomics.

Of the three proteins we characterized, BCMP11/hAG3 shows the highest prevalence in breast cancers (74%) with a strong positive correlation with estrogen receptor status (20). We have observed no mRNA or protein expression in normal breast epithelia or stromal tissue. The finding that BCMP11 is potentially localized in secretory organelles is consistent with it having a putative signal sequence and thus the prediction that it is a secreted extracellular protein. Consistent with this, yeast two-hybrid cloning identified the human homolog of a rat glycosylphosphatidylinositol-anchored metastasis-associated protein, C4.4A, as a BCMP11-interacting protein. C4.4A may therefore represent an autocrine receptor for BCMP11. Further *in vitro* and *in vivo* studies will be required to determine the biological role of BCMP11 in breast tumor cell growth and/or metastasis.

No clues as to the function of BCMP101 could be gained from its primary sequence, but its mRNA expression was highly restricted to breast cancer-derived cell lines and clinical breast cancer where it was up-regulated more than 3-fold in six of seven breast cancer tissues relative to the donor matched normal tissues. Fluorescently tagged BCMP101 showed a localization in the plasma membrane, particularly in areas of cell-cell contact, and a key finding with respect to this is its interaction with α1-catenin. α1-Catenin is known to associate with the cytoplasmic domains of multiple plasma membrane-localized cadherins and as such are thought to play an important role in cell-cell adhesion. Interestingly, α1-catenin is mutated in the invasive human colon cancer cell family HCT-8 and is therefore an invasion suppressor gene in human colon cancer (24). We can speculate that BCMP101 may play a role in breast tumor development by binding to α1-catenin and blocking its tumor suppressor functions.

BCMP84 is a member of the S100 family of proteins, and we

[2] R. Boyd, unpublished observations.

have shown here plasma membrane localization in both breast cancer-derived cell lines and clinical breast cancer tissues but cytosolic expression in non-tumor cells. The translocation of BCMP84 from cytosol to plasma membrane in 17% of breast cancers is the lowest prevalence event of the three proteins described here. However, BCMP84 shows this proliferation and differentiation-associated event in other cancers such as tonsil and bladder. The prevalence and significance of this translocation to cancer remain to be established, but this 17% may define molecularly a subset of breast cancers that have commonalities with BCMP84-positive tumors derived from other tissues. Other members of the S100 family of calcium-binding proteins are known to be expressed in cancer tissues (25). In particular, S100A4 (p9Ka), whose expression has been shown to induce metastasis in rodent models of breast cancer (26, 27), is expressed in human breast cancers and tightly correlates with poor prognosis (28, 29). Stradal and Gimona (30) have demonstrated that S100A6 (calcyclin) exhibits a calcium-dependent association with the plasma membrane and nuclear envelope in porcine smooth muscle and human CaKi-2 cells. These data suggest that the translocation of BCMP84 from the cytosol of normal cells to the plasma membrane in tumor cells may be regulated in a calcium-dependent manner. Indeed, further evidence for a calcium-dependent localization of BCMP84 was discovered through its interaction with nucleobindin. Nucleobindin is found in both cytosolic and membrane fractions and has been strongly associated with several different subfamilies of Gα proteins on the luminal surface of Golgi membranes where it is thought to act as a major calcium-binding protein (21, 31). The finding that BCMP84 can interact with nucleobindin which in turn can bind G proteins in a calcium-dependent manner provides a possible mechanism by which BCMP84 can associate with the plasma membrane in cancer cells and suggests a role for BCMP84 in G protein-coupled signal transduction events. In support of this we have identified both nucleobindin and Gα_{i3} (and multiple other G proteins) in our breast cancer cell line membrane protein preparations (see supplemental Table I). The translocation of BCMP84 to the plasma membrane in carcinoma cells exemplifies the potential of our proteome-driven approach as disease-associated cellular translocation of a protein would not be detected at the level of the transcriptome.

In summary, we have demonstrated a process utilizing multiple proteomic methodologies for the *de novo* discovery of cancer cell membrane-associated proteins with potential clinical relevance to breast cancer. This process also shows the primary discovery value of cancer-derived cell lines where the ability to generate samples of high purity and quality for proteome analysis far outweighs the perceived problems of differences between cell lines and primary clinical material. Indeed, our characterization of three of the novel proteins identified in this study, BCMP11, BCMP84, and BCMP101, indicates their potential as targets for breast cancer therapy and/or diagnostic markers of the disease. Furthermore, the discovery potential of this data set (supplemental Table I) extends well beyond these three examples to include many uncharacterized proteins associated with the membranes of breast cancer cells.

REFERENCES

1. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. (2000) *Cancer J. Clin.* **50,** 7–33
2. (2001) *Expert Rev. Anticancer Ther.* **1,** 3
3. Brenner, T. L., and Adams, V. R. (1999) *J. Am. Pharm. Assoc.* **39,** 236–238
4. Abicht, A., and Lochmuller, H. (2000) *Curr. Opin. Mol. Ther.* **2,** 539–600
5. Ranson, M., Mansoor, W., and Jayson, G. (2002) *Expert Rev. Anticancer Ther.* **2,** 161–168
6. Hubert, R. S., Vivanco, I., Chen, E., Rastegar, S., Leong, K., Mitchell, S. C., Madraswala, R., Zhou, Y., Kuo, J., Raitano, A. B., Jakobovits, A., Saffran, D. C., and Afar, D. E. (1999) *Proc. Natl. Acad, Sci. U. S. A.* **96,** 14523–14528
7. Catimel, B., Ritter, G., Welt, S., Old, L. J., Cohen, L., Nerrie, M. A., White, S. J., Heath, J. K., Demediuk, B., Domagala, T., Lee, F. T., Scott, A. M., Tu, G. F., Ji, H., Moritz, R. L., Simpson, R. J., Burgess, A. W., and Nice, E. C. (1996) *J. Biol. Chem.* **271,** 25664–25670
8. Dhanasekaran, S. M., Barrette, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2001) *Nature* **412,** 822–826
9. Simpson, R. J., Connolly, L. M., Eddes, J. S., Pereira, J. J., Moritz, R. L., and Reid, G. E. (2000) *Electrophoresis* **21,** 1707–1732
10. Pasquali, C., Fialka, I., and Huber, L. A. (1999) *J. Chromatogr.* **722,** 89–102
11. Eng, J. K., McCormack, A. L., and Yates, J. R., III (1994) *J. Am. Soc. Mass Spectrom.* **5,** 976–989
12. Fromont-Racine, M., Rain, J. C., and Legrain, P. (1997) *Nat. Genet.* **16,** 277–282
13. Rain, J. C., Selig, L., De Reuse, H., Battaglia, V., Reverdy, C., Simon, S., Lenzen, G., Petel, F., Wojcik, J., Schachter, V., Chemama, Y., Labigne, A., and Legrain, P. (2001) *Nature* **409,** 211–215
14. Page, M. J., Amess, B., Townsend, R. R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M. J., Stein, R. C., Waterfield, M. D., Davies, S. C., and O'Hare, M. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **22,** 12589–12594
15. Thompson, D. A., and Weigel, R. J. (1998) *Biochem. Biophys. Res. Commun.* **251,** 111–116
16. Petek, E., Windpassinger, C., Egger, H., Kroisel, P. M., and Wagner, K. (2000) *Cytogenet. Cell Genet.* **89,** 141–142
17. Aberger, F., Weidinger, G., Grunz, H., and Richter, K. (1998) *Mech. Dev.* **72,** 115–130
18. Schafer, B. W., Wicki, R., Engelkamp, D., Mattei, M. G., and Heizmann, C. W. (1995) *Genomics* **25,** 638–643
19. Rosel, M., Claas, C., Seiter, S., Herlevsen, M., and Zoller, M. (1998) *Oncogene* **17,** 1989–2002
20. Fletcher, G. C., Patel, S., Tyson, K., Adam, P. J., Schenker, M., Loader, J. A., Daviet, L., Legrain, P., Parekh, R., Harris, A. L., Terrett, J. A. (2003) *Br. J. Cancer*, in press
21. Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, J. M., Jin, M., Hennemann, H., McQuistan, T., De Vries, L., and Farquhar, M. G. (1998) *J. Cell Biol.* **141,** 1515–1527
22. Koller, A., Washburn, M. P., Lange, M., Andon, N. L., Deciu, C., Haynes, P. A., Hays, L., Schieltz, D., Ulaszek, R., Wei, J., Wolters, D., and Yates, J. R., III (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11969–11974
23. Zhou, H., Ranish, J. A., Watts, J. D., and Aebersold, R. (2002) *Nat. Biotechnol.* **20,** 512–515
24. Vermeulen, S. J., Nollet, F., Teugels, E., Vennekens, K. M., Malfait, F., Philippe, J., Speleman, F., Bracke, M. E., Van Roy, F. M., and Mareel, M. M. (1999) *Oncogene* **18,** 905–915
25. Ilg, E. C., Schafer, B. W., and Heizmann, C. W. (1996) *Int. J. Cancer* **68,** 325–332
26. Davies, M. P., Rudland, P. S., Robertson, L., Parry, E. W., Jolicoeur, P., and Barraclough, R. (1996) *Oncogene* **13,** 1631–1637
27. Ambartsumian, N. S., Grigorian, M. S., Larsen, I. F., Karlstrom, O., Sidenius, N., Rygaard, J., Georgiev, G., and Lukanidin, E. (1996) *Oncogene* **13,** 1621–1630
28. Platt-Higgins, A. M., Renshaw, C. A., West, C. R., Winstanley, J. H., De Silva Rudland, S., Barraclough, R., and Rudland, P. S. (2000) *Int. J. Cancer* **89,** 198–208
29. Rudland, P. S., Platt-Higgins, A., El-Tanani, M., De Silva Rudland, S., Barraclough, R., Winstanley, J. H., Howitt, R., and West, C. R. (2000) *Cancer Res.* **60,** 1595–1603
30. Stradal, T. B., and Gimona, M. (1999) *J. Biol. Chem.* **274,** 31593–31596
31. Lin, P., Fischer, T., Weiss, T., and Gist Farquhar, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 674–679