Mammaglobin Is Found in Breast Tissue as a Complex with BU101

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ABSTRACT: The mammaglobin gene has been shown to be preferentially expressed in breast tissue. Few genes match its specificity. Mammaglobin has generated much interest, and studies are ongoing to develop diagnostic tests for breast cancer based on the detection of mammaglobin. While searching the Incyte Genomics Lifeseq database for tissue-specific markers, we observed a second secretoglobin, BU101, also known as lipophilin B. We report here that mammaglobin, in breast tissue, is found as a complex with BU101. The complex was isolated from breast cancer tissue and was characterized as the biologically relevant form of mammaglobin.

Our knowledge of the secretoglobin superfamily is rapidly expanding with the discovery of many new human genes. Five years ago known members of the family were hare and rabbit uteroglobin (UG) proteins and human, mouse, and rat homologues, which in these species were called the Clara cell 10 kDa (CC10kDa)[1] proteins (*1*). These proteins are homodimers of an approximately 70 amino acid polypeptide. Rat prostatein (*2*−*4*), cat major allergen (*5*), and RYD5 (*6*) were known but considered distantly related, partly due to reduced sequence homology and partly because they exhibit a more complex quaternary structure. These proteins are heterotetramers comprised of two heterodimeric subunits.

Currently, the superfamily includes an additional five human proteins: (a) mammaglobin (*7*) [also known as human steroid binding factor C2 (*8*)], (b) mammaglobin B (*9*) [also known as lacryglobin (*10*), lipophilin C (*11*), endometrial-specific steroid binding factor III (*12*), and human mammaglobin homologue (*13*)], (c) BU101 (*14*) [also known as lipophilin B (*11*), endometrial-specific steroid binding factor II (*12*), human steroid binding protein C1 (*8*), and human breast tumor associated protein 67 (*15*)], (d) endometrial-specific steroid binding factor I (*12*), and (e) lipophilin A (*11*).

The new secretoglobin members are known by a variety of names. Numerous groups have identified them from databases containing human genomic sequence. As is the case with some of the members, BU101 was published originally in the patent literature. As with many newly discovered sequences, a consensus name has yet to be identified.

Uteroglobins (UG/CC10kDa proteins) have been shown to exhibit a variety of in vitro and in vivo biological activities including a potent antiinflammatory activity (*16*−*19*), inhibitory effects on neutrophil migration (*20*, *21*), platelet aggregation (*22*), chemoinvasion (*23*−*25*), and anchorage-independent growth of transformed cell lines (*26*, *27*). Recently, Mukherjee proposed its role as a novel cytokine (*28*). A putative receptor for uteroglobin has been identified although the presumed pathway, by which binding to this receptor exerts its cellular effects, has not yet been clearly defined. A uteroglobin knock-out mouse model suffers from a predisposition to malignancies and severe renal disease (IgA nephropathy) (*29*). Another knock-out model demonstrates a null phenotype (*30*).

The more distantly related rat prostatein has been shown to be involved in experimental autoimmune prostatitis, a condition involving inflammation of the prostate. It serves as an autoantigen and perhaps as an inducing antigen of the disease itself (*31*). Cat major allergen also elicits an immune response. This protein complex is responsible for causing cat-induced asthma in humans (*32*).

Considerable effort has been made to identify a function common to all members of the secretoglobins, but to date, that function has remained elusive. Whether these proteins have receptors and are involved in signaling pathways has yet to be definitively substantiated.

As its name suggests, mammaglobin, one of the newly discovered members of this family, exhibits highly breast-specific expression. This tissue specificity has provided the impetus for investigations evaluating mammaglobin as a marker for the detection of metastasizing breast tumor cells (*7*, *33*−*37*). This specificity also makes it an ideal candidate as an immunohistochemical marker in diagnosing tumors of unknown origin or detecting spread of disease to the lymph nodes (*38*). As a serum marker, it has potential for assessing response to therapy or recurrence (*39*).

In this work, we demonstrate that, in breast tissue, mammaglobin is present as a complex with another secretoglobin, BU101. We have named this complex MamBu. To determine if this association is an intrinsic property of the

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[1] Abbreviations: BU101, breast uteroglobin 101; CC10kDa, Clara cell 10 kDa protein; EST, expressed sequence tag; ATCC, American Type Culture Collection; TBE, Tris−borate−EDTA; MOPS, morpholinopropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEK293, human embryonic kidney 293 cells; DMEM, Dulbecco's modified Eagle's medium; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; GCG, Genetics Computer Group; ESSBF I, endometrial-specific steroid binding factor I.

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two proteins, or if, alternatively, breast tissue somehow induces this association, we have expressed, individually and in concert, these two proteins in a cell line normally devoid of secretoglobins. We find that coexpression of mammaglobin and BU101 dramatically increases the level of secreted protein and that the protein so produced exists as a MamBu complex. This, as well as our detection of the complex in breast tissue, indicates that MamBu may be the normal form of mammaglobin.

MATERIALS AND METHODS

Identification of BU101 from the Lifeseq Database. Lifeseq is a database of expressed sequence tags (ESTs) available by subscription from Incyte Genomics Inc. A feature of these database is that it is organized by tissue type, such that a representation of the expression patterns of the proteome is provided. The database has not been culled for degeneracy, so that highly expressed sequences are represented several times in the database. This organization can be viewed in terms of a "transcript image" which may be regarded as an approximate representation of the distribution of mRNAs in the chosen tissue. We searched for sequences that were preferentially represented in various tissues, in particular, for this work, breast tissue. Clusters of overlapping sequences were identified by comparing the Lifeseq cDNA sequences with each other and with those in public databases. In this manner we identified BU101 and mammaglobin as being putatively specifically expressed in breast tissue.

Clones, Cell Lines, and Tissues. Clones 603148 and 899895, containing the full coding sequence for BU101 and mammaglobin, respectively, were obtained from Incyte Genomics. All cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained according to the ATCC instructions. Tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects (*40*).

Ribonuclease Protection Assay. Synthesis of both the labeled cRNA hybridization probe and unlabeled sense strand was achieved from the plasmid containing either the mammaglobin or BU101 cDNA sequence insert flanked by opposed SP6 and T7 polymerase promoters. The ^{32}P-labeled hybridization probe was generated by in vitro transcription from the T7 promoter using the Riboprobe in vitro Transcription System (Promega) as recommended by the manufacturer. The unlabeled sense strand was generated by in vitro transcription from the SP6 promoter. Hybridization of the probe to sample or control (diluted sense strand) was performed using the DirectProtect Lysate RNase Protection kit (Ambion) as recommended by the manufacturer. After overnight hybridization at 37 °C, the unhybridized RNA was digested, and the hybridized fragments were precipitated. The precipitates were electrophoresed in 6% polyacrylamide TBE and 8 M urea denaturing gels, exposed to storage phosphor screens, and analyzed using the STORM storage phosphor autoradiography system and ImagQNT software (Molecular Dynamics).

Northern Analysis. Briefly, 5−10 μg of total RNA isolated using the lithium chloride/urea technique was incubated in 15 μL of a solution containing 40 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, and 50% v/v formamide for 15 min at 65 °C. The denatured RNA was mixed with 2 μL of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and loaded into a denaturing 1.0% agarose gel containing 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. The gel was electrophoresed at 60 V for 1.5 h and rinsed in RNase free water. RNA was transferred from the gel onto nylon membranes (Brightstar-Plus, Ambion Inc.) for 1.5 h using the downward alkaline capillary transfer method. The filter was rinsed with 1× SSC, and RNA was cross-linked to the filter using a Stratalinker (Stratagene Inc.) on the auto-cross-linking mode and dried for 15 min. The membrane was then placed into a hybridization tube containing 20 mL of preheated prehybridization solution (5× SSC, 50% formamide, 5× Denhardt's solution, 100 μg/mL denatured salmon sperm DNA) and incubated in a 42 °C hybridization oven for at least 3 h. While the blot was prehybridizing, a ^{32}P-labeled random-primed probe was generated using the BU101 insert fragment (obtained by digesting clone 603148H1 with *Xba*I and *Not*I) using the Random Primer DNA Labeling System (Life Technologies, Inc.) according to the manufacturer's instructions. Half of the probe was boiled for 10 min, quick chilled on ice, and added to the hybridization tube. Hybridization was carried out at 42 °C for at least 12 h. The hybridization solution was discarded, and the filter was washed in 30 mL of 3× SSC and 0.1% SDS at 42 °C for 15 min, followed by 30 mL of 3× SSC and 0.1% SDS at 42 °C for 15 min. The filter was wrapped in plastic wrap and exposed to Kodak XAR-Omat film for 8−96 h, and the film was developed for analysis.

RT-PCR. Total mRNA was purified using Ultraspec, as recommended by the manufacturer. A two-step RT-PCR reaction was performed. Briefly, 0.5 μg of extracted mRNA was reverse transcribed in a 20 μL reaction mixture containing 1× PCR II buffer (Perkin-Elmer), 5 mM $MgCl_2$, 1 mM dNTP, 20 units of RNasin, 2.5 μM random hexamers, and 50 units of MMLV (Moloney murine leukemia virus) reverse transcriptase (RT). Reverse transcription was performed at room temperature for 10 min, 42 °C for 60 min in a PE-480 thermal cycler, and followed by further incubation at 95 °C for 5 min to inactivate the RT. PCR was performed using 2 μL of the cDNA reaction in a final PCR reaction volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTP, a 0.5 μM amount of each sense (5′-TTCTGCCCAGCTCTTGTTTCTGAG-3′) and antisense (5′-TGCAGTGAAGATCAGGGTG-3′) primer, respectively, and 2.5 units of Taq polymerase. The reaction was incubated in an MJ Research Model PTC-200 as follows: 40 cycles of amplification (94 °C, 20 s; 58 °C, 30 s; 72 °C, 30 s), a final extension (72 °C, 10 min), and a soak at 4 °C.

Transient Expression. A plasmid for the expression of secretable mammaglobin or BU101 protein was constructed by inserting the appropriate polynucleotide sequence from clone 899895 or 603148 into the pcDNA3.1/Myc-His vector (Invitrogen). A construct was obtained such that the desired sequence would have an attached myc epitope and His_6 tag for facile purification. For transfection of human embryonic kidney 293 cells (HEK293), these plasmids were purified from DH5 alpha cells using a QIA filter Maxi kit (Qiagen). Transfection was carried out using the cationic lipofectamine-

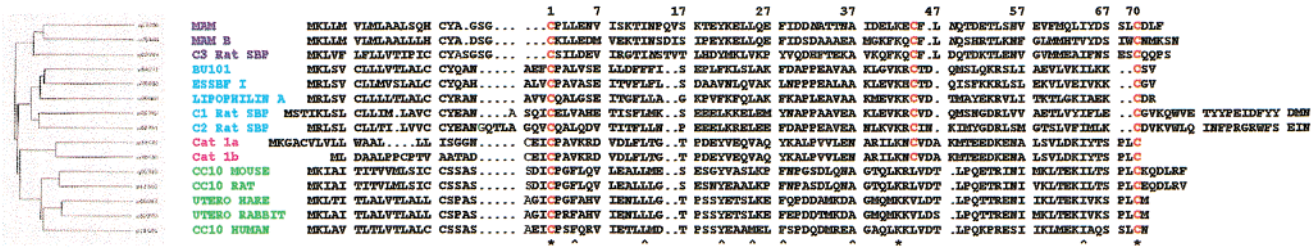

FIGURE 1: Sequence alignment of secretoglobin family members. Sequence alignment was performed using the GCG program PILEUP. (*) indicates a conserved residue over all sequences, (∧) indicates a highly conserved residue, and *N* indicates a potential N-linked glycosylation site.

mediated procedure (*41*). Supernatants and cell extracts were analyzed for the mammaglobin and BU101 gene products 72 h posttransfection by Western analysis using a monoclonal antibody developed against a myc epitope (Invitrogen). A three-step development was used involving the primary antibody, a biotinylated antispecies secondary antibody, and a streptavidin−alkaline phosphatase conjugate.

Creation of the Stable Cell Line MB8. Transfection of the mammaglobin m/h and BU101 m/h expression plasmids was performed using the cationic lipofectamine-mediated procedure (*41*). At 72 h posttransfection, the cells were released from the dish by limited trysinization and reseeded into 100 mm culture dishes in DMEM, 10% FBS, and 400 μg/mL G418 at dilutions of 1:100, 1:1000, and 1:10000. These cultures were allowed to grow for 5−7 days, until well-isolated foci of cells were identified by microscopy. These foci were isolated by cloning cylinders, their cells released by limited trypsinization, and clonal lines created from each foci. After growth to near confluence the supernatants of each clonal line were analyzed for mammaglobin m/h and BU101 m/h expression by Western blot analysis using an anti-myc monoclonal antibody, anti-BU101, and anti-mammaglobin polyclonal antisera. The clonal line labeled MB8 was found to express both mammaglobin m/h and BU101 m/h in the supernatant. This line was expanded and then passaged 1:30 three times, following which expression of mammaglobin m/h and BU101 m/h was again reconfirmed to ensure stability of the insertion event. The final product of this procedure was a cell line derived from HEK293 cells that express mammaglobin m/h and BU101 m/h, which we have labeled HEK293-MB8.

Western Analysis. Extracts of human tissue were prepared for SDS−PAGE and Western analysis by homogenization in 0.1 M Tris-HCl pH 7.5, 15% glycerol, 0.2 mM EDTA, 1 mM DTT, 10 μg/mL leupeptin, and 1 mM PMSF. The homogenates were centrifuged, and the supernates were analyzed for protein concentration using the bicinchoninic acid reagent (Sigma). Ten micrograms of total protein was run on 10−20% precast tricine gels (Novex) and transferred to nitrocellulose for Western development using polyclonal antisera developed against either a BU101 or mammaglobin peptide, specifically BU101.3 and Mam.1.

Purification of Breast Tissue. Breast cancer tissue was obtained from a patient with invasive, poorly differentiated adenocarcinoma of the breast. Two-tenths of a gram of the tissue, which had been snap frozen and stored at −70 °C, was homogenized in 0.1 M Tris-HCl (pH 7.5), 15% (w/v) glycerol, 0.2 mM EDTA, 10 μg/mL leupeptin, and 1.0 mM phenylmethanesulfonyl fluoride (*42*). Following homogenization, the homogenates were centrifuged at 5000*g* at 4 °C for 5 min to separate supernatant from debris. The supernatant was purified using a Mono Q 5/5 column (Pharmacia) running with 20 mM piperazine, pH 6.0, buffer and a gradient to 1 M NaCl. Fractions were pooled and analyzed by dot blots to detect both BU101 and mammaglobin epitopes. Positive fractions were pooled and concentrated to 100 μL by ultrafiltration (using a Centriplus 30; 30 000 MWCO). The retentate was applied to a 10 mm × 30 cm column of Superose 12 (Pharmacia) and eluted with PBS (50 mM phosphate, 150 mM sodium chloride, pH 7.4) at a flow rate of 0.4 mL/min. The column was calibrated with molecular mass standards available from Pharmacia.

Antisera Production. Polyclonal antisera were obtained from female white New Zealand rabbits immunized with peptide, either conjugated or unconjugated to KLH given in complete Freunds adjuvant (CFA). One week prior to the first immunization, 5−10 mL of blood was obtained from the animal to serve as a nonimmune prebleed sample. Booster immunizations in incomplete Freunds adjuvant (IFA) were given in 4 week cycles following the primary immunization. Bleeds were taken 2 weeks after the boosters.

ELISA. ELISA assays were performed using various dilutions of antigen in phosphate-buffered saline passively coated on the microtiter plates overnight. The polyclonal antisera were used at 1:1000 dilution, and detection was accomplished using a goat anti-rabbit alkaline phosphatase conjugate.

RESULTS

Sequence Homology. Mammaglobin and BU101 are both members of the secretoglobin superfamily. Thus far, 15 members have been identified from various organisms including human, feline, mouse, rat, hare, and rabbit. Sequence alignment was performed on the translated or known protein sequences using the GCG program PILEUP (Figure 1). Three amino acids are conserved among all of the members. These include cysteines at positions 1 and 70, as well as lysine at position 42. A number of other residues are highly homologous including positions 4, 12, 21, 25, 28, 38, and 64 (Figure 1). These positions are occupied by hydrophobic residues.

Some differences among the sequences are evident. A third cysteine residue, at position 44, is common among the sequences that are not a CC10kDa or uteroglobin protein. Also, three members possess N-linked glycosylation sites: mammaglobin, mammaglobin B, and the C3 chain of rat prostatein. Each of these asparagines is located between putative helices, identified by alignment of the primary

Table 1: Tissue Distribution of Secretoglobins in the LifeSeq Database[a]

(A) EST Abundance Levels of Secretoglobins

						institution
	Mam	Mam B				Wash U
		ESSBF III	ESSBF II	ESSBF I		HGS
		lipophilin C	lipophilin B		lipophilin A	UCLA
	SBP C2		SBP C1			Incyte
	Mam	EU250	BU101	TU104		
tissue type						total no. of ESTs
breast	178	1	41	0	0	240308
prostate	0	2	3	0	0	250816
uterus	3	20	13	10	0	175253
all						3440324

(B) Tissue Libraries Containing Secretoglobins

						institution
	Mam	Mam B				Wash U
		ESSBF III	ESSBF II	ESSBF I		HGS
		lipophilin C	lipophilin B		lipophilin A	UCLA
	SBP C2		SBP C1			Incyte
	Mam	EU250	BU101	TU104		
tissue type						no. of libraries
breast	27	1	12	0	0	59
prostate	0	2	2	0	0	68
uterus	1	9	6	4	0	34
all						808

[a] Secretoglobin sequences were blasted against the LifeSeq database. (A) The number of ESTs representing the sequence of interest in the different tissue libraries is shown. The total number of ESTs from those libraries is shown in the last column. (B) The number of libraries containing the ESTs representing the sequence of interest is shown. The total number of libraries is shown in the last column.

sequence with rabbit uteroglobin and rat CC10kDa protein, of which there are crystal structures (*43*).

Mammaglobin and BU101 did not cluster together. Mammaglobin was found to cluster with mammaglobin B and the C3 chain of rat prostatein. BU101 was found to cluster with ESSBF I (endometrial-specific steroid binding factor I), lipophilin A, and the C1 and C2 chains of rat prostatein. The CC10kDa proteins and the uteroglobins clustered together, while the cat allergen protein chains, Ch1a and Ch1b, were further removed.

Homology levels within these clusters were found to be above 40% and dropped to below 30% between clusters. BU101 shares 59% identity with ESSBF I and lipophilin A, its two closest homologues. Identity levels drop to 51% and 45% between BU101 and the two other members of the cluster, rat steroid binding proteins, C1 and C2, respectively. Similarly, mammaglobin shares 58% identity with its closest homologue, mammaglobin B, and 42% identity with the other member of the cluster, rat steroid binding protein C3. BU101 and mammaglobin share only 27% identity.

Tissue Distribution. We queried Incyte Genomics Lifeseq EST database with the secretoglobin sequences to investigate their tissue specificity. Table 1A shows the number of ESTs present in the database that represent the sequence of interest, and Table 1B shows the libraries that contain these ESTs. Those tissue types that contained only one EST were not included. As other investigators have observed, mammaglobin was predominantly found in mammary tissue. The database was found to contain 178 ESTs representing mammaglobin in breast tissue and 3 ESTs in uterine tissue.

Table 2: Ribonuclease Protection Assay Results[a]

		no. of mRNA molecules/cell	
sample ID	status	mammaglobin	BU101
breast			
96-12-C007G	normal	24	7
96-11-C016R	normal	4	2
96-12-C027R	normal	9	8
96-10-C135R	normal	0.3	0.2
96-10-C157	normal	37	10
96-09-C030	cancer	0	0
96-09-C033R	cancer	≫76	≫87
C011G	cancer	≫41	≫46
C023G	cancer	0.5	0.3
C012G	cancer	0	0
lung			
97-01-C005R	normal	0	0
C037G	cancer	0	0
colon			
C027R	normal	0	0

[a] The amount of BU101 and mammaglobin mRNA at 318 and 244 bp, respectively (hybridized probe size), was quantitated using a standard curve generated with the unlabeled sense strand. The amount of sequence-specific mRNA is recorded as molecules per cell from the sample. The number of cells was approximated from the DNA content of the tissue. The sample IDs and status are those designated by the Cooperative Human Tissue Network.

BU101, likewise, was found to be abundant in breast tissue with 41 ESTs observed and was present at lower levels in uterine tissue (13 ESTs) and prostate tissue (3 ESTs).

The LifeSeq database also provides a tissue distribution profile for other members of the secretoglobin family. Mammaglobin B is most prevalent in the uterus, with lower levels of expression found in the prostate and breast. ESSBF I was present only in the uterus. Lipophilin A was not found in the database. For comparison, CC10kDa protein had an abundance level of 205 in respiratory tissue and cytoplasmic β-actin had an average level of 250 in each of the tissue categories. In each tissue type only some of the members are prevalent. In this database, in breast tissue, mammaglobin, and BU101 are expressed at measurable levels whereas the other secretoglobin members are not.

Mammaglobin and BU101 mRNA and protein were detected in breast tissue using RT-PCR, Northern blots, ribonuclease protection assays, and Western blots. Quantitative analysis of the mRNA levels by ribonuclease protection assays demonstrated a correlation between levels of mammaglobin and BU101 mRNA (Table 2 and Figure 2). Those breast tissue specimens that had high levels of one of this pair (mammaglobin or BU101) also had high levels of the other. Similarly, tissues with low or undetectable levels of one showed corresponding levels of the other.

Northern analysis for BU101 was performed on a variety of tissues. BU101 mRNA was observed in 1 of 2 breast, 0 of 2 colon, 0 of 2 lung, 0 of 1 ovary, 0 of 1 prostate, and 0 of 1 spleen (data not shown). RT-PCR was performed on breast, colon, and lung specimens for both mammaglobin and BU101. RT-PCR products of the correct size were observed for 9 of 10 breast, 0 of 5 lung, and 0 of 5 colon samples in both cases. The placental DNA control was negative since the forward and reverse primers were located on exons 2 and 3, respectively.

The Western blots of 9 breast tissues (reduced) probed for each sequence are shown in Figure 3. Those samples

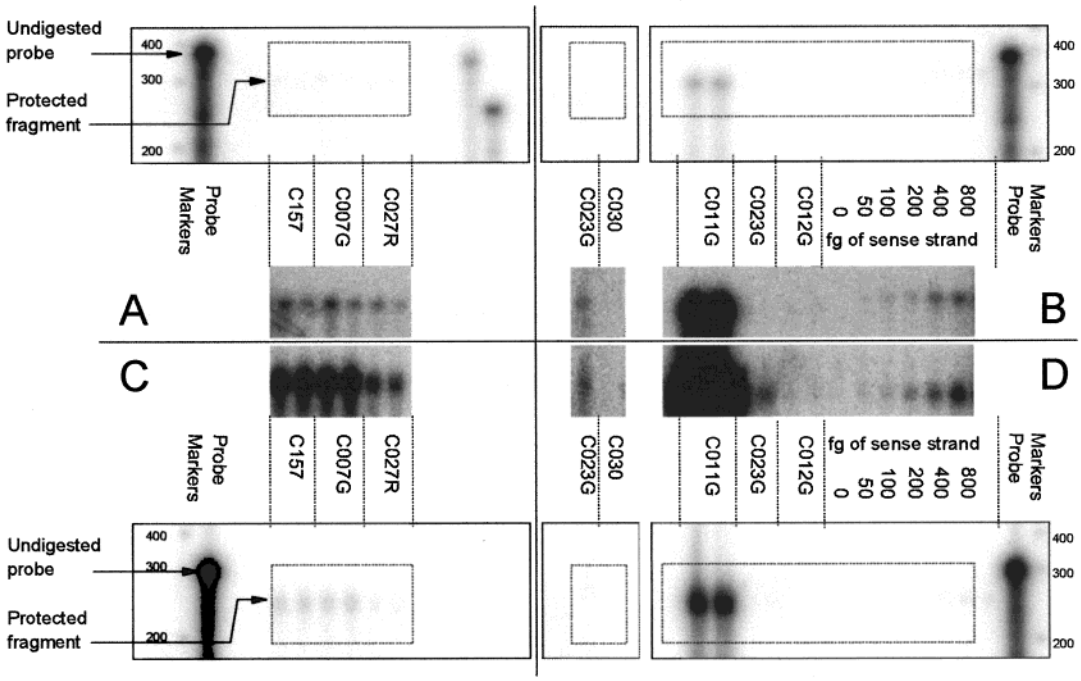

FIGURE 2: Ribonuclease protection assays. RNA extracts prepared from snap frozen tissue were hybridized overnight at 37 °C to ^{32}P-labeled probe (generated by in vitro transcription from the T7 promoter). The unhybridized RNA was then digested. The hybridized fragments were precipitated and electrophoresed in 6% polyacrylamide TBE and 8 M urea denaturing gels, exposed to storage phosphor screens, and analyzed using the STORM storage phosphor autoradiography system and ImagQNT software. (A) BU101-protected fragment (318 bp) in three normal breast tissue extracts. (B) BU101-protected fragment (318 bp) in four breast cancer tissues. C023G was run a second time due to the interference of the large signal from C011G. Also shown are the sense strand standards. (C) Mammaglobin-protected fragment (244 bp) in three normal breast tissue extracts. (D) Mammaglobin-protected fragment (244 bp) in four breast cancer tissues and the sense strand standards. The original image is shown at the top and bottom of the figure and shows the relative intensities of all of the bands. An electronically contrast and intensity enhanced region (enclosed by dotted lines in the original) showing the weaker signals is shown near the middle for clarity of reproduction.

containing detectable levels of BU101 also contain mammaglobin, and the levels of each protein appear to be roughly equivalent. BU101 has a predicted MW of 7.7 kDa, and the Western blot shows the corresponding species observed in tissue. The gene for mammaglobin is similarly small, resulting in a protein with a predicted MW of 8.4 kDa, but the protein contains two N-linked glycosylation sites. Each of these has the potential to undergo processing and result in the heterogeneous pattern of molecular masses observed spanning ~9−25 kDa. The bands on the blots developed with preimmune sera are recognized by the streptavidin−alkaline phosphatase conjugate and are believed to be endogenous biotinylated proteins.

Isolation and Identification of a Complex. The presence of a complex comprising BU101 and mammaglobin could best be shown by isolating the species from human tissue. Breast tissue was homogenized and fractionated by ion-exchange chromatography, specifically a Mono Q column run with 20 mM piperazine buffer, pH 6.0. These conditions were selected on the basis of the calculated isoelectric points of the individual sequences. The mature sequence of mammaglobin has a p*I* of 3.8 and a net charge of −10 at pH 6.0. The mature sequence of BU101 has a p*I* of 8.4 and a net charge of +2 at pH 6.0. Under these conditions, the sequences would be expected to separate during chromatography. Each fraction from the chromatography run was analyzed by dot blot with either anti-BU101.3 polyclonal antisera (Figure 4A) or anti-mammaglobin.1 polyclonal antisera (Figure 4B). Fractions 42−48 were positive for BU101 and mammaglobin. These fractions were then analyzed by Western blot under reducing and nonreducing conditions. Using the anti-BU101.3 polyclonal antisera (Figure 4C), BU101 is clearly visible under reducing conditions at approximately 7 kDa, the predicted molecular mass. Under nonreducing conditions, a higher molecular mass species is observed at approximately 24 kDa, and the 7 kDa band is lost. Using the anti-mammaglobin.1 polyclonal antisera (Figure 4D), mammaglobin, at approximately 18 kDa (under reducing conditions), is observed in the same fractions. Under nonreducing conditions the 18 kDa band is lost, and the ~24 kDa band is detected. This simple experiment demonstrates the presence of a disulfide-linked heterodimer consisting of mammaglobin and BU101 in human breast tissue.

The material in these fractions was further analyzed by gel filtration chromatography, specifically Superose 12 (Figure 5A). The Superose 12 fractions were analyzed by probing with anti-BU101.3 and anti-mammaglobin.1 polyclonal antisera. The resultant dot blots (Figure 5C,D) and the elution profile generated with the spot density data (Figure 5B) show that the same fractions were positive for both BU101 and mammaglobin. Again, BU101 and mammaglobin coelute, and the apparent molecular mass of the species observed was 45 kDa, as determined by calibration of the Superose column with appropriate molecular mass standards (inset, Figure 5A). This molecular mass is consistent with a heterotetramer composed of two disulfide-linked MamBu dimers in which mammaglobin is glycosylated. This composition is typical of the secretoglobin family of proteins.

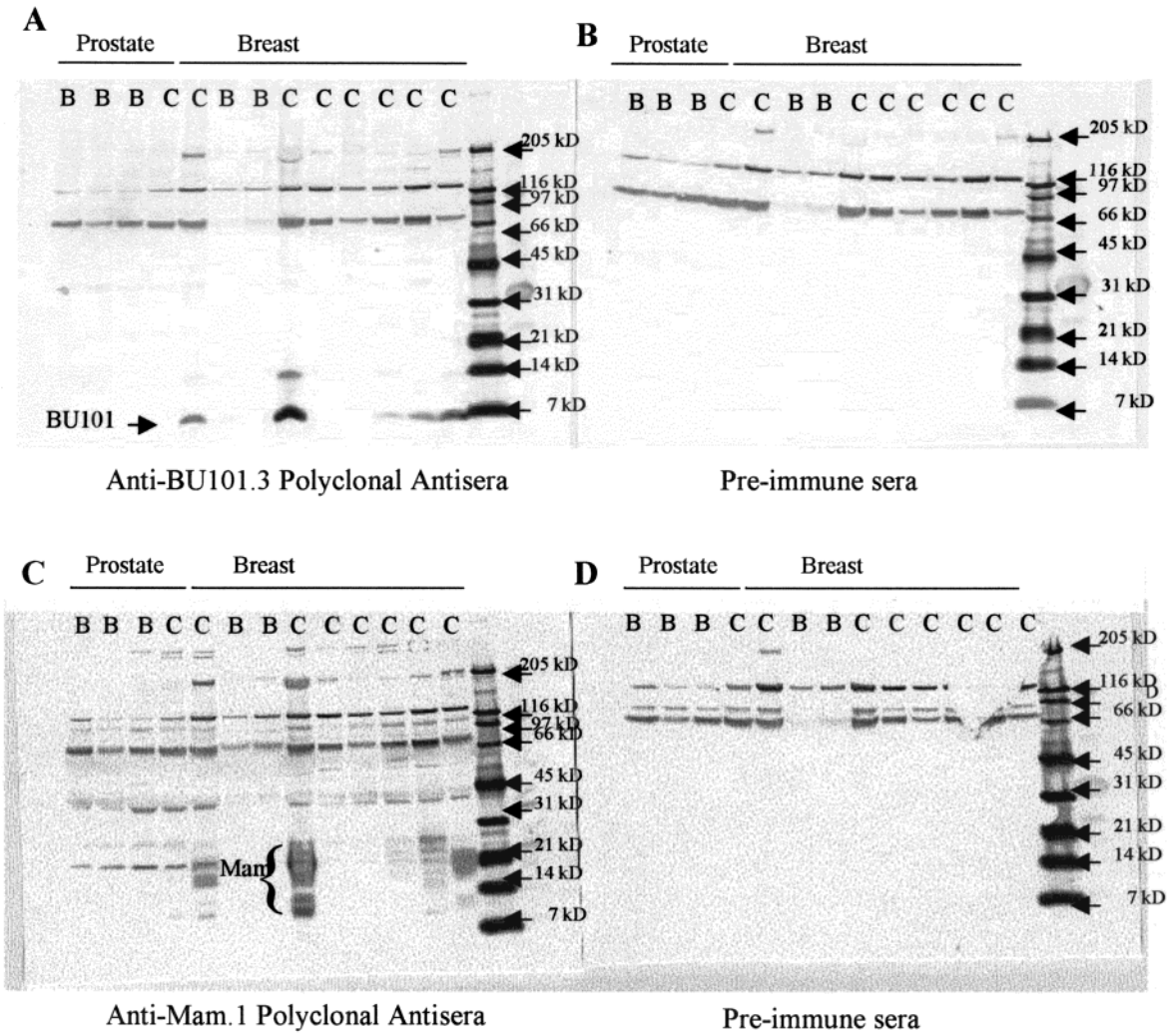

FIGURE 3: Western blot analysis of human tissue extracts. Protein extracts were prepared under reducing conditions from snap frozen tissue specimens. Western blots were developed with (A) anti-BU101.3 peptide polyclonal antisera, (B) preimmune sera for blot A, (C) anti-mammaglobin.1 peptide polyclonal antisera, and (D) preimmune sera for blot C. A different specimen is run in each lane. Specimens that have benign disease are designated with a B in the lane, and specimens with cancer are designated with a C in the lane. BU101 has a molecular mass of approximately 7 kDa. Mammaglobin has a molecular mass spanning 9−25 kDa.

Cell Model for the MamBu Complex. The BU101 and mammaglobin genes were obtained from Incyte Genomics (clones 603148 and 899895, respectively) and were cloned separately into the pcDNA 3.1 Myc/His expression vector (Invitrogen). Initially, these sequences were expressed transiently in HEK293 cells. Analysis of the supernatants and cell lysates revealed that transfection with the vector containing BU101 m/h (MW 11.4 kDa) alone resulted in accumulation of BU101 m/h inside the cell. Figure 6A, blot 1, shows a band at ~12 kDa present in the cell lysate (lane 1) which is absent in the supernatant (lane 2). Other bands at approximately 24 and 36 kDa in the lysate are believed to be multimers of BU101 m/h which were incompletely reduced during sample preparation. These same bands are observed in the nonreduced sample (lane 5 of blot 1) and are also detected by the anti-myc monoclonal antibody (blot 3). Comparison of the samples reduced (lane 1) and nonreduced (lane 5) with the anti-myc monoclonal antibody shows the increase in monomeric BU101 m/h in the reduced lane, but the reduction is incomplete.

Mammaglobin m/h expressed alone was secreted into the media (Figure 6A, blot 2, lane 3). Mammaglobin m/h has a predicted molecular mass of 12.1 kDa but has two N-linked glycosylation sites which may be modified and increase the predicted molecular mass to be consistent with the bands at approximately 17 and 24 kDa. Coexpression of BU101 m/h with mammaglobin m/h reversed BU101's inability to be secreted as observed in Figure 6A, blot 1, lane 4. The polyclonal antisera used for these experiments do not cross-react, as can be seen clearly in Figures 4 and 6B.

A stable cell line was created from HEK293 cells by transfecting the mammaglobin m/h and BU101 m/h genes and using antibiotic selection. Clonal selection was achieved with cloning cylinders, and a number of lines were grown and tested for production of both BU101 m/h and mammaglobin m/h. The highest expressing line, MB8, was chosen for further analysis. Mammaglobin m/h appeared to be more fully glycosylated, as judged by the relative abundances of the various bands on Western blots, when expressed from the MB8 cell line (Figure 6B, blot 2, reduced) compared to its transient expression (Figure 6A, blot 2, lane 3).

The supernatant harvested from the growth of these MB8 cells was purified using a nickel column (taking advantage of the His_6 tag placed at the C-terminus of both mammaglobin and BU101). Demonstration of the MamBu m/h complex involved reduction experiments followed by West-

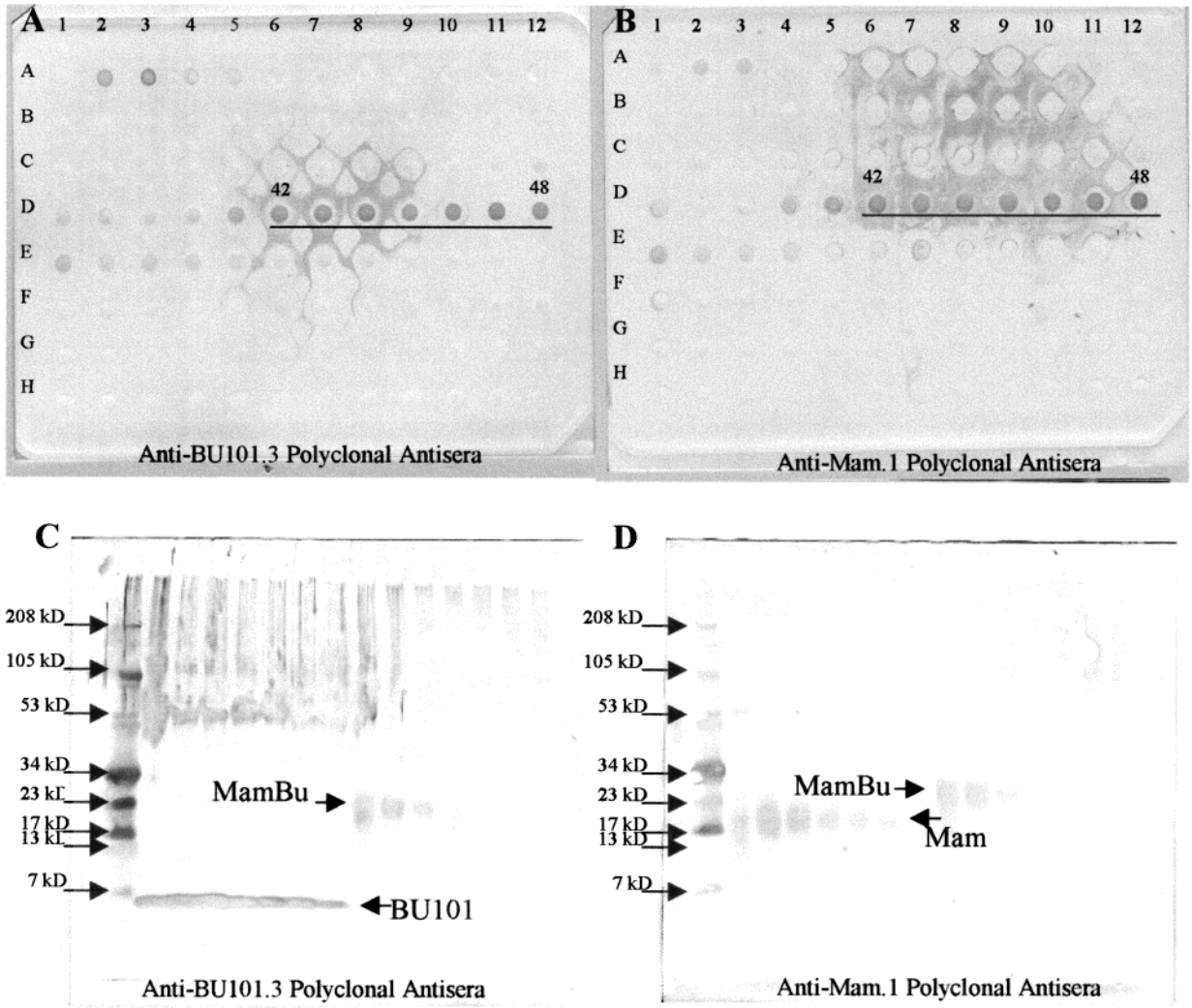

FIGURE 4: Isolation of complex from tissue by ion-exchange chromatography. Tissue extract was applied to a Mono Q column at pH 6. Material was eluted with increasing salt. Fractions were assayed for BU101 and mammaglobin using (A) anti-BU101.3 peptide polyclonal antisera and (B) anti-mammaglobin.1 peptide polyclonal antisera. Fractions 42−48 were prepared for SDS−PAGE under reducing and nonreducing conditions. Fractions run under reducing conditions are closest to the molecular mass markers. (C) Western blot of fractions 42−48 developed with anti-BU101.3 peptide polyclonal antisera. (D) Western blot of fractions 42−48 developed with anti-mammaglobin.1 peptide polyclonal antisera.

ern blot analysis (Figure 6B). The nickel-purified material was reduced and run in lane 1 of Figure 6B. BU101 m/h and mammaglobin m/h are observed at slightly higher molecular masses, 11 and 22 kDa, respectively, due to the presence of a 3.7 kDa myc/his tag on each protein. These bands are replaced by the disulfide-linked MamBu m/h dimer observed at 33 kDa by all antisera under nonreducing conditions (Figure 6A, all blots).

Further analysis of the MamBu m/h complex involved ion-exchange chromatography and gel filtration chromatography, as was done with the breast tissue extract. The MamBu m/h complex, having a p*I* of 5.9, eluted from the Mono Q column at 60 mM NaCl compared to 150 mM NaCl for the MamBu complex (p*I* 4.4). Gel filtration chromatography of the myc/his-tagged complex resulted in a native molecular mass of 60 kDa (Figure 5B). Again, these results support the structural model of two disulfide-linked heterodimers existing as a noncovalently associated heterotetramer. The molecular mass of this recombinantly produced MamBu m/h complex correlates with that observed from breast tissue (45 kDa) with the addition of four myc/his tags (15 kDa).

Antibodies were developed against peptides from the sequence of either BU101 or mammaglobin (Figure 7). On the basis of sequence alignment with rabbit uteroglobin and rat CC10kDa proteins, the four putative helices of BU101 and mammaglobin, involved in the uteroglobin fold (*40*), may be identified. The peptides were designed to span one or more of these potential helices with the aspiration that the antisera would be useful in recognizing the native material. For BU101, peptides 1, 2, 4, and 5 were designed to represent the four putative α-helices and peptide 3, the *β*-turn between helices 2 and 3. For mammaglobin, peptide 1 represents helix 2−turn−helix 3, peptide 2 represents helix 4, and peptide 5 represents helix 1. Both peptides 1 and 2 encompass an N-linked glycosylation site for mammaglobin.

All peptides used to generate polyclonal antisera to BU101 and mammaglobin gave a good antigenic response against the peptide immunogen. Each polyclonal antiserum was then tested for its ability to bind the MamBu m/h complex. Antisera generated against the *β*-turn region of BU101 recognized the complex, as did antisera raised against helices

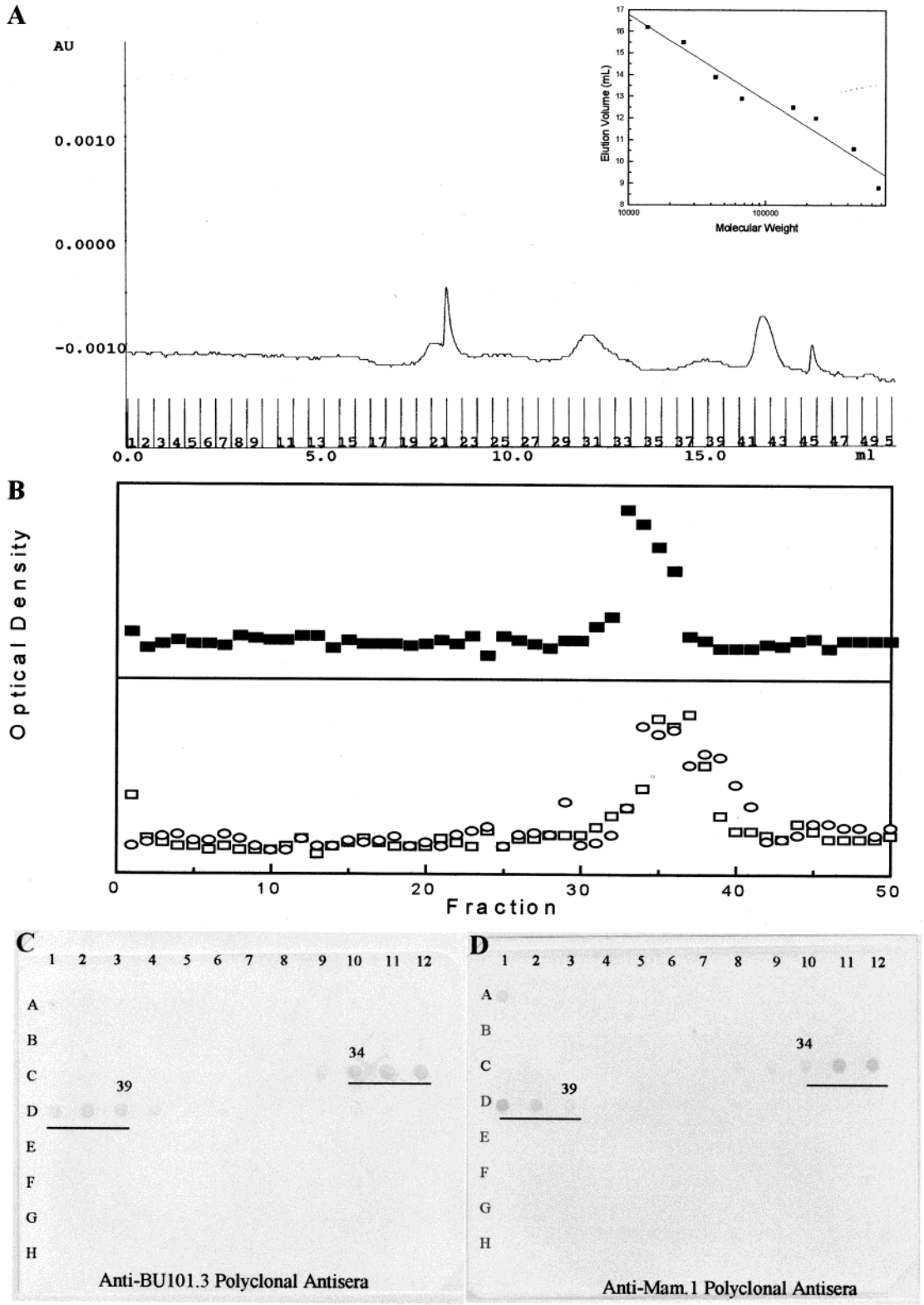

FIGURE 5: Isolation of complex by size exclusion chromatography. (A) Elution profile at 280 nm of the partially purified tissue extract (fractions 42−48 from the Mono Q column pooled and concentrated) from the Superose 12 column. The calibration curve using Pharmacia molecular mass standards is shown in the inset. (B) Elution profile showing the immunological reactivity of each fraction from the Superose 12 column. The average optical density of a 2 mm × 2 mm area representing each spot (fraction) was measured, and the background value (no spot) was subtracted. Open squares and open circles represent mammaglobin- and BU101-positive fractions from the tissue extract. The peak (fraction 36) has an elution volume of 14.4 mL, which corresponds to a molecular mass of ~45 kDa. Solid squares represent myc-positive fractions from the elution of MamBu myc/his. The peak (fraction 34) has an elution volume of 13.6 mL, which corresponds to a molecular mass of ~60 kDa. (C) Dot blot of fractions assayed for BU101 using anti-BU101.3 peptide polyclonal antisera. (D) Dot blot of fractions assayed for mammaglobin using anti-mammaglobin.1 peptide polyclonal antisera.

2 and 3, with diminishing recognition by antisera raised against peptides closer to the terminii (Figure 7). The same was true for mammaglobin. Peptide Mam.1, encompassing the β-turn, recognized the MamBu m/h complex, unlike the

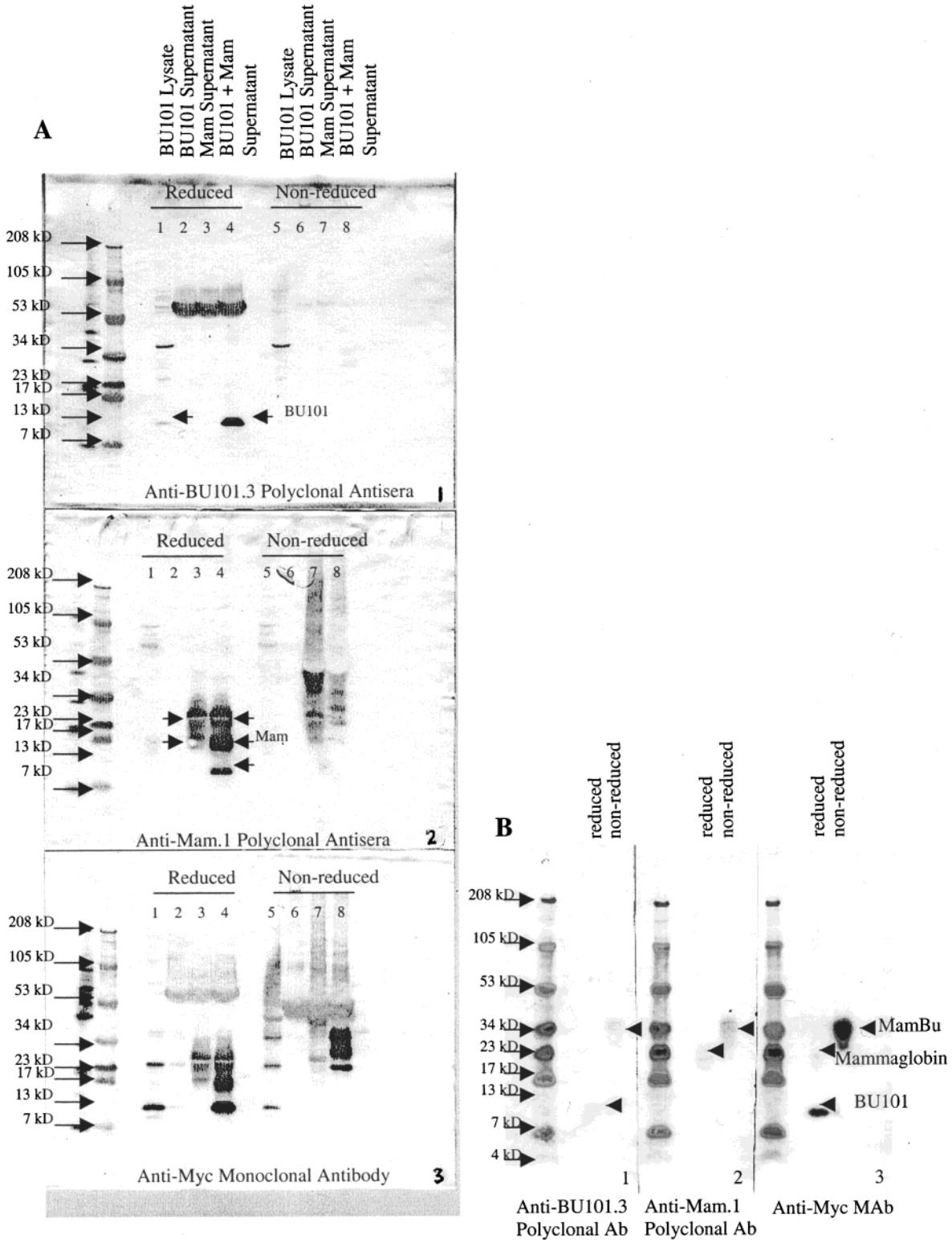

FIGURE 6: Recombinant expression of mammaglobin m/h and BU101 m/h. (A) Transient expression of BU101 m/h, mammaglobin m/h, and MamBu m/h. For all blots, lane 1 is the cell lysate from transient transfection of BU101 m/h, reduced lane 2 is the supernatant from transient transfection of BU101 m/h, reduced, lane 3 is the supernatant from transient transfection of mammaglobin m/h, reduced, lane 4 is the supernatant from transient transfection of BU101 m/h and mammaglobin m/h, reduced, lane 5 is the cell lysate from transient transfection of BU101 m/h, not reduced, lane 6 is the supernatant from transient transfection of BU101 m/h, not reduced, lane 7 is the supernatant from transient transfection of mammaglobin m/h, not reduced, and lane 8 is the supernatant from transient transfection of BU101 m/h and mammaglobn m/h, not reduced. Blot A was developed with anti-BU101.3 polyclonal antisera. Blot B was developed with anti-mammaglobin.1 polyclonal antisera. Blot C was developed with an anti-myc monoclonal antibody. (B) Stable expression of mammaglobin m/h and BU101 m/h from the MB8 cell line. For all blots, lane 1 is the supernatant from MB8 cell line, reduced, and lane 2 is the supernatant from MB8 cell line, not reduced. Blot 1 was developed with anti-BU101.3 polyclonal antisera. Blot 2 was developed with anti-mammaglobin.1 polyclonal antisera. Blot 3 was developed with an anti-myc monoclonal antibody.

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                                    Helix 1     Helix 2   Turn   Helix 3          Helix 4
                                   HHHHHHHHHHH  HHHHHHHHHHHHTTTTHHHHHHHHHHHHHHH  HHHHHHHHHHHHHHH
Peptide
BU101  mklsvclllvtlalccyqanaEFCPALVSELLDFFFISEPLFKLSLAKFDAPPEAVAAKLGVKRCTDQMSLQKRSLIAEVLVKILKKCSV
.1     --------------------EFCPALVSELLDFFF
.2     -----------------------------------ISEPLFKLSLAKFDAc
.3     ------------------------------------------SLAKFDAPPEAVAAKc
.4     ----------------------------------------------------EAVAAKLGVKRCTDQ
.5     ------------------------------------------------------------------MSLQKRSLIAEVLVKc

                                  Helix 1      Helix 2    Turn   Helix 3          Helix 4
                                 HHHHHHHHHHH  HHHHHHHHHHHHHTTTTHHHHHHHHHHHHHH  HHHHHHHHHHHHHHH

Mam mkllmvlmlaalsqhcyagSGCPLLENVISKTINPQVSKTEYKELLQEFIDDNATTNAIDELKECFLNQTDETLSNVEVFMQLIYDSSLCDLF
.1  ---------------------------------PQVSKTEYKELLQEFIDDNATTNAIDELKECFL
.2  ------------------------------------------------------------------LNQTDETLSNVEVFMQLIYDSSLCDLF
.5  -------------------SGCPLLENVISK
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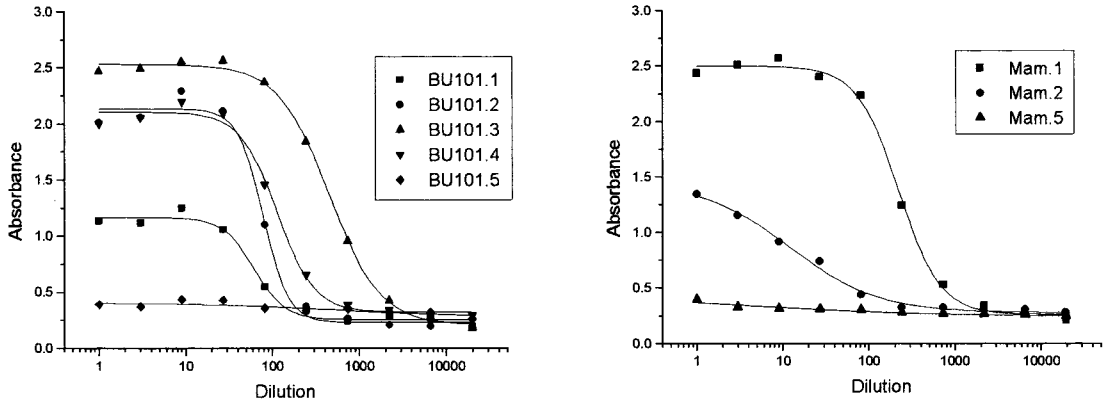

FIGURE 7: Synthetic peptides and recognition of complex by peptide polyclonal antisera. The complete sequences of BU101 and mammaglobin are shown. The mature protein sequence is shown in capital letters, and the signal sequence is shown in lower case letters. The positions of the four helices and the β-turn are indicated and were derived by sequence comparison to rabbit uteroglobin and human CC10kDa protein, which have published crystal structures. The peptide indicated with bold lettering spans the β-turn, and antisera raised with these peptides showed the best binding characteristics to the MamBu complex. ELISA assay detecting the complex from the MB8 cell line. Polyclonal antisera were diluted 1:1000. The complex was coated at various dilutions.

antisera raised against the N-terminus (Mam.5) or the C-terminus (Mam.2).

DISCUSSION

Much attention has been given recently to mammaglobin because of its specificity for breast tissue. The data from the LifeSeq EST database support this claim. BU101 is a second secretoglobin that was found to be abundant in breast tissue. It does not exhibit as high a specificity as mammaglobin as it was found not only in 12 breast libraries but also in 6 uterus libraries and in 2 prostate libraries. Although its expression profile extends beyond breast into the reproductive organs, no other tissue type was found to contain comparable levels of BU101 in this database.

Tissue types expressing BU101 (in the database) also express other secretoglobin members. In breast tissue, mammaglobin is expressed with BU101. In prostate tissues, mammaglobin B is observed as well as BU101, and in uterine tissues, mammaglobin, mammaglobin B, BU101, and ESSBF I are all expressed, suggesting the possibility of other secretoglobin complexes.

Analysis of breast tissue specimens, both neoplastic and normal, using RT-PCR, ribonuclease protection assays, and Western blots showed a consistent correlation between the expression level of BU101 and the expression level of mammaglobin. Of the 20 breast specimens analyzed, not one was found that expressed mammaglobin and not BU101, or vice versa.

Examination of the family of secretoglobins determined that each known member is involved in a protein complex, either with another copy of itself (as homodimer) or with other members of the family (as heterotetramers). The first group includes the members of the Clara cell 10 kDa proteins including mouse, rat, and human CC10kDa proteins as well as rabbit and hare uteroglobin (*44*−*49*). These sequences contain two highly conserved cysteine residues, one near the amino and one near the carboxyl end of the sequence, which are involved in intermolecular disulfide bonds linking the two chains in a head-to-tail orientation. The second cluster of secretoglobins includes mammaglobin, mammaglobin B, lipophilin A, BU101, ESSBF I, rat prostatein, and cat major allergen.

Rat prostatein and cat major allergen are known to form protein complexes of multiple protein chains whereas mammaglobin, mammaglobin B, lipophilin A, BU101, and ESSBF I are individual proteins with as yet unknown quaternary structure. Rat prostatein is a tetramer of three protein chains, C1, C2, and C3, with C3 being present twice. Each of these chains contain three cysteines in their mature form, of which two are homologous to the two conserved cysteines in the CC10kDa family. Biochemical characterization of the complex revealed that it is composed of two

disulfide-linked subunits, C1:C3 and C2:C3, which then associate noncovalently to form the tetramer (*49*).

A similar composition was found for the major cat allergen, Fel d I, which consists of two noncovalently associated subunits, each composed of two disulfide-linked proteins. Unlike rat prostatein, there are just two chains involved in the complex, Ch1 and Ch2. However, variants do exist for both genes, creating mixtures of Ch1a and Ch1b with Ch2L (long) and Ch2S (short). There appears to be tissue-dependent expression of these variants. In both the salivary glands and the skin, Ch1a is preferentially expressed over Ch1b, whereas Ch2L is preferentially expressed in the salivary glands, and Ch2S is preferentially expressed in the skin (*50*).

The clustering of mammaglobin and mammaglobin B with this branch of the family suggests that they may be involved in higher order complexes with one or more proteins. Indeed, there are differences between those sequences that form homodimers and those that form heterotetramers: those that form heterotetramers have an extra cysteine residue located at position 44. Using the crystal structure of the rabbit uteroglobin homodimer, we were able to model using Homology (Silicon Graphics) the putative structure of a BU101 and mammaglobin heterodimer. The cysteine at position 44 was located at the interface, in proximity to form an intermolecular disulfide bond. Whether this disulfide link results in a functional difference between the homodimeric and heterotetrameric secretoglobins remains to be seen. Amino acids that are conserved among the sequences are predominantly hydrophobic with the exception of lysine at position 42. It would appear that this residue is critical to the function of all secretoglobins.

Evidence for a heterotetrameric MamBu complex was provided from human breast tissue. Demonstration of a disulfide-linked heterodimer of 24 kDa was accomplished using ion-exchange chromatography followed by Western blot analysis and reduction experiments, whereas the native molecular mass of 45 kDa was determined by gel filtration chromatography. The most likely explanation for this is that the species exists as a $mammaglobin_2BU101_2$ tetramer in solution. However, other more esoteric possibilities, such as an exotic tetramer consisting of a mammaglobin−BU101 dimer interacting with an as yet unknown species to form the 45 kDa species, cannot be ruled out.

A model of this complex was produced recombinantly from HEK293 cells. A stable transfectant expressing both mammaglobin and BU101 (each containing a myc/his tag at the C-terminus) was produced. Analysis of this material provided the same compositional results as the complex from breast tissue. The disulfide-linked dimer composed of mammaglobin m/h and BU101 m/h was demonstrated by SDS−PAGE. The molecular mass (33 kDa) was elevated from the added weight of the myc/his tags. The native material ran as a 60 kDa species by gel filtration chromatography, consistent with the heterotetramer. The 60 kDa species was shown to consist only of mammaglobin m/h and BU101 m/h by SDS−PAGE and Coomassie Blue staining.

The finding that mammaglobin is involved in a tetrameric complex with BU101 is consistent with the structural homology of the secretoglobin family and the tissue distribution information provided by the Incyte Genomics LifeSeq database. The identification of the MamBu complex provides evidence for a model in which human secretoglobin proteins form heteromeric complexes, which may demonstrate tissue-specific expression. Furthermore, each secretoglobin may be involved in more than one complex. This model may prove of great utility as investigators continue to evaluate mammaglobin for its potential clinical applications as a diagnostic marker for breast cancer management as well as a novel target for breast cancer treatment (*33*−*39*).

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