Butyrophilin Is Expressed in Mammary Epithelial Cells from a Single-sized Messenger RNA as a Type I Membrane Glycoprotein*

(Received for publication, July 9, 1997, and in revised form, October 30, 1997)

Lisa R. Banghart‡§, Clayton W. Chamberlain‡§, Jorge Velarde‡, Igor V. Korobko‡¶, Sherry L. Ogg‡, Lucinda J. W. Jack§, Vikram N. Vakharia†, and Ian H. Mather‡**

From the ‡Department of Animal and Avian Sciences, and the ||Center for Agricultural Biotechnology, University of Maryland, College Park, Maryland 20742

We investigated the expression of butyrophilin in eukaryotic cells with a view to determining the number of mRNA species, the incorporation of the peptide chain into microsomes, and the topology of the processed protein in biological membranes. Butyrophilin is synthesized from a single sized mRNA in both bovine and murine lactating mammary tissue and associates with microsomal membranes with a type I topology (Nexo-Cyto) via a single hydrophobic anchor in the middle of the sequence. Several isoelectric variants of the protein were detected in cellular membranes from lactating bovine mammary tissue and in the milk-fat-globule membrane. We found no evidence for soluble forms of butyrophilin in postmicrosomal supernatants. The 66-kDa protein appears to be subjected to limited proteolysis, giving rise to a 62-kDa fragment lacking the C terminus and to other more minor fragments of lower Mr in the milk-fat-globule membrane. Antipeptide antibodies to epitopes within the N- and C-terminal domains were used to show that butyrophilin retains a type I topology in plasma membranes when expressed in insect cells from a baculovirus vector, and in secreted milk-fat globules. These data do not agree with previous suggestions that butyrophilin may exist in cytoplasmic soluble forms, or be reorganized in the plane of the lipid bilayer during secretion in lipid droplets from mammary cells. The results are discussed with reference to the role butyrophilin may play as the principal scaffold for the assembly of a complex with xanthine oxidase and other proteins that functions in the budding and release of milk-fat globules from the apical surface during lactation.

Several years ago we cloned a cDNA encoding the major milk-fat-globule membrane (MFGM)† protein, butyrophilin (BTN) (1) with a view to understanding the function of this mammary-specific protein in milk-fat secretion (for reviews of this process, see Refs. 2–4). From the derived amino acid sequence we predicted that BTN is an integral protein with a single membrane anchor and a glycosylated exoplasmic N terminus (type I orientation) (1). Subsequent comparisons with more recently cloned cDNAs have established that BTN is a member of the immunoglobulin superfamily (5) with two N-terminal immunoglobulin domains, one of the intermediate type (IgI) (6) toward the N terminus and one of the constant C1 type (7) toward the membrane bilayer (8). A large proportion of the C-terminal region comprises the RFP or B30.2 domain, a sequence which is present in a subfamily of zinc-finger proteins (9, 10), and a group of recently identified BTN-like genes (11, 12).

Several workers have proposed that BTN may function as an integral receptor for cytoplasmic fat droplets and that budding of the droplets at the cell surface is initiated by interactions between the cytoplasmic tail of BTN and other proteins, notably the redox enzyme xanthine oxidase (1, 13–16). However, the assumption that BTN behaves as an integral protein in mammary epithelial cells has been confounded by several observations. There are persistent reports of “soluble” forms of BTN in postmicrosomal supernatant fractions of lactating mammary tissue. BTN has been identified by immunoblotting techniques associated with cytoplasmic lipid droplets (17), which are the presumed precursors of secreted milk-fat globules (17, 18), and with a recently described lipoprotein particle which also contains xanthine oxidase, fatty acid synthase, GTP-binding proteins, and lipids (19).2 BTN is secreted in association with the MFGM in several forms which include not only the isoelectric variants expected for a heterogeneous N-linked glycoprotein (22, 23), but also smaller forms (22, 24, 25) which could be the soluble products of alternatively spliced mRNAs lacking the membrane anchor.

Morphological analysis of the structure of MFGM has also yielded ambiguous results. In previous immunocytochemical studies, antibodies prepared against purified bovine or guinea pig BTN predominantly decorate the protein coat (24, 26) on the cytoplasmic face of the MFGM (13, 14), suggesting that BTN is largely located there possibly as a “peripheral” cytoskeletal-like component (13). Furthermore, selective removal of the

* This work was supported by Grants C-RFP91-002 and ANSC-93-078 from the Competitive Grants Program of the Maryland Agricultural Experiment Station, Fogarty International Fellowship 1 F06TW1508-01 from the National Institutes of Health, and Grant DCB 9119264 from the National Science Foundation (to I. H. M.). 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.

‡ Contributed equally to the results of this work.
§ Present address: Institute of Gene Biology, 111334, Vavilov Str. 34/5, Moscow, Russia.
¶ To whom correspondence and reprint requests should be addressed: Dept. of Animal and Avian Sciences, University of Maryland, College Park, MD 20742. Tel.: 301-405-1380; Fax: 301-314-9059; E-mail: im2@email.umd.edu.
** To whom correspondence and reprint requests should be addressed: University of Maryland, College Park, MD 20742. Tel.: 301-405-1380; Fax: 301-314-9059; E-mail: im2@email.umd.edu.
† The abbreviations used are: MFGM, milk-fat-globule membrane; BTN, butyrophilin; IgI, intermediate type immunoglobulin domain; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction.

2 In preliminary experiments we also apparently identified a soluble form of BTN in postmicrosomal supernatants by immunoblotting techniques (20). These results could not be repeated when Tween 20 was omitted from the washing buffers used for immunoblotting, and subsequently when a different batch of peroxidase-conjugated second antibody was used to detect bound antibody on the blots, either in the presence or absence of Tween 20. This component may have been the heavy chain of endogenous immunoglobulins identified as a nonspecific band on blots of immunoprecipitates developed with chemiluminescence reagents by other workers (21).
lipid bilayer from fat globules by physical agitation leaves a substantial amount of BTN associated with the droplet surface in coat structures (17), which is not consistent with the predicted integral properties of BTN (1). These data parallel the results of freeze-fracture electron microscopy from several laboratories which show that the fracture faces of MFGM are depleted of the intramembranous particles typically associated with integral proteins (27–29).

The situation has been further complicated by the discovery of at least six BTN-like genes in the major histocompatibility complex which are widely expressed in many tissues, possibly including the mammary gland (11, 12). Some of these BTN-like proteins (with amino acid identities to BTN approaching 50%) appear to be synthesized from alternatively spliced mRNAs (11). This raises the possibility that earlier studies employing polyclonal antibody to purified BTN have mistakenly identified other BTN-like proteins from this gene family in mammary tissue.

To resolve these issues, we have reinvestigated the expression of BTN in mammary cells using a combination of immunochemical and molecular approaches with epitope-specific antibodies. The results indicate that BTN is synthesized as an integral membrane protein from a single size class of mRNA (11) folds in the exoplasmic domain and the B30.2 region in the cytoplasmic domain are indicated.

EXPERIMENTAL PROCEDURES

Materials

Trypsin type XIII and soybean trypsin inhibitor type 1-S were purchased from Sigma. Moloney murine leukemia virus and avian myeloblastosis virus reverse transcriptase, Taq DNA polymerase, and in vitro protein translation kits were from Promega Corp. (Madison, WI). Neuraminidase isolated from *Arthrobacter ureafaciens* was from Boehringer Mannheim. The pCRII and pBlueBacII vectors were obtained from Stratagene (La Jolla, CA). Materials for insect cell culture were from JRH BioSciences (Lenexa, KS). Materials for electrophoresis and immunoblotting including goat anti-rabbit IgGhorseradish peroxidase were from Bio-Rad. Other antibodies were from ICN Biochemicals (Costa Mesa, CA). Primers were custom ordered from either Midland Certified Reagent Co. (Midland, TX) or Life Technologies, Inc. (Gaithersburg, MD). Radionucleotides were from either DuPont (Boston, MA) or Amersham Corp. (Arlington Heights, IL). RIBI was purchased from Immunotech. Research Inc. (Hamilton, MT).

Methods

Preparation of Milk and Tissue Fractions

Washed fat globules for topological studies were prepared from fresh uncooled milk (pooled from three cows) by a method designed to limit physical damage to the outer surface of the droplets (30). Bovine MFGM was prepared as described (31). Soluble (whey) fractions of bovine milk were obtained by centrifuging 200 ml of the pooled skim milk from three cows at 100,000 × g for 2 h at 4 °C. The residual layer of fat was removed by aspiration and the opalescent subnatant carefully separated from the white pellet of casein. Cell fractions were prepared from mammary glands obtained from lactating cows at slaughter using either a Brinkman PT 10/35 homogenizer (Westbury, NY) or a Sorvall Omnimixer, essentially as described (25).

Preparation of Antibodies

Antipeptide antibodies were prepared with peptides synthesized by Dr. Thomas Kempe (Department of Chemistry and Biochemistry, University of Maryland, College Park). For the exo-1 and cyto-1 antibodies (Fig. 1), antigen was treated with immunogen enhancer (CYTImmune Sciences, Inc., College Park, MD) (32) and mixed 1:1 (v/v) with either RIBI or Freund’s complete adjuvant. Antigen/adjuvant mixtures were injected subcutaneously into multiple sites on the back of New Zealand White rabbits. For the exo-2 and cyto-2 antibodies (Fig. 1), antigen was coupled to keyhole limpet hemocyanin (33), mixed with Freund’s complete adjuvant, and injected into the thigh lymph nodes and subcutaneously into the back of the neck. Subsequent injections of antigen mixed with either Freund’s incomplete adjuvant or Tris-buffered saline (140 mM NaCl in 10 mM Tris/HCl buffer, pH 7.4) were administered in the subcapsulam, the thigh muscles, and the ear vein.

Antibody to bovine BTN was prepared by using antigen purified from MFGM by preparative SDS-polyacrylamide gel electrophoresis. The purified protein was coupled to keyhole limpet hemocyanin (33) by standard procedures. With the exception of exo-1, antibody was purified by adsorption to, and elution from, the respective antigen (peptide or protein) covalently coupled to keyhole limpet hemocyanin (33), mixed with Freund’s complete adjuvant, and injected into the thigh lymph nodes and subcutaneously into the back of the neck. Subsequent injections of antigen mixed with either Freund’s incomplete adjuvant or Tris-buffered saline (140 mM NaCl in 10 mM Tris/HCl buffer, pH 7.4) were administered in the subcapsulam, the thigh muscles, and the ear vein.
For immunoblots, proteins separated by one- or two-dimensional gel electrophoresis were transferred to nitrocellulose (36) and the blots developed according to the procedures of Bio-Rad. Antibody-peroxidase conjugates were detected by enhanced chemiluminescence (Amersham).

In some immunoblots Tween 20 was omitted from the washing buffers.

In Vitro Synthesis and Translation of Butyrophilin mRNA

The full-length cDNA clone BT1 (1) encoding bovine BTN was inserted into Bluescript and sense mRNA was synthesized as described by Zerial et al. (37). The synthesized mRNA was translated in the presence of [35S]methionine (1,000 Ci/ml) (30 μCi), a mixture of 19 unlabeled amino acids (without methionine) and rabbit reticulocyte lysate, using the system of in vitro transcription kit. In some experiments, microsomal membranes from dog pancreas, pretreated with 50 mM EGTA (38) were incubated overnight at 4 °C in an end-over-end stirrer.

Translation mixtures prepared as described above, either in the presence or absence of microsomal membranes, were treated with proteinase K at a final concentration of 0.5 mg/ml for 1 h on ice. In some experiments, the membranes were permeabilized by the addition of Triton X-100 to a final concentration of 1% (v/v). Proteolysis was stopped by the addition of 12.5 mM phenylmethylsulfonyl fluoride and further incubation for 5 min at 0 °C.

Immunoprecipitation of BTN Translated in Vitro

Translation mixtures (25 μl) prepared in the presence of microsomal membranes were incubated overnight in an end-over-end stirrer with 20 μl of antipeptide antibody or control immunoglobulin at 4 °C. The microsomes were washed twice with 200-μl aliquots of Tris-buffered saline, recovered by centrifugation at 100,000 × g in a Sorvall RC120 mini-ultracentrifuge at 4 °C, and resuspended in 50 μl of Tris-buffered saline. Goat anti-rabbit IgG was added at a final dilution of 1:50 and a final volume of 100 μl in Tris-buffered saline. Immunoprecipitated membranes were recovered by centrifugation at 1,000 × g after incubating overnight at 4 °C in an end-over-end stirrer.

Preparation of Recombinant Baculovirus, vBT

The sequence of DNA encompassing nucleotides 43 through 1655 of clone BT1 (1) was amplified using the PCR, and primer pairs 5′-CCCAAGAAGCTCTAGGCGACGCTT-3′ and 5′-TGGCTGGAGTATSCTTACGACCC-3′ to prepare a 1.6-kilobase fragment encoding 99- and 3′-terminal amino acids (without methionine) and rabbit reticulocyte lysate, using the system of in vitro transcription kit. In some experiments, microsomal membranes from dog pancreas, pretreated with 50 mM EGTA (38) were incubated overnight at 4 °C in an end-over-end stirrer.

Translation mixtures prepared as described above, either in the presence or absence of microsomal membranes, were treated with proteinase K at a final concentration of 0.5 mg/ml for 1 h on ice. In some experiments, the membranes were permeabilized by the addition of Triton X-100 to a final concentration of 1% (v/v). Proteolysis was stopped by the addition of 12.5 mM phenylmethylsulfonyl fluoride and further incubation for 5 min at 0 °C.

Immunoprecipitation of BTN Translated in Vitro

Translation mixtures (25 μl) prepared in the presence of microsomal membranes were incubated overnight in an end-over-end stirrer with 20 μl of antipeptide antibody or control immunoglobulin at 4 °C. The microsomes were washed twice with 200-μl aliquots of Tris-buffered saline, recovered by centrifugation at 100,000 × g in a Sorvall RC120 mini-ultracentrifuge at 4 °C, and resuspended in 50 μl of Tris-buffered saline. Goat anti-rabbit IgG was added at a final dilution of 1:50 and a final volume of 100 μl in Tris-buffered saline. Immunoprecipitated membranes were recovered by centrifugation at 1,000 × g after incubating overnight at 4 °C in an end-over-end stirrer.

Preparation of Recombinant Baculovirus, vBT

The sequence of DNA encompassing nucleotides 43 through 1655 of clone BT1 (1) was amplified using the PCR, and primer pairs 5′-CCCAAGAAGCTCTAGGCGACGCTT-3′ and 5′-TGGCTGGAGTATSCTTACGACCC-3′ to prepare a 1.6-kilobase fragment encoding the entire open reading frame of BTN with Nhel and BglII restriction sites at the 5′- and 3′-ends, respectively. This fragment of DNA, after cloning into the pCR1 vector, was directionally subcloned into the baculovirus transfer vector pBlueBac II to obtain plasmid pBTBacII.

Spodoptera frugiperda (Sf9) insect cells were cultured in Hink’s TNM-FH-medium (39) and cells were supplemented with 10% fetal calf serum, 50 μg/ml gentamicin sulfate, and 2.5 μg/ml amphotericin B in either Falconware tissue culture flask at 28 °C or spinner flasks (Belco) at room temperature. Cells grown in spinner flasks contained 0.1% Pluronic F-68 in addition to the above supplemented medium. A recombinant baculovirus, with the BTN cDNA under the control of the polyhedrin promoter, was generated by homologous recombination as described (40). Briefly, 2 × 107 Sf9 cells were cotransfected with 2 μg of pBTBacII DNA and 1 μg of wild-type Autographa californica nuclear polyhedrosis virus DNA. Recombinant virus was isolated by blue/white selection (40) and plaque purified three times. The presence of the BTN cDNA was confirmed by PCR and the recombinant virus designated vBT.

Determination of the Orientation of BTN in Sf9 Cells

Sf9 cells were grown on microscope coverslips in Hink’s medium (39) supplemented with 5% (w/v) fetal calf serum. Individual wells of 12-well tissue culture plates were seeded with 0.9 × 106 Sf9 cells in 2 ml of medium. After 24 h growth at room temperature, the cells were infected with 0.3 ml of 10^7 plaque-forming units/ml vBT. Twenty-four hours post-infection, intact cells on coverslips were washed twice with 50 mM EGTA (38) were added at a final concentration of 4.0 A(280 nm) units/ml. Mixtures were dissolved in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Gels were soaked in ENHANCE (Amersham), dried, and exposed to x-ray film.

Expression of Butyrophilin in Mammary Tissue

Total RNA was prepared from lactating bovine mammary tissue obtained at slaughter from the USDA Beltsville abattoir (Beltsville, MD) or from pooled mammary tissue from Balb/c mice (3 mice from day 1, or 3 mice from day 14 of lactation). The RNA was reverse transcribed into cDNA by incubation with either Moloney murine leukemia virus or avian myeloblastosis virus reverse transcriptase and oligo dT primers at 37 or 42 °C, respectively, for 15 min, following the protocol described in the Perkin-Elmer RT-PCR kit (Perkin-Elmer Corp., Branchburg, NJ). Specific regions of the synthesized cDNA, indicated by Arabic numbers in Fig. 2, were amplified by the PCR using Tag DNA polymerase and the primer pairs listed in the legend to Fig. 2. The amplified products obtained with primer pairs 5′ (bovine) and 10′ (mouse) were sequenced to confirm identity (see also Ref. 41).

Topological Analysis of BTN in Fat Globules and MFGM

Washed cream and MFGM samples were suspended in PBS in 0.5-ml aliquots in 1.5-ml Eppendorf tubes with or without trypsin. Each mixture contained 1.0 mg of protein and 0–10 μg of trypsin. Samples were incubated at 35 °C for 30 min and proteolysis was stopped by the addition of 250 μg of soybean trypsin inhibitor in 100 μl of PBS on ice. The cream and MFGM samples were washed once with PBS containing trypsin inhibitor (1 mg/ml). Fat globules were separated at 5 °C by centrifuging in a bench-top Eppendorf microcentrifuge set at maximum speed for 5 min. The bottom of the tube was then cut with a razor blade and the subnatant carefully removed from the packed cream layer with a micropipette. Cream was quantitatively recovered from the tube by centrifuging the contents into a secondary 1.5-ml Eppendorf tube containing within a 15-ml centrifuge tube. MFGM was recovered by centrifugation at 100,000 × g in a Sorvall RC120 mini-ultracentrifuge. Cream and MFGM samples were prepared for SDS-polyacrylamide gel electrophoresis and immunoblotting by heating in SDS sample buffer at 95 °C for 3 min.

Protein Determination

Protein was assayed, either with the bicinchoninic acid reagent (42), or by the method of Lowry et al. (43) using bovine serum albumin as a standard. Soluble whey fractions from milk were dialyzed against 62.5 mM Tris/HCl buffer, pH 6.8, overnight to remove lactose before protein analysis.

RESULTS

We first investigated the expression of BTN in mammary cells by RT-PCR to establish whether BTN is synthesized from a single sized mRNA or from alternatively spliced mRNAs. Total RNA from the mammary tissue of one lactating cow and two samples of pooled RNA each from three Balb/c mice were reverse-transcribed into cDNA and amplified using the PCR. Primer pairs for the PCR were selected to amplify BTN cDNA (mRNA) across all exons (Fig. 1A). Boundaries of exons 41, 42) paying special attention to the exon encoding the membrane anchor (exon 4). In every case and in both species, single major amplified products were obtained of the sizes predicted from the published cDNAs (1, 16, 41), indicating that BTN is synthesized from one major size class of mRNA as originally suggested from Northern analysis of bovine RNA (Fig. 2).
Expression of Butyrophilin in Mammary Tissue

To study the distribution and orientation of BTN in cells and tissues, four antipeptide antibodies were produced, two specific for sequences in the N-terminal, exoplasmic domain (exo-1 and exo-2), and two to sequences in the C-terminal, cytoplasmic domain (cyto-1 and cyto-2) (Fig. 1). The antipeptide antibodies were determined to be monospecific by blotting to MFGM proteins that had been separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. All four antibodies bound to the major band of BTN protein of approximately 66 kDa in lactating mammary tissue, and that it is conserved from bovine MFGM (data not shown).

The distribution of BTN in milk and tissue fractions from lactating mammary gland was examined further by immunoblotting techniques. A 66-kDa immunoreactive protein was detected in the microsomal membrane fractions from three cows (example from one shown in Fig. 3, a and b, lanes 3) and in MFGM (Fig. 3, a and b, lanes 1). The soluble postmicrosomal supernatant fractions from bovine liver, lung, or heart, with either the exo-2 or cyto-2 antibodies. Similar results were obtained with an affinity purified preparation of rabbit antibody prepared against BTN purified from bovine MFGM (data not shown).

The forms of BTN in MFGM and microsomal membranes were further analyzed by two-dimensional gel electrophoresis (Fig. 3, c and d). Four major isoelectric variants of approximate Mr, 66,000 with pI values close to the ovalbumin standard (pI 5.1) were detected in the microsomal membrane fraction (Fig. 3, c and d). These same variants were also the major forms of BTN in MFGM detectable with antibody to the whole protein (Fig. 4a). Besides the aggregated material of high Mr, (Fig. 4a), several other immunoreactive components were detected in MFGM with similar or more basic pI values (numbered 1–5 in Fig. 4a). Epitope mapping with the four antipeptide antibodies showed that variants labeled 2 in Fig. 4a lack the C terminus, since they bound all antibodies except cyto-2 (data not shown and Fig. 4b). Sequencing by Edman degradation confirmed that these variants have an intact N terminus. The identities of other immunoreactive proteins (labeled 3–5 in Fig. 4a) are summarized in Table I. These data indicate that BTN is expressed primarily as a series of acidic variants of approximately 66 kDa in lactating mammary tissue, and that it is subjected to limited proteolysis during or following secretion in association with MFGM.

We next investigated the association of BTN with cellular membranes to establish whether the membrane anchor predicted from the cDNA sequence is functional, and to determine the orientation of BTN in cellular membranes and in secreted fat globules. BTN mRNA was synthesized in vitro from the cDNA (1) and the mRNA translated in the presence or absence of dog pancreas microsomes. A 35S-labeled protein of Mr, 59,000 was synthesized in the absence of membranes (Fig. 5, lane 1), which is very close to the predicted Mr, of 59,260 for the pre-

3 The N-terminal sequence obtained was identical to the processed form of BTN (1) through the first 10 residues. However, both Q (expected) and N residues were detected at position 9. Since the protein was prepared from the milk of three animals, we assume that this is due to allelic polymorphism, either within or between cows.
protein estimated from the cDNA sequence. Translation of BTN in the presence of microsomes caused a shift in \( M_r \) to 62,500 which is close to the size expected for the protein after incorporation into microsomal membranes, followed by removal of a 2.8-kDa signal sequence and addition of two 2.5-kDa N-linked oligosaccharides (Fig. 5, lane 2). To establish that the synthesized protein had associated with microsomes via the predicted membrane anchor (1), samples of BTN synthesized in the presence or absence of sealed microsomes were digested with proteinase K. In the presence of membranes, a 36.5-kDa fragment of BTN was protected from proteolysis (Fig. 5, lanes 3 and 4). Addition of Triton X-100 to permeabilize the membranes in the presence of proteinase K led to complete digestion of the protected fragment (Fig. 5, lanes 5 and 6). This result is exactly as expected if BTN associates with microsomal membranes via the hydrophobic region of 27 contiguous amino acids in the middle of the protein sequence.

The topology of BTN in microsomes was established using the domain-specific antipeptide antibodies. Immunoprecipitates recovered with cyto-2 antibody were enriched in membranes containing the 62.5-kDa form of BTN (Fig. 5, inset, lane 2, double arrowheads). This form was not recovered with exo-2 antibody (Fig. 5, inset, lane 1), which is as expected if the protein is incorporated into microsomes with a type I orientation. Both samples contained the 59-kDa unglycosylated form of BTN (Fig. 5, inset, lanes 1 and 2, single arrowhead), which we presume is untranslocated protein, precipitated with either antibody. No labeled protein or vesicles were recovered in the absence of antibody (Fig. 5, inset, lane 3). These data therefore confirm that BTN is incorporated into microsomal vesicles with the N terminus facing the exoplasmic space.

That BTN is also associated with plasma membranes in type I orientation was established using a recombinant baculovirus, vBT, to express BTN in insect cells. Sf9 cells were infected with vBT and 24 h post-infection, labeled with the exo-2 and cyto-2 antibodies before or after permeabilizing the plasma membrane with saponin. As expected for a type I glycoprotein the surface was readily labeled with exo-2 antibody in either intact membranes.
or permeabilized cells (Fig. 6, a and b). In contrast, the cyto-2 antibody only bound to the cells following permeabilization with detergent (Fig. 6, c and d).

Finally, we determined the topology of BTN in MFGM and secreted milk-fat globules. Fat droplets were removed from bovine milk by a mild flotation procedure (30) and treated with graded amounts of trypsin. Proteolysis was stopped by the addition of trypsin inhibitor and the membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot (Fig. 7, a-d). Under these conditions, BTN was cleaved from the N terminus into fragments of Mr 40,000–55,000 (Fig. 7, a and c, brackets) that remained associated with the lipid bilayer and that bound to antipeptide antibodies specific for either the C terminus (cyto-2) or to epitopes within the B30.2 domain (cyto-1) (Fig. 7d, brackets). The exo-2 antibody to epitopes within the constant type C1 immunoglobulin domain (Fig. 7e) also bound to the 53–55-kDa fragments. Exo-1 antibody (Fig. 7e) only recognized undigested BTN (Fig. 7d).

The pattern of peptide fragmentation is consistent with cleavage between the IgI domain and constant type C1 immunoglobulin domain and partial digestion of the remaining exoplasmic sequence. This occurred in the absence of any significant penetration of the lipid bilayer by the protease, since xanthine oxidase which is localized on the cytoplasmic face (14, 45, 46) remained intact during trypsin digestion (Fig. 7b). In contrast, digestion of isolated MFGM under identical conditions led to the cleavage of xanthine oxidase into a 130-kDa fragment and extensive fragmentation of BTN (Fig. 7, b and c). This is a stringent test of the orientation of BTN in fat droplets since the C-terminal sequence of BTN contains 33 trypsin-sensitive sites, including 11 within the first 30 residues distal to the membrane anchor (Fig. 1).

That the N terminus of BTN is exposed on the exoplasmic face of the MFGM was confirmed by digesting intact fat droplets with neuraminidase. Terminal sialic acid residues were cleaved from glycans in the N-terminal region (23) as shown by a slight shift in the electrophoretic mobility of BTN and the production of more basic isoelectric variants after enzyme treatment. Similar results were obtained with isolated MFGM (data not shown).

**DISCUSSION**

The above results clearly show that BTN is synthesized from a single size class of mRNA in mammary tissue and is incorporated into membranes as an integral glycoprotein of type I orientation in a number of contexts, including dog pancreas microsomes, the plasma membrane of insect cells, and the membrane of secreted milk-fat droplets. BTN is therefore a classical member of the immunoglobulin superfamily (7) with a single glycosylated exoplasmic domain comprising two immunoglobulin-like folds (5, 8) and, a functional membrane anchor approximately in the middle of the sequence. Smaller Mr forms of BTN in MFGM are proteolytic fragments either produced in situ or during isolation of MFGM in vitro. The most abundant of these forms which comprise 62-kDa fragments of BTN lacking the C terminus (labeled 2 in Fig. 4e) are the ones most consistently identified in other laboratories (see, e.g., Refs. 4, 22, and 24). Whether these forms have functional significance is unknown.

The results also confirm that the major immunoreactive proteins labeled I and 2 in Fig. 4a are not bovine homologs of the recently discovered family of human BTN-like proteins (11, 12) since all the variants labeled I reacted with all four anti-
peptide antibodies to epitopes within the exoplasmic and cytoplasmonic domains of BTN, and three of the four antibodies reacted with the variants, labeled 2 in Fig. 4a (Table I). Furthermore, all peptide sequences obtained by Edman degradation of the 66- and 62-kDa protein bands are within the sequence derived from bovine BTN cDNA (Refs. 1 and 23, and this study). We cannot exclude the possibility that other BTN-like proteins are associated with the MFGM. However, such proteins will at most comprise a minor fraction of the total membrane-associated protein.

These conclusions are in agreement with some, but not all previous biochemical and morphological studies. The results are at variance with some freeze-fracture studies which showed that MFGM and areas of the apical plasma membrane in close apposition with budding fat droplets are depleted of the intramembranous particles associated with the MFGM. However, such preparations that are depleted of phospholipid and other membrane proteins (24). It was reasoned that if BTN remains associated with morphologically identifiable coat structures after removal of the membrane bilayer, then BTN must be entirely incorporated into the coat either as a cytoskeletal-type protein or, as noted above, because of a rearrangement of an integral form of BTN during lipid secretion (13). Since BTN retains a type I orientation following secretion (Fig. 7), it is most likely that earlier coat preparations consisted of a stable complex of BTN and detergent-resistant proteins denuded of membrane phospholipid.

The structure and constitution of the protein complex intrinsic to the MFGM coat is unclear. Regions of the coat are organized in a highly ordered crystalline lattice with hexagonal symmetry in the milk-fat droplets of some species (49–51). Because the major coat components are the integral proteins BTN (1, 24) and the peripheral proteins, xanthine oxidase (14, 24, 52) and adipocyte differentiation-related protein (53), the protein lattice may be composed of the cytoplasmic tail of BTN cross-linked in a supramolecular complex with xanthine oxidase and adipocyte differentiation-related protein. A recent study has shown that a recombinant fusion protein of the C-terminal domain of mouse BTN specifically associates with xanthine oxidase from mammary epithelial cells cultured in the presence of lactogenic hormones (16). Since xanthine oxidase is a homodimer (54), divalent binding between xanthine oxidase dimers and the cytoplasmic tails of BTN molecules may serve to stabilize coat protein interactions. Associations between the cytoplasmic tail of BTN, xanthine oxidase, and fat globules must be substantial since both proteins remain associated with the fat globule surface following physical removal of the bilayer (17). We are currently testing the hypothesis that BTN and associated proteins form a hydrophobic complex which binds to the surface of cytoplasmic fat droplets and drives expulsion of lipid from the cell.
Our data do not rule out the possibility that some BTN associates as an integral protein with lipid droplets during their formation in the endoplasmic reticulum (17), or with lipoprotein particles containing fatty acid synthase (19) during the subsequent accretion of lipid droplets in the cytoplasm (17–19). However, these sources are unlikely to contribute significant quantities of BTN to the protein coat of MFGM because BTN comprises a minor fraction of the total protein associated with either lipoprotein particles or cytoplasmic lipid droplets (17, 19) and it is not detectable by sensitive immunoblotting techniques in postmicrosomal supernatants (Fig. 3, a and b, this study).

Demonstration that BTN retains a type I orientation following secretion in association with milk-fat globules is an essential step toward understanding the possible role of BTN in milk-fat secretion and determining the molecular architecture of the MFGM and its associated protein coat. Furthermore, the topology of BTN should allow the selective proteolytic release of the exoplasmic immunoglobulin domains from intact washed fat globules for structural and functional studies.

Acknowledgments—We thank Liane Langbehn for animal care and help with antibody production and Gerard Edwards for technical assistance in producing and maintaining recombinant baculovirus stocks. Dr. Marino Zerial (European Molecular Biology Laboratory, Heidelberg, Germany) is gratefully acknowledged for generous help in initiating the in vitro translation experiments with I. H. M. during a sabbatical leave in his laboratory. The staff of the Milk Secretion and Mastitis Laboratory, USDA, Beltsville, MD, are thanked for help in producing and maintaining recombinant baculovirus stocks.

FIG. 7. Determination of the orientation of BTN in milk-fat globules. Washed cream (lanes 1–7), or MFGM (lanes 1–7), as indicated, were incubated without trypsin (lanes 1 and 2) or with trypsin (lanes 3–7), Each mixture contained 1 mg of membrane protein and 0 (lanes 1 and 2), 0.2 (lanes 3), 2.0 (lanes 4), 4.0 (lanes 5), 10.0 (lanes 6), or 20.0 (lanes 7) μg/ml trypsin. Incubation conditions were as described under “Experimental Procedures.” Controls in lanes 1 contained no trypsin. Controls in lanes 2 contained 20 μg of trypsin added after the addition of trypsin inhibitor. All samples were separated by SDS-polyacrylamide gel electrophoresis in either (a and c) 12% or (b) 8% gels. Proteins were stained with Coomassie Blue in a, or transferred to nitrocellulose in b and c and blotted with 5,000-fold dilutions of antibodies to (xo) xanthine oxidase (b), or BTN (c). d, cream samples incubated with 10 μg/ml trypsin, separated by SDS-polyacrylamide gel electrophoresis and blotted with antipeptide antibodies, exo-1 (1,000 × dilution), exo-2 (5,000 × dilution), cyto-1 (1,000 × dilution), or cyto-2 (10,000 × dilution). The positions of the epitopes for these antibodies in BTN are shown in e. Arrowheads, undigested BTN. The positions of protein M, standards are indicated by dots to the left of a and were from top to bottom, phosphorylase b (97,400), bovine serum albumin (66,300), glutamate dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), and trypsin inhibitor (21,500). Note the 40–55-kDa fragments of BTN which accumulate in the cream fractions on treatment with trypsin (brackets in a and c) which react with cyto-1 and cyto-2 antibodies (bracket in d). The 53/55-kDa fragments also bind to exo-2 antibody in d, but no fragments other than undigested BTN are recognized by exo-1 antibody. In contrast, BTN in isolated MFGM is extensively degraded (a and c). Immunoblot in b shows that xanthine oxidase is protected from trypsin digestion in cream compared with isolated MFGM.
acquiring tissue from lactating cows and Margaret Kempf as always provided excellent secretarial assistance.

REFERENCES

1. Jack, L. J. W., and Mather, I. H. (1990) J. Biol. Chem. 265, 14481–14486
2. Patton, S., and Keenan, T. W. (1975) Bioclinh. Biophy. Acta 415, 273–309
3. Keenan, T. W., Mather, I. H., and Dylewski, D. P. (1988) in Fundamentals of Dairy Chemistry (Wong, N. P., ed) 3rd Ed., pp. 511–582, Van Nostrand Reinhold Co., New York
4. Keenan, T. W., and Patton, S. (1995) in Handbook of Milk Composition (Gensen, R. G., ed) pp. 5–56, Academic Press, Inc., New York
5. Gardinier, M. V., Amiguet, P., Linington, C., and Matthieu, J.-M. (1992) J. Neurosci. Res. 33, 177–187
6. Harpaz, Y., and Chothia, C. (1994) J. Mol. Biol. 238, 528–539
7. Williams, A. F., and Barclay, A. N. (1988) Ann. Rev. Immunol. 6, 381–405
8. Linsley, P. S., Peach, R., Gladstone, P., and Bajorath, J. (1994) Protein Sci. 3, 1341–1343
9. Bellini, M., Lacroix, J.-C., and Gall, J. G. (1993) Exp. Cell Res. 211, 377–382
10. Tazi-Ahnini, R., Henry, J., Offer, C., Bouissou-Bouchouata, C., Mather, I. H., Vernet, C., Boretto, J., Mattei, M.-G., Takahashi, M., Jack, L. J. W., Mather, I. H., and Jack, L. J. W. (1993) J. Dairy Sci. 76, 600–612
11. Dylewski, D. P., Dapper, C. H., Valivullah, H. M., Deeney, J. T., and Keenan, T. W. (1994) In Vitro Cell Dev. Biol. 30, 29–36
12. Dylewski, D. P., Dapper, C. H., Valivullah, H. M., Deeney, J. T., and Keenan, T. W. (1994) In Vitro Cell Dev. Biol. 30, 29–36
Butyrophilin Is Expressed in Mammary Epithelial Cells from a Single-sized Messenger RNA as a Type I Membrane Glycoprotein

Lisa R. Banghart, Clayton W. Chamberlain, Jorge Velarde, Igor V. Korobko, Sherry L. Ogg, Lucinda J. W. Jack, Vikram N. Vakharia and Ian H. Mather

J. Biol. Chem. 1998, 273:4171-4179.
doi: 10.1074/jbc.273.7.4171

Access the most updated version of this article at http://www.jbc.org/content/273/7/4171

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 9 of which can be accessed free at http://www.jbc.org/content/273/7/4171.full.html#ref-list-1