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BUTYROPHILIN, AN APICAL PLASMA MEMBRANE-ASSOCIATED GLYCOPROTEIN CHARACTERISTIC OF LACTATING MAMMARY GLANDS OF DIVERSE SPECIES

HANS W. HEID [a], STEFANIE WINTER [a], GERDA BRUDER [a], THOMAS W. KEENAN [b] and ERNST-DIETER JARASCH [a]

[a] *Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg (F.R.G.) and* [b] *Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 (U.S.A.)*

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Lipid globule membranes were isolated from human and bovine milk and from the milk of sheep, goat, pig, rat and guinea pig, and their polypeptide compositions were analyzed. The major polypeptides with molecular weights similar to that of bovine butyrophilin were separated by gel electrophoresis, isolated and characterized with respect to isoelectric point, molecular weight, immunological cross-reactivity and peptide composition after proteolytic cleavage. We show that in all species examined these proteins are similar to bovine butyrophilin in (i) their relative insolubility in buffers of low and high ionic strength and in non-denaturing detergents, (ii) the occurrence of several isoelectric variants, and (iii) patterns of peptides obtained by protease digestion. It is concluded that closely related proteins are major constituents of the cytoplasmic coat structures associated with milk lipid globule membranes of many species, and we propose the name butyrophilins for this group of proteins. Bovine and human butyrophilins are glycosylated with relatively large amounts of glucosamine, mannose, glucose and galactose but little fucose, sialic acids or galactosamine. Most if not all of the sugar residues are associated with an acetone-soluble peptide fragment of M_r 12000–16000 focusing at about pH 4.0. We suggest that this fragment contains a membrane-spanning peptide sequence and is involved in the attachment of the cytoplasmic coat to the membrane of the milk lipid globule.

Introduction

Milk lipid droplets are formed in the cytoplasm of the lactating mammary epithelial cell, are enveloped by apical plasma membrane and then secreted into the alveolar lumen of the mammary gland, by a process morphologically similar to the budding of RNA-viruses from plasma membrane [1–4]. The secreted milk lipid globule consists of (i) a lipidic core mainly of triacylglycerols [5], (ii) a rim of cytoplasm which is usually very thin (less than 50 nm) in globules of bovine milk but can be larger in globules from other species [6,7], and (iii) a membrane covered by a 10–50 nm thick, dense fuzzy coat on its internal, cytoplasmic face [6,8] and a glycocalyx on its external surface [9,10].

The proteinaceous coat [11] attached to the inner face of the membrane has been isolated and shown to be enriched in two polypeptides of molecular weights 155000 and 67000 [8,12]. In bovine milk lipid globules, the predominant coat protein is a polypeptide of M_r 67000, for which we have proposed the name butyrophilin because it is found in close association with butter lipids [12]. Using specific antibodies to purified butyrophilin we have shown that this protein is located prim-

arily, if not exclusively, on the cytoplasmic aspect of the apical plasma membrane of the lactating mammary epithelial cells and of milk lipid globules [12]. Recent reports have indicated that butyrophilin is acylated [13] and glycosylated [8,12,14].

As the location of glycoproteins in the cytoplasm is in conflict with present views of glycosylation mechanisms [15], we have studied various biochemical properties of butyrophilin in more detail. We also show that proteins similar to bovine butyrophilin occur in the milk of other ruminants (goat, sheep), of nonruminant artiodactyls (pig), of rodents (guinea pig, rat) and man.

Methods

Fresh bovine milk was obtained from Holstein cows. Milk samples were also collected from lactating Nubian goats, Anglo-Merino sheep, Yorkshire pigs, Sprague-Dawley rats and Pirbright White guinea pigs. Milk from women about 1 week after delivery was kindly provided from volunteers at the St. Elisabeth Women's Hospital in Heidelberg. Preparation of milk lipid globules and milk lipid globule membranes from all species examined was as described from bovine material [7,16].

Polypeptides were separated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to Laemmli [17]. Two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis was according to O'Farrell [18]. Gels were stained with Coomassie brilliant blue. Glycoproteins were identified on the gels with the periodic acid/Schiff reaction (cf. Ref. 8).

Polypeptides of butyrophilin from milk lipid globule membranes of the various species were excised and eluted electrophoretically from unstained, unfixed SDS-polyacrylamide gels of 3 mm thickness. The position of the specific polypeptide bands in the gels was examined by co-electrophoresis of fluorescamine-labelled (Fluram, Hoffmann-La Roche, Basel, Switzerland) proteins (cf. Ref. 19). The isolated polypeptides were dialyzed against 0.15 M NaCl buffered with 10 mM potassium phosphate (pH 7.4) to remove the excess of detergent, concentrated by membrane filtration, and examined for homogeneity by one- and two-dimensional gel electrophoresis.

Carbohydrates were analyzed as alditol acetate derivatives by combined gas chromatography-mass spectrometry as described [20]. Quantitation of data was by addition of 2-deoxy-D-glucose as an internal standard. Protein was determined by the method of Lowry et al. [21] using bovine serum albumin as standard.

Limited proteolysis of polypeptides using *Staphylococcus aureus* V8-protease (Miles Laboratories, Elkart, IN, U.S.A.) and the separation of the polypeptide fragments by one- and two-dimensional gel electrophoresis was as described (Ref.

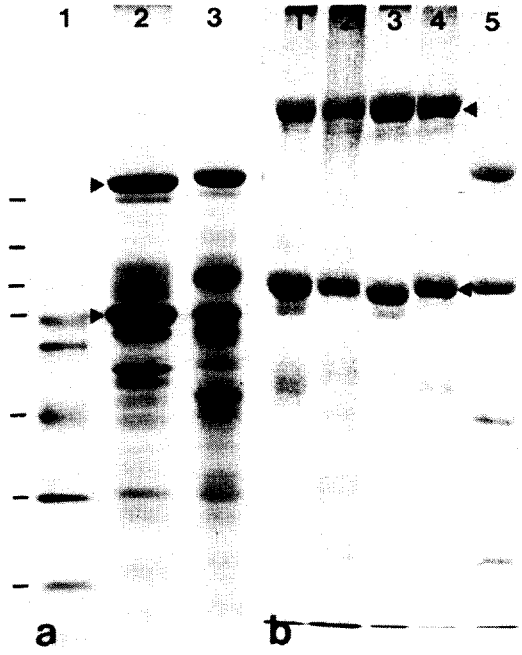

Fig. 1. One-dimensional gel electrophoresis of milk lipid globule membranes from various species. (a) Gradient gel (6–20% acrylamide) of bovine (lane 2) and human (lane 3) milk lipid globule membranes and molecular weight marker proteins, indicated by horizontal bars (lane 1; from top to bottom: β-galactosidase, phosphorylase *a*, transferrin, bovine serum albumin, catalase, alcohol dehydrogenase, chymotrypsinogen, ribonuclease). (b) 9% acrylamide gel of milk lipid globule membranes of cow (lane 1), goat (lane 2), sheep (lane 3), pig (lane 4), and of molecular weight marker proteins (lane 5; from top to bottom: phosphorylase *a*, bovine serum albumin, actin, chymotrypsinogen). Arrowheads designate the two major polypeptide bands of milk lipid globule membranes of all species examined: xanthine oxidase (M_r 155000) and a polypeptide with a molecular weight similar to that of bovine butyrophilin (M_r 67000).

22; see also Ref. 23). Two-dimensional peptide mapping of radioiodinated proteins after complete digestion with trypsin was by the method of Elder et al. [24]: Individual spots separated by two-dimensional gel electrophoresis were excised and labelled with 0.2 mCi ^{125}I in 2 μl NaI (Amersham International, U.K.). Peptides were digested with 50 μg trypsin (217 U/mg Trypsin-TPCK, Millipore Corporation, Freehold, NJ, U.S.A.) per gel piece for 24 h at 37°C, lyophilized and analyzed by combined electrophoresis and chromatography on cellulose-coated thin-layer plates. Autoradiography was performed at −70°C using Kodak XAR-5 X-ray films.

Antibodies to butyrophilin of bovine milk lipid globule membranes raised in guinea pigs have been described [12]. Immunological identification of polypeptides on gels was by electrophoretic transfer of the polypeptides to nitrocellulose paper and binding of the specific antibodies and ^{125}I-labelled protein A from *Staphylococcus aureus* (New England Nuclear, Boston, MA, U.S.A.) as described [25,26].

Results

Electrophoresis of polypeptides from milk lipid globule membranes

Lipid globule membranes were prepared from human and bovine milk and milk from goats, sheep, pigs, guinea pigs and rats, and their polypeptide compositions were analyzed and compared by SDS-polyacrylamide gel electrophoresis (Fig. 1). In all species, predominant polypeptide bands were at M_r 155 000 (xanthine oxidase, cf. Refs. 27,28) and M_r 65 000–68 000, i.e., the molecular weight of bovine butyrophilin [12]. In human (Fig. 1a, slot 3), guinea pig and rat (not shown) membranes and, to lesser amounts, also in those of the cow, goat, and sheep, a band at about M_r 64 000 was

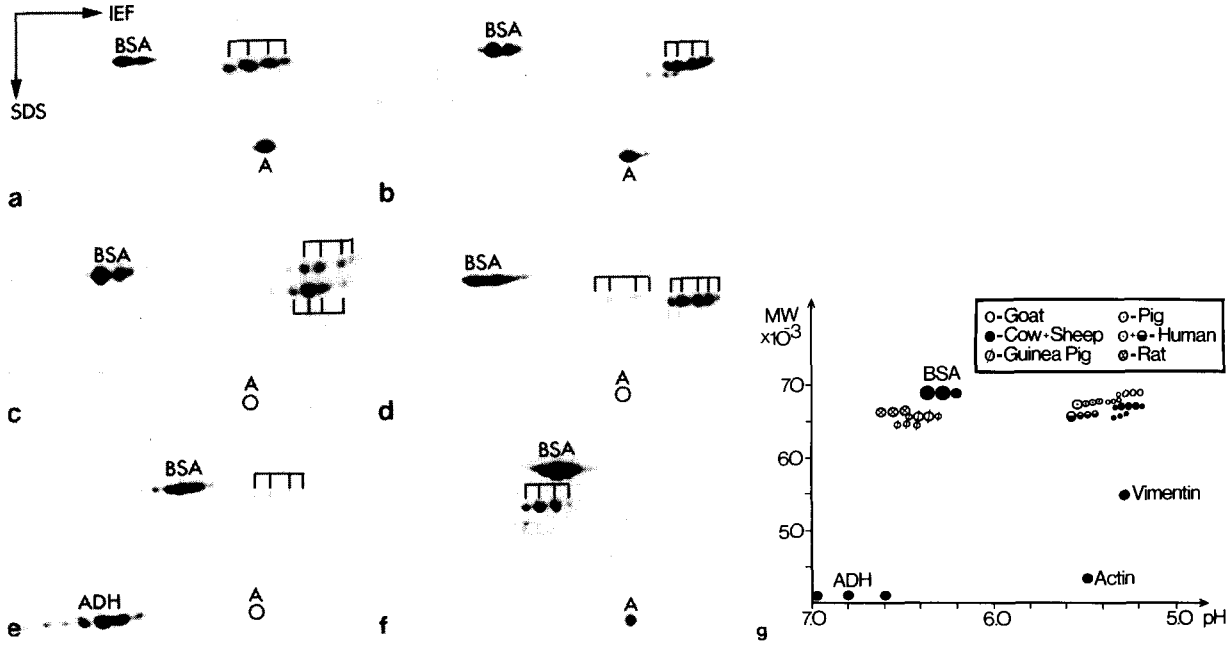

Fig. 2. Two-dimensional gel electrophoresis of milk lipid globule membranes from various species. Isoelectric focusing (IEF) was from basic to acidic; the range from pH 7 to pH 4.5 is shown. SDS-polyacrylamide gel electrophoresis (SDS) was from top to bottom; the range M_r 80 000–35 000 is shown. The following polypeptides were added as markers: bovine serum albumin (BSA), α-actin (A), vimentin (V), alcohol dehydrogenase (ADH). In c, d and e, the relative position of α-actin is indicated by O. (a) from pig; (b) from sheep; (c) mixture from cow and goat; (d) mixture from pig and sheep; (e) human; (f) guinea pig. (g) Summary of the positions of the M_r 65 000–68 000 major polypeptides from milk lipid globule membranes of seven species, as determined by coelectrophoresis of mixtures of these and marker proteins. Whereas the isoelectric positions of the specific polypeptides from pig, the ruminants and man were similar (pH 5.2–5.6), those from the rodents were less acidic (pH 6.2–6.6).

found on the gels which was probably a proteolytic fragment of the M_r 65000–68000 polypeptide (see below). Like bovine butyrophilin (see Ref. 8,15), the polypeptides of similar molecular weight in other species were enriched in the insoluble sediment after treatment with buffers containing 1.5 M KCl or 0.5 M $MgCl_2$ and 1% Triton X-100 and centrifugation at $100000 \times g$ for 1 h (Heid, H.W., unpublished data). We found, by two-dimensional gel electrophoresis, that these polypeptides consisted of 3–5 isoelectric variants focusing at pH 5.2–5.6, except for the polypeptides of guinea pig and rat which focused at about pH 6.2–6.6 (Fig. 2).

We proposed to extend the name butyrophilin to these proteins in view of their similarity to the butyrophilin of bovine milk lipid globule membranes. By co-electrophoresis of membrane proteins from the various species it was shown that the molecular weight and isoelectric pH range of butyrophilin differed among the species. Only bovine and ovine butyrophilin were indistinguishable on the two-dimensional gels (Fig. 2g). The electrophoretic properties of butyrophilin as analyzed in total milk lipid globule membranes were not significantly altered when the polypeptide was excised and eluted from the gels (compare Fig. 2 with Fig. 4a below). The minor polypeptides of approx. M_r 64000 mentioned above also consisted of about three isoelectric variants that were slightly less acidic (by about 0.02 pH units) than the corresponding butyrophilin.

Limited proteolysis of bovine and human butyrophilin

Bovine butyrophilin, purified by excision and elution from gels, was digested for varying periods of time with a protease from *Staphylococcus aureus* strain V8 ('V8-protease') as shown in Fig. 3. This protease specifically cleaves peptide bands on the carboxyl terminal side of glutamic and aspartic acid residues [29]. By short incubation with low amounts of protease, bovine butyrophilin was cleaved into four major fragments of molecular weight about 45000, 42000, 35000 and 16000. Upon prolonged proteolytic digestion, the M_r 45000 fragment was further cleaved into the M_r 35000 fragment and possibly some minor components of lower molecular weight.

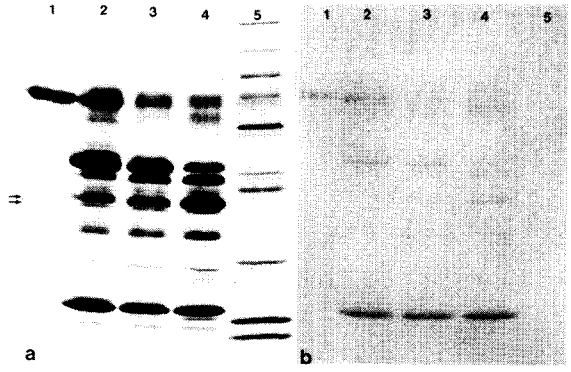

Fig. 3. One-dimensional gradient gel electrophoresis (12–20% acrylamide) of polypeptide fragments from purified bovine butyrophilin obtained by limited proteolysis with V8-protease. (a) Coomassie blue stain. (b) Periodic acid/Schiff stain. Lane 1: 5 μg purified butyrophilin. Lanes 2–4: Polypeptide fragments from 50 μg butyrophilin after treatment with 5 μg V8-protease at 25°C for 5 min (lane 2), 20 min (lane 3) and 60 min (lane 4). The protease activity was stopped by boiling the samples for 5 min. Lane 5: Molecular weight marker proteins (from top to bottom: β-galactosidase, phosphorylase *a*, transferrin, bovine serum albumin, catalase, alcohol dehydrogenase, aldolase, chymotrypsinogen, ribonuclease, cytochrome *c*). The double arrow indicates the position of V8-protease as determined from gels run in parallel. Note the intense staining for carbohydrates of the small polypeptide fragment (M_r 16000) of butyrophilin after treatment with V8-protease.

Butyrophilin reacted with the periodic acid/Schiff reagent (see also Ref. 8,12,14). The larger fragments at M_r 35000–45000 showed only very weak reaction for carbohydrates; however, the M_r 16000 polypeptide strongly reacted with the periodic acid/Schiff reagent (Fig. 3b). In two-dimensional gels, the three major proteolytic fragments at M_r 35000–45000 had 4–6 isoelectric variants and focused at higher pH values than the uncleaved butyrophilin. The strongly periodic acid/Schiff-positive M_r 16000 fragment, however, focused as a single spot at a much more acid pH value (Fig. 4c,e). This fragment remained soluble in mixtures of acetone/water (4 : 1), even at 0°C; it was therefore missing from routine preparations (Fig. 4b) in which the peptides had been precipitated by acetone prior to isoelectric focusing (cf. Ref. 26). With higher acetone concentrations and lower temperatures (−20°C) this glycopeptide was sedimented by centrifugation at $10000 \times g$ for 15 min (Fig. 4c). In contrast to the polypeptide frag-

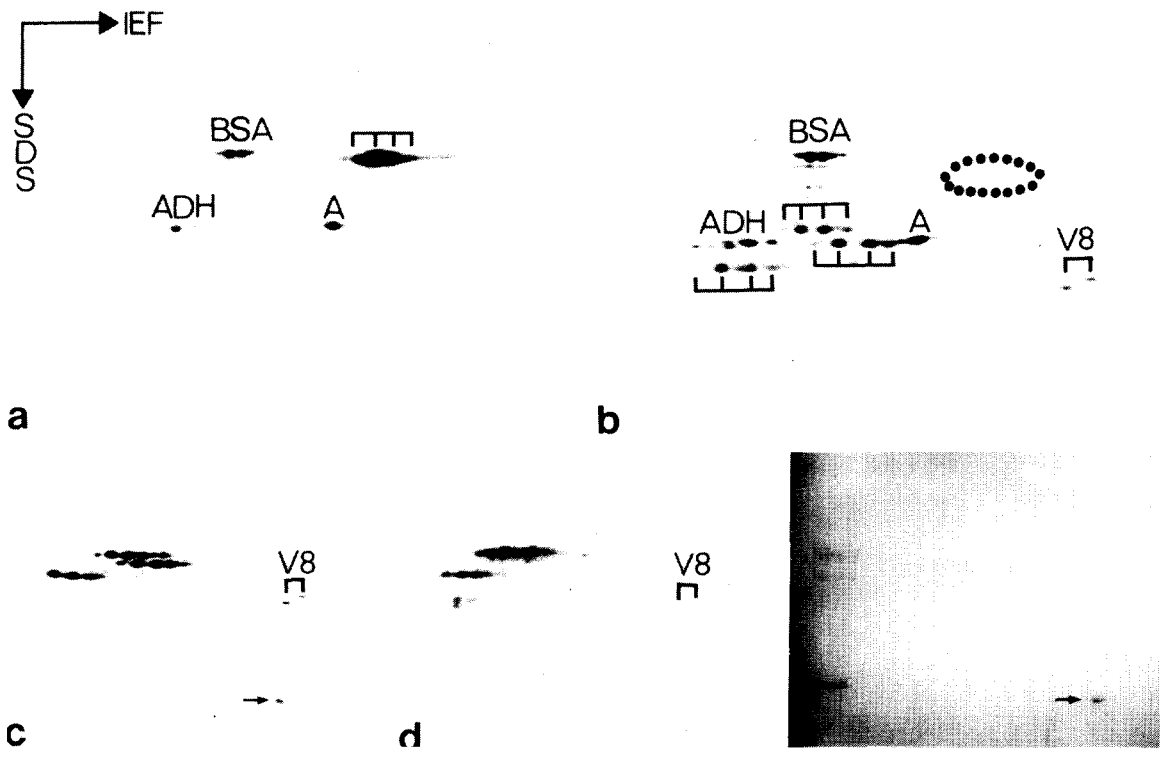

Fig. 4. Two-dimensional gel electrophoresis of purified bovine butyrophilin (a) and of polypeptide fragments of butyrophilin derived by proteolysis with V8-protease for 5 min (b–e). Isoelectric focusing (IEF) was from basic to acidic. Gel electrophoresis (14% acrylamide) was from top to bottom (SDS). Bovine serum albumin (BSA), α-actin (A), and alcohol dehydrogenase (ADH) were co-electrophoresed as marker proteins (a,b). The position of V8-protease is indicated (V8). Gels in a–c were stained with Coomassie blue. (d) Autoradiograph of an immunoblot reaction of peptides transferred to nitrocellulose paper from a gel corresponding to that in c. (e) Periodic acid/Schiff stain of a gel corresponding to that in c with an additional one-dimensional separation of butyrophilin fragments. Like the uncleaved butyrophilin (a), the three major polypeptide fragments of M_r 45000, 42000 and 35000 exhibited at least four isoelectric variants (brackets in b); these focused at higher pH values than the intact protein (position indicated by a dotted line). The peptide fragment of M_r 16000 (arrow) focused at a more acidic pH (c). This peptide was soluble in acetone/water (4:1, v/v) at 0°C and was therefore missing from preparations obtained by this precipitation method (b). Antibodies to butyrophilin reacted with the three fragments of M_r 45000–35000 (brackets in b) but not with the acidic M_r 16000 fragment (d). This fragment is the only peptide with strong staining for carbohydrates (arrow in e).

ments of M_r 45000, 42000 and 35000 which reacted with the antibodies raised against intact butyrophilin, the acetone-soluble, acidic (p*I* 4.0), glycosylated peptide fragment of M_r 16000 was not recognized by these antibodies (Fig. 4d).

Similar results were obtained with human butyrophilin after treatment with V8-protease (Fig. 5). Two large fragments of M_r 40000 and 37000 with four isoelectric variants each have been detected. Like the corresponding fragments of bovine butyrophilin (Fig. 4b), these fragments focused at slightly higher pH (p*I* 5.3–6.2) than the uncleaved protein (p*I* 5.3–5.5; see also Fig. 2). A small acidic peptide fragment (M_r 12000, p*I* 3.8) corresponding to the bovine fragment of M_r 16000 and p*I* 4.0 (Fig. 4b,c) was also soluble in mixtures of acetone/water (4:1) at 0°C (Fig. 5b) but was precipitated at −20°C with higher acetone concentrations (Fig. 5c). It was also glycosylated as judged from periodic acid/Schiff staining (not shown). Two additional acidic (p*I* 4.0 and 4.1), glycosylated fragments of M_r 13000 and 11000 present in the same gel (Fig. 5c) were demonstrated by the peptide mapping technique to be closely related to this acetone-soluble fragment (see below).

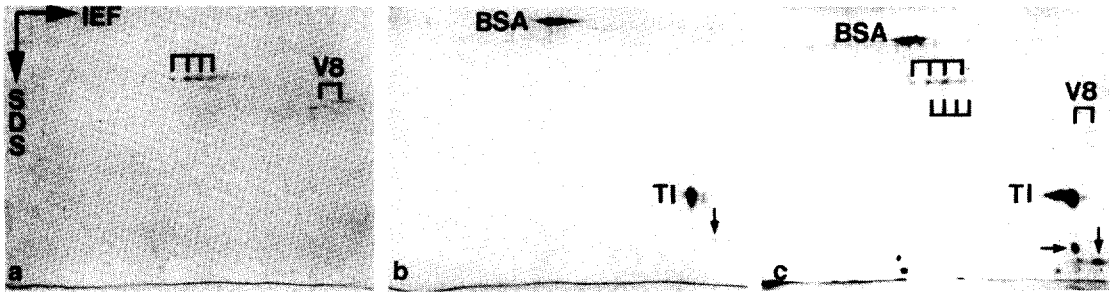

Fig. 5. Two-dimensional gel electrophoresis of polypeptide fragments of human butyrophilin derived by limited proteolysis. 20 μg of the purified protein were digested with 2 μg of V8-protease for 5 min at 25°C. Isoelectric focusing (IEF) was from basic to acidic. Gel electrophoresis (14% acrylamide) was from top to bottom (SDS). Gels were stained with Coomassie blue. Bovine serum albumin (BSA) and soybean trypsin inhibitor (TI) were co-electrophoresed as marker proteins; the position of the V8-protease is indicated (V8). (a) Fragments of human butyrophilin precipitated by acetone/water mixtures (4 : 1, v/v) at 0°C by centrifugation at 10000 × *g* for 5 min. (b) Fragment of human butyrophilin, not sedimented by acetone/water (4 : 1, v/v) at 0°C by centrifugation at 10000 × *g* for 5 min. The material was dried under a stream of nitrogen gas. (c) Fragments of human butyrophilin precipitated in higher concentrations of acetone (8 vol : 1 vol H_2O) at −20°C and by centrifugation at 10000 × *g* for 5 min. A major fragment of M_r 40000 with four isoelectric variants (indicated by brackets) at p*I* 5.5–6.2 is present in the gels of (a) and (c). A minor fragment of M_r 37000 with isoelectric variants at p*I* 5.3–6.0, indicated by the lower bracket in c), is also occasionally found. The acetone/water-soluble supernatant (b) contains a very acidic (p*I* 3.8) fragment of M_r 12000 (vertical arrow). In addition, the material precipitated at high acetone concentrations and low temperature (c) revealed two other acidic fragments, the larger one (M_r 13000, p*I* 4.0) is indicated by the horizontal arrow.

Carbohydrate contents of bovine and human butyrophilin

Analysis of bovine and human butyrophilin for carbohydrates revealed relatively large amounts of glucosamine, mannose, glucose and galactose (Table I), whereas fucose, galactosamine and sialic acids were not detected at significant concentrations. The data suggest that two residues of *N*-acetylglucosamine (hydrolyzed to glucosamine by the analytical procedure) and six residues of galactose are present in one butyrophilin molecule of both species. The corresponding numbers for mannose and glucose residues were six and five in human butyrophilin and nine and three in bovine butyrophilin, respectively.

TABLE I

CARBOHYDRATE CONTENTS OF PURIFIED HUMAN AND BOVINE BUTYROPHILIN

Numbers indicate molar ratios of carbohydrate to butyrophilin.

	Human	Bovine
Glucosamine	1.0	1.7
Mannose	6.1	8.9
Glucose	5.3	3.0
Galactose	6.1	6.2
Fucose	< 0.2 [a]	< 0.2 [a]

[a] Limit of detection.

Peptide mapping of bovine butyrophilin

Bovine butyrophilin and its major polypeptide fragments obtained by limited V8-protease treatment have been further characterized by two-dimensional peptide mapping after complete trypsin digestion. Fig. 6 shows autoradiographs of such peptide maps of (a) total butyrophilin, (b) the M_r 45000 polypeptide fragment, and (c) the M_r 16000 polypeptide fragment. All the identified isoelectric variants of butyrophilin (see Fig. 2) and its M_r 45000 fragment were analyzed in this way and their tryptic peptide patterns shown to be indistinguishable from those of Figs. 6a and b. In addition, peptide maps of isoelectric variants of the minor component of M_r 64000 (cf. Fig. 2) were similar or identical to those of butyrophilin.

About ten fragments were clearly identified, apart from numerous small spots, in the autoradiographs of total butyrophilin. Four of these fragments, having almost identical electrophoretic and chromatographic mobilities, were also present in the M_r 45000 polypeptide fragment. The peptide patterns of the M_r 42000 and 35000 fragments closely resembled that of the M_r 45000 fragment (not shown). The remaining six identified peptides

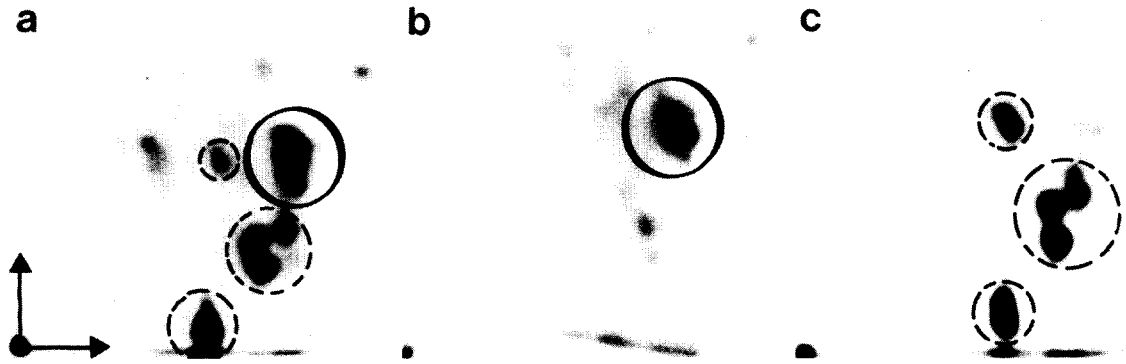

Fig. 6. Two-dimensional separation of peptide fragments from bovine butyrophilin obtained by complete proteolysis with trypsin. Electrophoresis was from left (acidic) to right (basic) and chromatography was from bottom to top (arrows). (a) Total butyrophilin. (b) The M_r 45000 fragment and (c) the M_r 16000 fragment obtained by limited V8-protease treatment. The prominent spots characteristic for the M_r 45000 peptide are encircled by a solid line and those characteristic for the M_r 16000 peptide by dashed lines. The M_r 42000 and 35000 fragments had tryptic peptide patterns very similar to that of the M_r 45000 fragment.

were all present in the acidic, glycosylated M_r 16000 fragment described above (Fig. 6c). No overlapping of spots from the M_r 35000–45000 fragments and the small acidic fragment was observed. When the trypsin-digested peptide from the M_r 45000 and M_r 16000 fragments were mixed and then analyzed on thin-layer plates, a pattern resulted which was virtually identical to that of total butyrophilin.

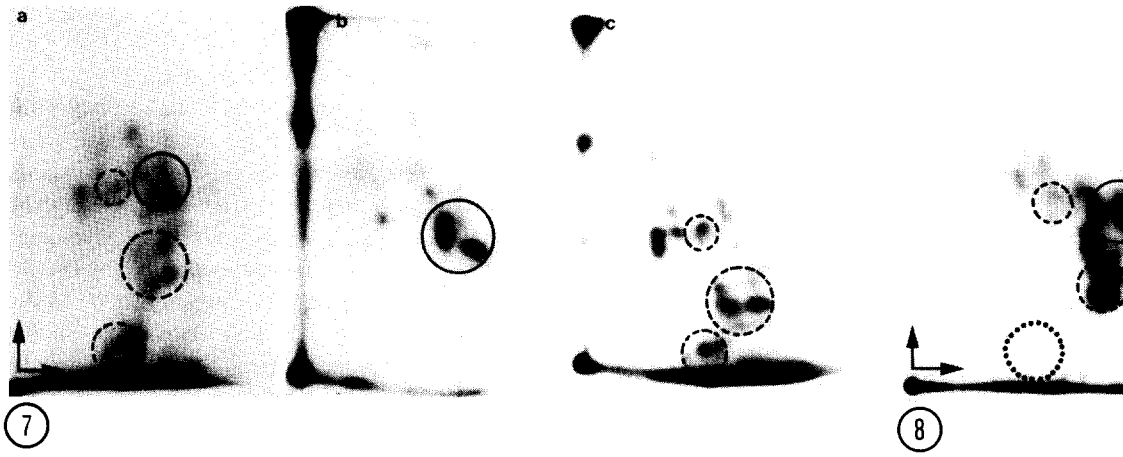

Fig. 7. Two-dimensional separation of peptide fragments from human butyrophilin obtained by complete proteolysis with trypsin. Electrophoresis was from left (acidic) to right (basic) and chromatography was from bottom to top (arrows). (a) Total human butyrophilin; (b) its M_r 40000 fragment, and (c) its M_r 13000 acidic fragment obtained by limited V8-protease treatment. The prominent groups of spots characteristic for the M_r 40000 peptide are encircled by a solid line and those characteristic for the M_r 13000 (*pI* 4.0) peptide by dashed lines. Note that the peptide pattern of the M_r 40000 fragment (b) is similar to that of the M_r 45000 fragment of bovine butyrophilin (Fig. 6b), and the pattern of the M_r 13000 acidic, glycosylated fragment (c) is similar to that of the M_r 16000 acidic, glycosylated fragment of bovine butyrophilin (Fig. 6c).

Fig. 8. Two-dimensional separation of peptide fragments from guinea pig butyrophilin obtained by complete proteolysis with trypsin. Prominent groups of radioactive spots similar to those of bovine and human butyrophilin (cf. 6a and 7a) are encircled by solid and dashed lines; however, the group of acidic peptides with low chromatographic mobility, present in human and bovine butyrophilin, was missing (the corresponding area is indicated by a dotted circle).

Peptide mapping of butyrophilin from other species

The overall similarity of butyrophilins from milk lipid globules of various species, as suggested from their relative insolubility, molecular weights and isoelectric points, was confirmed by the similarity of their tryptic peptide patterns.

For comparison, the peptide maps of the M_r 67000 polypeptide from human milk lipid globule membranes and its proteolytic fragments of M_r 40000 and M_r 13000 obtained by V8-protease digestion are shown in Fig. 7. Clearly, the peptide patterns of the uncleaved protein as well as the major fragment of M_r 40000 and the acidic, glycosylated fragment of M_r 13000 resembled the patterns of bovine butyrophilin and its M_r 45000 and 16000 fragments, respectively (cf. Fig. 6). The four isoelectric variants of the M_r 40000 fragment of human butyrophilin had identical patterns. Likewise, the peptide maps of the acidic M_r 13000 and 12000 fragments (Fig. 5c) were indistinguishable. As controls, two-dimensional peptide mapping of bovine serum albumin, soybean trypsin inhibitor and *S. aureus* V8-protease were also performed. Peptide maps of these proteins as well as those of two spots of p*I* 6.5 and approx. M_r 12000 excised from the gel of Fig. 5c had no similarity to butyrophilin or its fragments (data not shown here). Butyrophilins of milk lipid globule membranes from guinea pig and rat exhibited the least correspondence in electrophoretic properties to bovine butyrophilin (cf. Fig. 2). As shown in Fig. 8 for guinea pig butyrophilin, even here the resemblance of tryptic peptide patterns was remarkable. The only pronounced difference to bovine and human butyrophilin was the absence of relatively acidic peptide fragments with low chromatographic mobilities in the peptide pattern of guinea pig butyrophilin. We suggest that this apparent absence of acidic amino acid sequences is the reason that the butyrophilins of guinea pig and rat have more basic isoelectric points.

Discussion

The milk lipid globule membrane which is derived from the apical plasma membrane of the lactating mammary epithelial cell [1,2] has a number of characteristics typical of plasma membranes from a variety of cells. These include similar lipid composition [30], the presence of gangliosides and sialic acids [31,32], of 5′-nucleotidase and alkaline phosphatase [33–35], and of similar electron transport systems [16,36]. Therefore, milk lipid globule membranes can be regarded as a model system for a selected domain of plasma membrane and as a readily available source of apical plasma membrane from the mammary epithelial cell [2,7,37]. However, the two most prominent proteins of milk lipid globule membranes, xanthine oxidase and butyrophilin [7,12,27,28], are not characteristic of other plasma membranes studied so far.

Butyrophilin is enriched in the dense fuzzy coat associated with the cytoplasmic side of bovine milk lipid globule membrane, and, quantitatively, it is the major polypeptide of this coat [8,12,38]. Electron microscopic and immunolocalization studies have revealed that both the coat structure and the butyrophilin antigen are located over the entire apical plasma membrane, but not the basolateral plasma membrane, of the lactating epithelial cell; quantitatively, however, the coat appears to be concentrated and condensed in those plasma membrane domains where the lipid globules are budded from the cell apex [3,8,12].

In this study we show that proteins similar to the butyrophilin from bovine milk globule membranes not only occur in the corresponding membranes from goat and sheep but also in other, taxonomically more distant mammals such as pig, rat, guinea pig and man. Properties in common for all these major membrane-associated proteins include: (i) relative insolubility in buffers containing low (0.15 M NaCl) and high concentrations of salt (e.g., 0.5 M $MgCl_2$ or 1.5 M KCl); (ii) insolubility in non-denaturing detergents; (iii) similar molecular weights; (iv) appearance as an acidic polypeptide revealing 3–5 isoelectric variants; (v) similar patterns after proteolytic cleavage. Because of these similarities we propose to extend the name butyrophilin to the corresponding proteins of other species as well. Recently, monoclonal and polyclonal antibodies against human and guinea pig butyrophilins have been raised. Some of these antibodies cross-reacted with bovine butyrophilin as shown by immunoblotting on one-dimensional gels. Like bovine butyrophilin [12], immunofluorescence microscopy revealed an apical localization of human and guinea pig butyrophilin in

mammary epithelial cells (Johnson, V.G. and Mather, I.H., personal communication; Heid, H.W., unpublished results). We conclude that butyrophilin is a major constituent of the insoluble cytoplasmic coat associated with milk lipid globule membranes of many mammalian species.

Butyrophilin from bovine and human milk has been studied in more detail. Both are glycoproteins with similar carbohydrate composition and from the data it is suggested that *N*-acetylglucosamine (measured as glucosamine), mannose and glucose are present in stoichiometric amounts corresponding to those of the asparagine-linked oligosaccharide 'core' of other glycoproteins (e.g., Ref. 39). Of typical 'terminal' sugar residues, believed to be added to the carbohydrate core in the 'trans' cisternae of the Golgi apparatus after trimming of the peptide-linked mannose-rich oligosaccharide [15,40], only galactose is found in stoichiometric amounts in both human and bovine butyrophilin. Fucose, sialic acids and *N*-acetylgalactosamine have not been detected at significant concentrations. Whereas the amounts of mannose, galactose and glucose as well as the very low levels of fucose are similar to previous reports on preparations of bovine 'band 12' (including butyrophilin [8]), the relatively high amounts of glucosamine, galactosamine and sialic acids reported in that study are at variance with our data. As the membrane-associated coat of bovine milk lipid globule membrane is known to contain relatively high concentrations of these sugars [8], these differences may be due to the possible presence of other glycoprotein components in the excised gel fractions used in the previous study (for the occurrence in bovine milk lipid globule membrane of glycoproteins of similar molecular weight as butyrophilin, see Ref. 38). Recently, a glycoprotein from human milk lipid globule membranes was isolated which had a similar molecular weight to butyrophilin [41]. However, the carbohydrate composition of this protein preparation (isolated by $MgCl_2$ extraction) was strikingly different from that of butyrophilin, showing higher contents of both fucose and sialic acids. The sugar contents of this protein, therefore, resembled that of the high molecular weight glycoproteins prepared by extraction with $MgCl_2$, phenol or lithium diiodosalicylate [42–44]. In our hands, butyrophilin is not solubilized with the $MgCl_2$ extraction procedure [14] employed by Imam et al. [41]. The absence of sialic acids from butyrophilin is also confirmed by our finding that the isoelectric points of the polypeptide variants are not affected by treatment with neuraminidase (data not shown).

It is generally accepted that glycosylation of proteins occurs in the cisternae of the endoplasmic reticulum and the Golgi apparatus, but not in the cytoplasm. Consequently, the carbohydrate moieties of membrane-bound glycoproteins are located on the cisternal side of endomembranes and on the external side of the plasmalemma [15]. In agreement with this view, Horisberger et al. [10] have described binding of lectin-labelled gold granules as markers for carbohydrates exclusively on the external surface of bovine milk lipid globule membranes. Our data on the glycosylation of butyrophilin, the major protein of the dense, fuzzy coat attached to the internal, cytoplasmic side of the membrane, would not be contradictory to their observations, if butyrophilin is a membrane-spanning protein with its carbohydrate moiety oriented towards the external side. This possibility is supported by our findings that butyrophilin can be isolated from the membrane only by using membranolytic agents such as detergents or phospholipases and that most, if not all, of its sugar residues are associated with an acetone-soluble peptide fragment. We suggest that this peptide, which does not react with the antibodies against total butyrophilin, is the transmembrane fragment, whereas the major (antigenic) portion of the protein is located at the cytoplasmic face.

Previously, we have shown that bovine butyrophilin contains fatty acid residues in tight, possibly covalent, linkage to the protein [13]. It is possible that the fatty acids are bound to this hydrophobic peptide fragment and are involved in the insertion of butyrophilin in the membrane lipid bilayer (cf. Ref. 45). Composition and localization of the M_r 16000 and M_r 13000 peptide fragments of bovine and human butyrophilin are currently being studied in our laboratory.

To our knowledge, butyrophilin is specifically expressed in lactating mammary epithelial cells. It is not present, in amounts detectable by immunofluorescence (cf. Ref. 12) or immunoblotting techniques, in myoepithelial and mesenchymal cells

from the mammary gland, in cells of the liver, intestine, white adipose tissue, heart skeletal muscle, thymus or brain. Butyrophilin is also not detected in mammary epithelial cells of 6-month-old calves and of 18-month-old heifers (unpublished data). Similarly, we have not found, using antibodies against the M_r 67000 butyrophilin polypeptide from human milk, significant amounts of butyrophilin in biopsy specimens of non-lactating human breast as well as ductal and lobular carcinomas. Imam and Tökés [46] have recently localized a glycoprotein of molecular weight 70000 not only in lactating and non-lactating human breast but also in ductal breast carcinoma (see also Ref. 47). As judged from the high sialic acid and fucose contents reported by these authors [41], their glycoprotein preparation appears to be different from the butyrophlin preparation described here.

The occurrence of butyrophilin in many mammalian species, its specificity to the lactating mammary epithelial cell, its restricted location on the apical plasma membrane, its concentration on the milk lipid globule membrane and its tightly protein-bound fatty acids are suggestive of special functions of butyrophilin in the process of budding and secretion of the milk lipid globule. Studies on the binding of butyrophilin in the membrane and its role in the formation and structure of the membrane-associated coat might help elucidate these functions.

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