ABSTRACT

The milk fat globule membrane-associated proteins adipophilin (alias adipocyte differentiation-related protein) and butyrophilin were purified from bovine milk by reverse-phase chromatography. The nucleotide sequence of bovine adipophilin was obtained via peptide mapping and sequencing of a mammary gland cDNA clone, which comprises 1841 nucleotides and has an open reading frame of 450 amino acids. By peptide mapping, 19% of the amino acid sequence was confirmed. The obtained amino acid sequence has 87 and 80% identical residues with human and mouse adipophilin, respectively. Alignment with the proteins perilipin and TIP47 revealed two highly conserved segments, which may assemble into amphipathic α-helices.

(Key words: adipophilin, adipocyte differentiation-related protein, butyrophilin)

Abbreviation key: ADRP = adipocyte differentiation-related protein; DTE = dithioerytritol 1,4; MFGM = milk fat globule membrane.

INTRODUCTION

Milk lipids are secreted as triacylglycerols surrounded by a membrane called the milk fat globule membrane (MFGM). The triacylglycerols are generated at the endoplasmatic reticulum in the epithelial cells of the mammary gland and assemble into small droplets. Around the droplet, an electron-dense material, referred to as the coat material is generated and consists of polar phospholipids and proteins. The droplets are transported to the apical plasma membrane. In transit the droplets grow in size, probably by droplet-droplet fusion (3, 5). Once at the apical plasma membrane, the globules are secreted from the epithelial cells by a budding mechanism in which the globule is enveloped by the plasma membrane and eventually pinched off into the milk channel (14). Details of assembly, transport, and secretion of the milk fat globule are still unknown. The coat material is enriched in three proteins: the redox enzyme xanthine oxidoreductase, the mammary gland specific butyrophilin, and adipophilin (9).

Adipophilin (or adipocyte differentiation-related protein, ADRP) is found in all lipid-containing cells but mostly in the mammary gland and in differentiating preadipocytes (8). Adipophilin is one of the earliest markers of adipocyte differentiation in which its mRNA is induced 100-fold within 24 h of the onset of differentiation of preadipocytes (12). During the differentiation the protein is located at the periphery of the intracellular lipid droplets. In the mature adipocyte, adipophilin is replaced by a group of proteins called perilipins. However, in the epithelial cells of the mammary gland, adipophilin is not replaced by the perilipin (2). Instead it is secreted into the milk channel associated with the milk fat globule (9).

It has been shown that the three coat proteins (adipophilin, butyrophilin, and xanthine oxidoreductase) are present in nearly constant proportions in the mild-detergent- or high-salt-insoluble MFGM material and thereby sharing physicochemical characteristics (9). Other experiments have illustrated binding between xanthine oxidoreductase and a recombinant fusion protein comprising the cytoplasmatic tail of butyrophilin (11). Therefore, it has been proposed that interactions between butyrophilin, xanthine oxidoreductase, and adipophilin mediate formation of the protein coat of the MFGM (13).

The present work describes a method to isolate two proteins, butyrophilin and ADRP, from the coat material. Partial amino acid sequence information about adipophilin was obtained by peptide mapping, allowing isolation of a full-length clone from a bovine mammary cDNA library. The deduced amino acid sequence for bovine adipophilin had two regions with a high degree of similarity to regions in other lipid-associated proteins, TIP47 and perilipins.

MATERIALS AND METHODS

Isolation of MFGM and Proteins in Coat Material

All steps were carried out at 4°C unless otherwise indicated. Preparation of MFGM was performed as pre-
viously described (10), using cream from fresh unpasteurized bovine milk. Protein coat material was obtained by treating purified MFGM with 1% Triton X-100 in 0.02 M Tris-HCl, pH 7.0, for 1 h and was precipitated by centrifugation at 20,000 × g for 60 min. The precipitate was resuspended in 0.01 M Tris-HCl, pH 7.0, and the protein solubilized by incubation with 10% SDS and 0.02 M DTE at 60°C for 4 h as previously suggested (9). The detergent was removed by precipitation with 10 volumes of acetone at −20°C overnight and centrifugation at 13,000 × g for 10 min. The precipitate was dissolved in 60% formic acid and applied to a 1-ml Resource RPC column (Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were separated with a linear gradient of 2-propanol in 60% formic acid. The collected samples were diluted to 5% formic acid and were freeze-dried.

Protein Identification and Peptide Mapping

Protein samples were analyzed by SDS-PAGE, electroblotting, sequence analysis, and Western blotting as previously described (1).

To obtain peptides, purified adipophilin (0.01 mg) was reduced, carboxymethylated, and digested with trypsin [1:50 ratio (% wt/wt), protease:substrate] for 1 h at 37°C following standard procedures. Resultant peptide mixtures were separated by narrow bore, reverse-phase HPLC on a 2.1 × 100 mm C2/C18 µRPC column (Amersham Pharmacia Biotech) with a gradient from 0 to 60% acetonitril in 0.1% trifluoroacetic acid at 25°C. Peptide fractions were further purified by changing the column to a 2.1 mm × 100 mm Sephacil C8 narrow bore, reverse-phase column (Amersham Pharmacia Biotech) using the same conditions and solvents as before. Finally, peptides were analyzed by automated Edman degradation, using an Applied Biosystems 477 protein sequencer (Perkin Elmer, Foster City, CA).

Screening of a Bovine Mammary Gland cDNA Library and Subcloning

By using degenerated oligonucleotides (DNA Technology, Aarhus, Denmark) corresponding to four of the peptide sequences, a bovine mammary gland cDNA library (Stratagene, La Jolla, CA) was screened to isolate the adipophilin cDNA. The oligonucleotides were labeled at the 5’ end with [γ-32P]-ATP by T4 polynucleotide kinase. Approximately 500,000 clones were screened by in situ hybridization using the radioactive oligonucleotides. Hybridization was performed overnight in 6x SSC (0.9 M NaCl, 0.09 M tri-natriumcitrate dihydrate, pH 7.0), 2x Denhart (50x Denhart contains 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), and 50 µg of salmon sperm DNA/ml. Positive colonies were isolated by subsequent screening. The insert was subcloned into the pBluescript SK(+) vector (Invitrogen Corporation, Carlsbad, CA) and was sequenced for identification, using the Bigdyce terminator cycle sequencing kit and an ABI Prism 310 Genetic analyzer (Perkin Elmer). One full-length clone was obtained and was sequenced on both strands using a total of 10 oligonucleotides.

RESULTS

The coat material from MFGM was obtained as the insoluble fraction after treatment with 1% Triton X-100. Coat proteins can be brought into solution by incubation with 10% SDS and 0.02 M dithioerytritol (DTE) at 60°C in 4 h. Analysis by SDS-PAGE (Figure 1) showed that the coat material contains major bands at 150 kDa, 66 kDa, and 52 kDa, in agreement with the estimated sizes of xanthine oxidoreductase, butyrphilin, and adipophilin. Furthermore, the two bands at 52 kDa and 47 kDa (PAS-6/7) appeared to be preferentially in MFGM material soluble in 2% SDS sample buffer and not in the coat material.

To separate the proteins present in the coat material, the sample was subjected to reverse-phase HPLC chromatography (Figure 2), which separated the coat material into four major peaks. Evaluation of the obtained fractions by SDS-PAGE revealed that peaks 3 and 4 each contained one major band and a few faint bands.
(Figure 1), and peaks 1 and 2 contained numerous faint bands (not shown). The major band of peak 3 was estimated to be 52 kDa in size, and peak 4 had a major band at 66 kDa.

A fraction representing peak 4 was electroblotted onto a polyvinylidifluoride membrane, and the major band was subjected to N-terminal amino acid sequencing, revealing the identity as butyrophilin. By using the same procedure, the major protein band in peak 3 turned out to be N-terminally blocked and, therefore, had to be determined from internal peptide sequences. The protein at 52 kDa in peak 3 was judged, by band intensity in SDS-PAGE, to be the major constituent (80 to 90%) of the fraction, and it was therefore subjected to peptide mapping without further purification. Upon reduction, carboxymethylation, and digestion with trypsin, the resulting peptides were separated on a C2/C18 µRPC column. Major peptide fractions were further purified on a Sephacil C8 column, and the fractions obtained were analyzed by N-terminal amino acid sequencing. The obtained sequence of seven peptides showed that the protein was adipophilin (9, 12).

Western blotting on the four fractions with antibodies toward xanthine oxidoreductase, CD36, and PAS-6/7 revealed that many of the faint bands were most likely fragments of these major MFGM proteins. The significance of all these fragments is not clear, but some of them might reflect the relatively rough treatment used. By screening a bovine mammary gland cDNA library, with degenerated oligonucleotides derived from the tryptic peptide sequences, a full-length cDNA coding for the bovine adipophilin was obtained. The corresponding amino acid sequence was deduced (Figure 3). The isolated cDNA clone contained 1841 nucleotides and encoded a protein of 450 amino acids, giving a calculated average mass of 49,363 Da. The translational start codon (ATG) was at positions 57 to 59 following an untranslated sequence of 56 nucleotides. The stop codon for translation (TGA) was at positions 1407 to 1409. After the stop codon was a 3′ untranslated region of 432 nucleotides, which comprised two polyadenylation signals (AATAAA), at 1656 to 1661 and 1795 to 1800, respectively. The poly(A) tail started at 1817. The deduced amino acid sequence had 87 and 80% identity when compared with human and mouse adipophilin, respectively. Four of the five cysteine residues were conserved in the human and mouse adipophilin.

**DISCUSSION**

A search in amino acid sequence databases showed that especially perilipins and the newly discovered protein TIP47 were similar to adipophilin. In fact, bovine adipophilin has 27 and 45% identical residues when aligned with the amino acid sequence from human perilipin and TIP47, respectively (Figure 4). Moreover, it...
Figure 3. Nucleotide sequence of the bovine adipophilin cDNA and the translated amino acid sequence. The start codon (ATG), the stop codon (TGA), and the two potential polyadenylation consensus sequences (AATAAA) are double-underlined. The partial amino acid sequences obtained by peptide mapping are underlined.
Figure 4. Alignment of bovine adipophilin with human perilipin and human TIP47. Regions with high similarity are boxed.
can be observed that the three proteins have the highest degree of similarity in their N-terminal regions, corresponding to residues 1 to 100 in adipophilin.

Perilipins are a family of proteins located at the periphery of the intracellular lipid droplets. They share a common N-terminal sequence, but their C-termini differ (6). Because of apparent structural similarities the function of adipophilin and the perilipins may be related. Both are located at the rim of the lipid droplet, which may indicate that the two proteins have a lipid-binding domain at the N-terminus. Human TIP47 is believed to be responsible for the rescue of the mannose 6-phosphate receptor from the endosomes and back to the trans-Golgi network (4). Therefore TIP47 could also have a lipid-binding domain interacting with the membrane of the late endosome when it binds the mannose 6-phosphate receptor.

The hypothesis of an N-terminal lipid-binding domain promoted a search for amphipathic α-helices or fusogenic α-helices, because these two structural motifs have been reported to be a common trait of lipid and membrane-binding proteins, such as the apolipoproteins (15). Special attention was paid to two specific regions located at residues 35 to 54 (region 1) and residues 83 to 100 (region 2) in bovine adipophilin. In comparison, region 1 has 40 and 50% identical residues with perilipin and TIP47, respectively, while region 2 has 61 and 66% identical residues. The presence of a fusogenic α-helix was unlikely because it would require an eventual internal fragment of very hydrophobic nature to be in contact with the core of the lipid droplet. A plot of the two sequences in a helical wheel revealed that Leu43, Cys47, and Met54 of region 1 may form a hydrophobic side. In region 2 Ala83, Leu87, Ile90, Leu94, and Leu97 may also form a hydrophobic side in an amphipathic α-helix. On both sides of the two regions there was a conserved proline residue (residues 41 and 100), which could introduce a bend in the backbone of the protein. In this way, the two proposed amphipathic helices could be exposed from the rest of the protein and thereby run parallel to the rim of the lipid droplet. This finding could explain why the perilipins and adipophilin only show similarity in their N-termini. This region may contain the common lipid-binding motif, whereas the C-termini are responsible for individual functions of the two proteins.

In contrast to perilipin, TIP47 has similarities to adipophilin throughout the entire amino acid sequence. One could speculate that a common function could be as a transport protein, either as a motor protein like kinesin and cytoplasmic dynein or as a ligand to a motor protein like kinecin (7). The role of adipophilin during intracellular transportation of lipid droplets to the apical plasma membrane has not yet been investigated, but functions of TIP47 and adipophilin might turn out to be parallel.

The mass of bovine adipophilin calculated from the isolated cDNA was lower than the mass of the protein determined by migration in SDS-PAGE. This finding could indicate the presence of posttranslational modification(s) of adipophilin. It has been reported (9) that adipophilin was highly acylated with up to 5 to 6 mol long-chain fatty acids/mol of protein. As there are only five cysteines in bovine adipophilin, the primary site of acylation, it is possible that all these residues were modified. There is a conserved cysteine residue at position 47, near the proposed amphipathic α-helix, and if this residue is acylated it may contribute to the lipid binding. It would be very interesting to investigate whether TIP47 and the perilipins have similar acylations on their cysteine residues (or some of them). Furthermore, there is a cysteine residue inside the proposed amphipathic α-helix of the perilipins and adipophilin. If this residue is acylated, it would be interesting to investigate in which way this acylation would affect the formation and the properties of the proposed α-helix.

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REFERENCES


