Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane

Hans W. HEID*, Martina SCHNÖLZER* and Thomas W. KEENAN†‡

*Division of Cell Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Federal Republic of Germany, and

†Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

Milk lipid globules from humans, cows and rats contained a protein identified as adipocyte differentiation-related protein (ADRP) associated with the globule surface membrane material. This protein, previously believed to be specific to adipocytes, was a major constituent of the globule surface and was present in a detergent-insoluble complex that contained stoichiometric amounts of butyrophilin and xanthine oxidase. Identification of ADRP was by sequence similarity of tryptic peptides from cow and human proteins with the sequence inferred from the cDNA for mouse ADRP. The putative ADRP of lipid globules from cow, human and rat milk was recognized specifically by antisera

raised against a peptide synthesized to duplicate the N-terminal 26 residues of the mouse protein. In homogenates of lactating mammary gland, ADRP was found only in endoplasmic reticulum and in lipid droplet fractions. ADRP was modified, apparently post-translationally, and one modification apparently was acylation, primarily with C₁₄, C₁₆ and C₁₈ fatty acids. Two isoelectric variants of ADRP were present in cow globule membrane material. *In vitro*, ADRP served as a substrate for protein kinases associated with milk lipid globule membrane, but this protein did not seem to become phosphorylated intracellularly.

INTRODUCTION

Adipocyte differentiation-related protein (ADRP) is one of the earliest markers of adipocyte differentiation [1,2]. The mRNA for ADRP increases at least 100-fold within 1 day after the induction of differentiation in cultured preadipocytes; this is earlier than the induction of fatty acid-binding protein and lipoprotein lipase, early markers of adipocyte differentiation. The biological function of ADRP is not known. Adipocytes are specialized for synthesis and storage of triacylglycerols for ultimate mobilization and transport to other tissues. Milk-secreting cells in many species are specialized for synthesis of triacylglycerols, their aggregation into droplets and their secretion for utilization by the nursing offspring. The presence of ADRP in both of these terminally differentiated cells implies a role for this protein in triacylglycerol deposition.

Milks of many species contain appreciable amounts of lipid, with a reported range from 0.2% of milk weight for black rhinoceros to 61% for the hooded seal [3]. In many species, milk lipids consist primarily of triacylglycerols contained in globules that have a lipid-rich core surrounded by a specialized membrane called the milk lipid globule membrane (MLGM). Precursors of milk lipid globules are formed at the endoplasmic reticulum and are released into cytosol as small (diameters less than 0.5 µm) triacylglycerol-rich droplets surrounded by a non-bilayer coat of proteins and polar lipids [4–6]. While resident in the cytosol, droplets can grow appreciably in volume, reaching diameters of 5 µm or more before secretion. One growth mechanism is apparently droplet–droplet fusion [7]. During secretion, droplets interact with areas of plasma membrane in the cell apex that are differentiated from bulk plasma membrane by the presence, on the inner membrane face, of a material that stains densely in specimens prepared for conventional electron microscopic exam-

ination [8]. This densely staining inner membrane coat material seems to be enriched with two proteins, butyrophilin [8,9] and xanthine oxidase [10]. Secretion occurs by progressive envelopment of the lipid droplet in plasma membrane up to the point where the droplet is ultimately pinched off from the cell into the luminal space, completely surrounded by plasma membrane. The surface material coating the triacylglycerol-rich core of milk lipid globules, the MLGM, is a complex consisting of plasma membrane-derived membrane plus the material that coated the lipid droplet surface within the cell [11]. In electrophoretic profiles of MLGM we noted the presence of a previously unobserved but major polypeptide constituent and identified this as ADRP. This identification, and some characteristics of ADRP in mammary gland and milk are reported here.

METHODS

Membranes and cell fractions

MLGMs were isolated from the milk of Holstein cows, two human volunteers, and Sprague–Dawley rats [9]. MLGM was fractionated into soluble and insoluble (coat) fractions by extraction with 1% (w/v) Triton X-100/1.5 M NaCl/10 mM Tris (pH 7) [9], by extraction with 1.5% (w/v) dodecyl-β-D-maltoside/0.75 M aminohexanoic acid/10 mM Hepes (pH 7.0) [12] or by sequential extraction with these two detergent-containing solutions. MLGM was suspended in the detergent-containing solution by using an all-glass homogenizer, and held on ice for 30 to 60 min, after which insoluble and soluble materials were separated by centrifugation for 60 min at 2 °C and 150 000 g. Mammary tissues were collected from rats between the seventh and fourteenth days of lactation, and from lactating cows slaughtered at a meat-packing plant. Endoplasmic reticulum [6], cytoplasmic lipid droplet [5], cytosol [6], plasma membrane [13]

and Golgi apparatus [14] fractions were isolated from tissue homogenates. Epididymal fat pads were collected from male Sprague–Dawley rats of 150 g body weight, and adipocytes were prepared from this tissue by dissociation with collagenase [15].

Protein separation, detection and sequence analysis

Electrophoretic separations were by the method of Laemmli [16], in 8–16% (w/v) acrylamide gradient gels, and staining was with Coomassie Blue. MLGM coat preparations, poorly dispersed by standard sample preparation conditions [heating for 5 min in 2% (w/v) SDS solution], were dispersed with ultrasound in sample fixation solutions containing up to 10% (w/v) SDS and 1 M 2-mercaptoethanol or 50 mM dithiothreitol, and were heated at 60 °C for 4–10 h. Two-dimensional electrophoretic separations were by the method of O'Farrell [17]. Polypeptides were transferred to nitrocellulose or to poly(vinylidene difluoride) (PVDF) membranes for immunodetection or for sequence analysis [18]. Protein was estimated with bicinchoninic acid reagent (Pierce Chemical Company, Rockford, IL, U.S.A.). Coomassie-stained gels were scanned with a Shimadzu flying-spot densitometer to estimate the proportions of polypeptides. For sequence analysis, peptides were generated by incubation of samples on membranes with trypsin (Promega) [19], peptides were separated in a reverse-phase (Spherisorb) column, and sequences were determined with an ABI Procise 494 sequencer [20]. Masses of peptides were determined by matrix-assisted laser desorption ionization mass spectrometry with a Finnegan Vision 2000 mass spectrometer.

Antibodies and ligand binding

Antisera recognizing ADRP were prepared by synthesizing a peptide corresponding to the first 26 residues predicted from the mouse cDNA sequence [1], adding cysteine as the C-terminal residue, and coupling this peptide to keyhole limpet haemocyanin. This construct was used to immunize rabbits and guinea pigs. Antisera to perilipin was prepared by the same procedure with a synthetic peptide prepared to duplicate the N-terminal 24 residues predicted from the rat perilipin cDNA [21]. Antibodies to butyrophilin [9] and to the MLGM coat fraction [5] also were used. Antibodies bound by polypeptides on nitrocellulose or PVDF membranes were detected by incubation with peroxidase-conjugated second antibody, with an enhanced chemiluminescence kit (Amersham).

Fatty acylation and phosphorylation

Covalently bound fatty acyl groups in coat-associated polypeptides were determined by excision of constituents from polyacrylamide gels that had been stained with Coomassie Blue. Gel strips were minced finely and extracted exhaustively with DMSO, methanol and chloroform/methanol mixtures to remove buffer salts, SDS and unbound lipids [22]. Samples were then incubated for 90 min at 37 °C in sealed tubes with 0.5 M KOH in 80% (v/v) methanol. After neutralization with HCl, released lipid material was recovered by three extractions with hexane. The minced gel was then recovered and incubated at 90 °C for 3 h with 3 M HCl in 80% (v/v) methanol, after which lipid material was recovered by three extractions with hexane. Methyl esters were formed with boron trifluoride in methanol [23] and were separated on high-performance TLC plates (Whatman) developed with hexane/diethyl ether/acetic acid (42:8:0.5, by vol.). Separated constituents were made visible by charring with cupric acetate in phosphoric acid [24], and plates were scanned with a densitometer operated at 562 nm in the reflectance mode. Methyl esters were purified by preparative TLC. Esters were

detected by spraying the edge of the plate with iodine spray, and the esters from unstained regions were recovered. Methyl esters were separated in a Perkin-Elmer Autosystem gas chromatograph equipped with flame ionization detectors. The 30 m × 0.25 mm column was coated with a 0.1 µm film of Stabilwax (Restek Corporation). The identities of major methyl esters were confirmed by mass spectrometric analysis with a VG Analytical 7070 instrument with a gas chromatograph inlet.

Protein kinase assays were made with freshly prepared, unfractionated cow MLGM and with freshly isolated endoplasmic reticulum and cytoplasmic lipid droplet fractions. Fractions suspended in buffer (1 mg of protein for MLGM and lipid droplets, 2 mg of protein for endoplasmic reticulum) were incubated for 30 min at 37 °C in 25 mM Hepes (pH 7.0)/25 mM KCl/2.5 mM magnesium acetate/1 mM EGTA/0.1 mM sodium orthovanadate/50 mM NaF/5 mM sodium pyrophosphate/20 µM ATP containing 200 µCi of [γ -³²P]ATP (DuPont–NEN). After incubation, the reaction mixture was made to 1.5% (w/v) Triton X-100 and 1.5 M NaCl, held on ice for 30 min and then centrifuged for 30 min at 2 °C and 200 000 g. The pellet material was washed with 1.5% (w/v) Triton X-100, separated by electrophoresis and transferred to a PVDF membrane. Radioactivity was detected by exposure to X-ray film at –70 °C. The band corresponding in mobility to ADRP was excised from the PVDF sheet and hydrolysed with HCl, after which amino acids were separated on a cellulose-coated plate by electrophoresis in one direction at pH 1.9, followed by electrophoresis in the perpendicular direction at pH 3.5. Detection was by exposure to X-ray film. Carrier phosphorylated amino acids, added to the hydrolysate, were detected with ninhydrin.

One lactating rat was injected intraperitoneally with 2 mCi of [³²P]P_i (DuPont–NEN) and held with the litter for 12 h. Milk was collected, the animal was killed and endoplasmic reticulum and cytoplasmic lipid droplets were isolated from the homogenate of the mammary glands. Acini were prepared from minced mammary tissue from a cow [25], suspended in phosphate-free Krebs–Ringer bicarbonate (Sigma Chemical Company) and 20 µCi/ml [³²P]P_i was added. After incubation for 90 and 180 min at 37 °C in air/CO₂ (19:1), acini were recovered, washed, homogenized, and endoplasmic reticulum and lipid droplet fractions were isolated. Proteins in these fractions were separated by electrophoresis and transferred to PVDF, after which the membrane was exposed to X-ray film.

RESULTS

When MLGM is extracted at high ionic strength with non-ionic or weak ionic detergents, an insoluble material is obtained that is enriched in butyrophilin and that has morphological characteristics like those of the densely staining material observed on the inner face of regions of apical plasma membrane with which lipid droplets seem to interact [8,9]. When examining this material via conventional SDS/PAGE, we observed a previously undescribed polypeptide with a molecular mass of approx. 52 kDa (Figure 1). This constituent was not apparent in SDS/PAGE patterns of untreated MLGM, as a glycosylated protein known as glycoprotein B is dominant in this molecular mass region. Glycoprotein B of cow MLGM migrates as a doublet because of differential glycosylation [26,27]. This glycoprotein, which in the mouse has epidermal growth factor-like domains [28], can be dissociated from the membrane quantitatively with non-ionic detergent plus high salt (Figure 1).

The detergent/high-salt-insoluble coat material of MLGM did not dissociate entirely during conventional sample preparation for SDS/PAGE, and we established that conventional prep-

Figure 1 Electrophoretic profiles of cow MLGM

Shown are the detergent/high-salt-insoluble coat material from cow MLGM (lanes 1 and 2), and the detergent/high-salt-soluble material from this membrane (lane 3). Xanthine oxidase (XO), butyrophilin (BU), and ADRP (AD) are identified. Arrows along lane 3 denote the glycoprotein B doublet bands. The positions of molecular mass markers are marked at the right in kDa.

aration methods resulted in an under-representation of the amount of the 52 kDa polypeptide in gel patterns. Heating samples for extended times (several hours to overnight at 60 °C) in solutions containing 10 % (w/v) SDS more effectively solubilized this coat-derived material, and in samples so prepared the 52 kDa polypeptide was a prominent constituent in material from both human and cow milks.

Tryptic peptides generated from the 52 kDa polypeptide displayed extensive similarity to the sequence inferred from the cDNA for mouse ADRP (Figure 2). With the protein from cow MLGM a sequence was obtained from nine tryptic peptides, and of the 133 residues sequenced, 110 were identical with residues predicted from the mouse cDNA. From the human milk protein, sequence information was obtained for six tryptic peptides. Of the 87 residues sequenced, 74 were identical with those predicted from the mouse cDNA sequence. Many of the mismatched residues were conservative substitutions. The size estimated for the cow MLGM-associated polypeptide of about 52 kDa was larger than the 50 kDa calculated from the mouse cDNA sequence. The human MLGM polypeptide migrated as a molecule approx. 1 kDa larger than from cow MLGM under SDS/PAGE (Figures 3a and 3b).

Antisera raised against a synthetic peptide duplicating the N-terminal 26 residues of mouse ADRP recognized the polypeptide identified from sequence similarity as ADRP in human and cow, as well as rat, MLGM (Figures 3a and 3b). Antibody recognizing the putative ADRP in cow, human and rat MLGM was obtained also by immunizing animals with the detergent/high-salt-insoluble residue of cow MLGM and subsequent immunoaffinity purification of antibody over a cow MLGM-derived ADRP-affinity matrix [29]. Both anti-peptide (Figure 3c) and anti-protein (results not shown) antibodies recognized a polypeptide present in endoplasmic reticulum and cytoplasmic lipid droplet fractions from cow mammary gland. Antisera against a synthetic peptide duplicating the N-terminal 24 residues of perilipin did not recognize the putative ADRP in cow or human MLGM material, but this antisera did recognize polypeptides migrating as perilipins A and B in adipocytes from rat epididymal fat pads (results not shown).

M:	R ¹⁵	V A N L P L V S S T Y D L V S
C:		V A N L P L V S S T Y D L V S
M:	K ³⁷	D Q Y P Y L R
H:		D Q Y P Y L K
M:	K ⁵⁵	T V T S A A M T S A L P I I Q K
C:		T I T S V A Y T S A L
H:		T I T S V A M T S A L P I I Q K
M:	K ⁷¹	L E P Q I A V
C:		L E P Q I A V
M:	R ⁸⁹	M E E R L P I L N Q P T S E I V A S A R
C:		I E E K L P I L N Q P T N Q V V A N A K
H:		X X X X L P I L N Q P S T Q I V A N A K
M:	G ¹⁶⁵	M V Q F M N S G V D N A I T K S E M L V D Q Y F P L T Q E E
C:		V M Q L M S G V E N A L T K S E L L V D Q Y L P L T Q E E
M:	K ²⁰¹	K V E G F D M V Q K P S N Y E R
C:		K V E G F D L V Q K P S Y Y Y R
H:		K V E G F D L V Q K P S Y Y Y R
M:	K ²²⁸	A Y H Q A L S R
C:		A Y Q Q A L X R
M:	K ²⁴³	S Q E T I S Q L H S T V H L I E F A R
C:		S Q E X I S Q L H
H:		S Q Q T I S Q L H S T V H L I E F A R
M:	R ²⁸⁸	S I G Y D D T D E S H C V E H I E S R
C:		S I G Y D D T D E S H C A E H I E S R
M:	K ³⁴²	H L G V M A G D I Y S V F R
H:		H M G V M A G D I Y S V F R

Figure 2 Sequence comparison of the ADRP-like polypeptide

Sequences of tryptic peptides from human (H) and cow (C) MLGM-derived ADRP are shown together with sequences predicted from the mouse (M) cDNA [1]. Non-identical residues are identified by underlined italics.

With cow-derived specimens, the putative ADRP of endoplasmic reticulum migrated as a 47 kDa molecule (Figure 3c), whereas the ADRP associated both with intracellular lipid droplets and with MLGM migrated as a 52 kDa polypeptide (Figure 3c). Migration patterns were suggestive of possible post-translational modification of ADRP in the endoplasmic reticulum. Despite the presence of ADRP in endoplasmic reticulum, no characteristic leader sequence or transmembrane domain can be predicted from the inferred amino acid sequence. Over half (28 of 50) of the residues in the N-terminal segment of ADRP are hydrophobic, and this domain could be used for membrane insertion. We could not detect ADRP, in Western blots, in polypeptides of cytosol, Golgi apparatus and plasma membrane fractions from mammary gland.

Mass spectral measurement of molecular masses of tryptic peptides provided results suggestive of post-translational modification of MLGM-associated ADRP. The tryptic peptide derived from human ADRP and corresponding in sequence with the 16 residues following Arg-93 in the mouse sequence (Figure 2) has a predicted mass of 1608.9 Da, but the mass determined was 1920.5 Da. This mass difference does not correspond to any single known post-synthetic protein modification. ADRP has 18 Ser/Thr-containing potential kinase recognition sites, but none of them is in this peptide. ADRP, in Western blots, did not give a positive reaction with two different commercial glycan detection

Figure 3 Electrophoretic comparison of cow, human and rat MLGM

(a) The detergent and salt-insoluble MLGM coats from cow (lane 1), human (lane 3) and rat (lane 4) are shown, along with unfractionated human MLGM (lane 2). Arrowheads denote the constituent identified as ADRP. The positions of molecular mass markers are indicated at the right. (b) Polyclonal antiserum against a synthetic peptide duplicating the N-terminal 26 residues of mouse ADRP recognized an ADRP-like polypeptide in MLGM coat material from cow (lane 1), human (lane 2) and rat (lane 3) milks. (c) Anti-peptide antibody recognized a single major polypeptide in endoplasmic reticulum (lane 1), cytoplasmic lipid droplets (lane 2), and MLGM (lane 3) specimens from cow. In both (b) and (c) the bar at the left denotes the position of the 53 kDa marker.

Table 1 Quantitative analysis of fatty acids released from ADRP and butyrophilin from cow milk lipid globule membrane

Results are percentages by weight of the total fatty acid methyl ester pool; acids accounting for less than 0.5% of the total in either protein were excluded. Identification of the major methyl esters (myristate, palmitate, stearate and oleate) from ADRP was confirmed by mass spectrometry.

Fatty acid methyl ester	Fatty acid source ...	Fatty acid released (%)	
		ADRP	Butyrophilin
Laurate		1.7	0
Tridecanoate		0.7	1.0
Myristate		9.7	5.0
Pentadecanoate		0.8	0.9
Palmitate		48.1	44.2
Palmitoleate		0	3.2
Heptadecanoate		0.4	1.1
Stearate		22.0	26.8
Oleate		16.1	16.5
Linoleate		0	1.2

kits, implying that it is not glycosylated. Prenylation or phosphatidyl inositol-glycan anchors cannot be predicted from the sequence inferred from the cDNA.

Among potential post-translational modifications, acylation with fatty acids seemed a possibility. ADRP recovered from polyacrylamide gels released fatty acids when treated with alkali under conditions that would release ester-bonded but not amide-bonded fatty acids. Little additional fatty acid was released by acid hydrolysis, to release fatty acids from amide bonds, after

Figure 4 Two-dimensional separation of cow MLGM coat proteins

(a) The gel was stained with Coomassie Blue after separation by non-equilibrium isoelectric focusing in the horizontal direction (arrow) followed by SDS/PAGE in the vertical direction (arrow). (b) Antisera against the N-terminal peptide of ADRP identified two isoelectric variants of ADRP in cow MLGM. Abbreviations: XO, xanthine oxidase; BU, butyrophilin; AD, ADRP. The bars and numbers along the bottom of (a) denote the pH values of those regions of the isoelectric focusing gel.

incubation with 0.5 M KOH. Of the total fatty acid recovered by alkali and then acid hydrolysis, 97% was released by the alkali treatment. Eight different fatty acids were present in material released from ADRP (Table 1), ranging in chain lengths from C_{12} to C_{18} . The major acids, accounting for approx. 98% of the total, were myristate, palmitate, stearate and oleate plus other $C_{18:1}$ isomers. The identities of these fatty acids were established by the coincidence of retention times with standards and by mass spectral analysis. A similar pattern was found with butyrophilin isolated in parallel with ADRP samples, as was also shown previously [23]. There were differences in acyl groups between ADRP and butyrophilin (Table 1). Measurable quantities of palmitoleate and linoleate were absent from ADRP but were found in butyrophilin. On a molar basis, about 3-fold more fatty acid was obtained from ADRP than from butyrophilin.

Perilipin is the only protein in the sequence database that has some similarity to ADRP. Through the first 105 residues, perilipin and ADRP have approx. 60% similarity. Perilipin, a protein associated with adipocyte lipid droplets, is not expressed in mammary epithelial cells [30]. Antiserum that we raised against the N-terminal peptide of ADRP did not recognize perilipin in adipocytes, and antiserum prepared against the N-terminal peptide of perilipin did not recognize any protein of MLGM (results not shown). This anti-perilipin antiserum did recognize polypeptides migrating with molecular masses of approx. 56 and 46 kDa, corresponding to perilipins A and B, in Western blots of material from rat adipocytes. Perilipins A and B, protein isoforms that arise by alternative RNA splicing [21], are phosphorylated on serine and threonine residues on the lipolytic stimulation of adipocytes [21]. Perilipin also is expressed in steroidogenic cells and is associated with the storage cholesterol ester droplets in these cells [31]. In an attempt to determine whether ADRP might be phosphorylated, we found that, on two-dimensional separation of cow MLGM material, ADRP focused as at least two isoelectric variants, with the same molecular mass but with isoelectric points between 7.5 and 7.8 (Figure 4). We found that

Figure 5 Phosphorylation of ADRP

(a) When MLGM from fresh cow milk was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, polypeptides corresponding in mobility to butyrophilin (top arrowhead), ADRP (middle arrowhead) and $\alpha_{\text{s}1}$ -casein (bottom arrowhead) were prominently labelled. (b) When the polypeptide corresponding to ADRP was hydrolysed, radiolabel was detected in phosphorylated derivatives of serine (S) and threonine (T). O denotes the origin, and the unlabelled constituents were partial hydrolysis products.

the ADRP of MLGM could serve as a substrate for kinase activity endogenous to this membrane.

When cow MLGM isolated from freshly obtained milk was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, one of the prominent radiolabelled constituents was a polypeptide that co-migrated with, and was recognized by antibodies to, ADRP (Figure 5a). Butyrophilin, identified with a specific antibody, and a constituent corresponding in mobility to $\alpha_{\text{s}1}$ -casein, the major phosphoprotein of cow milk, also were labelled. The casein, identified only by coincidence of mobility with the milk protein, was not detected in replicas of the blot stained with Coomassie Blue. Both ^{32}P -labelled Ser and ^{32}P -labelled Thr were identified in hydrolysis products from labelled ADRP (Figure 5b). The polypeptide corresponding to ADRP was not labelled when endoplasmic reticulum or cytosolic lipid droplets were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under identical conditions. The ADRP-like band in milk lipid globules and in endoplasmic reticulum and cytoplasmic lipid droplets was not labelled when $[\text{}^{32}\text{P}]\text{P}_i$ was injected into a lactating rat 12 h before sample collection. However, caseins were labelled extensively in the milk from this animal. With mammary acini (clumps of epithelial cells resembling alveoli) from a lactating cow [25], ^{32}P from P_i was not incorporated into ADRP of endoplasmic reticulum or lipid droplets on incubation in unlabelled phosphate-free Krebs–Ringer bicarbonate for 3 h. Under these conditions, caseins were labelled.

Only a small proportion of the ADRP associated with the membrane, as determined by probing blots with antibody, could be solubilized with detergents such as Triton X-100 or dodecyl- β -D-maltoside; however, larger proportions of the membrane-associated butyrophilin and xanthine oxidase were solubilized by these treatments. Heating this mild-detergent-insoluble residue in solutions containing SDS and a reducing reagent dissolved some of this residue, but not all was dissociated except under prolonged incubation at elevated temperature. As judged by densitometric scanning of electrophoretic gels stained with Coomassie Blue, the material that dissolved at each of several extraction steps with SDS and β -mercaptoethanol contained nearly constant proportions of xanthine oxidase, butyrophilin and ADRP. By weight, butyrophilin, xanthine oxidase and ADRP accounted for approx. 45%, 25% and 20% respectively of the total protein in the mild-detergent/high-salt-insoluble

MLGM coat material. As calculated from polypeptide molecular masses predicted from cDNA sequences, butyrophilin, ADRP and xanthine oxidase were present in respective molar ratios of approx. 5:3:1 in this coat material.

DISCUSSION

Although the function of ADRP remains unknown, the expression of this protein in milk-secreting cells, as well as adipocytes, provides a tantalizing suggestion that it might be involved in the deposition of triacylglycerols into droplets in the cytosol, a phenomenon known to occur both in adipocytes and in mammary epithelial cells. In mammary cells, ADRP seems to be concentrated specifically in cellular components involved in the generation of milk lipid globules. The hydrophobic N-terminus of this protein, coupled with an apparent covalent attachment of fatty acids, seems to make it well suited for interaction with lipid droplets. Indeed, myristoylation and palmitoylation of hydrophobic peptides increased peptide partitioning into lipid micelles in model systems [32]. On the basis of the ratio of butyrophilin and ADRP in Coomassie Blue-stained gels, and the amount of fatty acid recovered directly from PAGE bands, we estimated an acyl group content of 5–6 mol per mol of protein for ADRP. That several fatty acyl groups were recovered from ADRP provides a suggestion that acylation of this protein, as in butyrophilin [23], is not specific.

That there are at least two charge forms of ADRP in cow MLGM was evident from isoelectric focusing. How these forms differ remains to be established. ADRP was phosphorylated by a kinase associated with MGLM, but this phosphorylation did not seem to be an intracellular event, as ADRPs in mammary gland and milk from a rat injected with $[\text{}^{32}\text{P}]\text{P}_i$ remained unlabelled. Thus the phosphorylation of ADRP *in vitro* might be fortuitous. The ADRP of endoplasmic reticulum and lipid droplets from cow mammary acini incubated with $[\text{}^{32}\text{P}]\text{P}_i$ were not labelled. We conclude from this that ADRP is probably not phosphorylated intracellularly. One possibility that we cannot exclude is that ADRP might be phosphorylated after milk secretion. Freshly secreted milk does contain a supply of ATP that could potentially be used for this purpose [33].

Our amino acid sequence information, although incomplete, provides evidence that ADRP might be highly conserved among mouse, cow and human, and the immunological cross-reactivity noted suggests that this conservation might extend to the rat as well. In contrast, although a butyrophilin-like protein is a major constituent of lipid globule membranes from several species, there is a much more limited immunological cross-reactivity among species for this protein [34]. Although a role for butyrophilin in the secretion of milk lipid globules has been suggested on the basis of its properties and localization in the apical plasma membrane and in MLGM [8,34], the inferred primary sequence [35] provided no clues as to how it might be involved in secretion.

Xanthine oxidase is a cytosolic protein, but in milk-secreting cells a proportion of the xanthine oxidase interacts with the apical cell surface and is secreted with milk lipid globules [10,36]. Some of the MLGM-associated xanthine oxidase is bound tightly to the mild-detergent/high-salt-insoluble inner membrane coat structure. Xanthine oxidase complexes specifically with the cytoplasmic portion of butyrophilin [37], but the inferred primary sequence of xanthine oxidase [38,39] provided no clue as to what specific role this enzyme could play in lipid globule secretion.

Because ADRP is apparently synthesized without a signal peptide, and lacks a transmembrane sequence [1], secretion of this protein would not be predicted. That it is secreted must be due to its interaction with the lipid droplets secreted as milk lipid

globules. In both adipocytes [30] and milk-secreting cells [37], triacylglycerol-rich droplets form in or on the endoplasmic reticulum membrane, and the surface coat on forming droplets in both cell types seems to be contiguous with endoplasmic reticulum membrane leaflets. Both perilipin [30] and ADRP ([1], and H. W. Heid and T. W. Keenan, unpublished work) are present on lipid droplet surfaces in adipocytes, but perilipin is not expressed in milk-secreting cells [30].

Recently it was shown that treatment of rats with etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase I, induced the expression of ADRP in liver [40]. Lipid droplets also accumulated in livers of etomoxir-treated rats. This finding, together with the finding that ADRP is associated with lipid droplets in milk-secreting cells and in adipocytes, provides the basis for suggesting that ADRP might be involved in lipid deposition into droplets. We speculate that in milk-secreting cells a function of ADRP additional to a potential role in triacylglycerol deposition might be as an adaptor molecule involved in interaction between the droplet surface and the butyrophilin/xanthine oxidase-containing inner membrane coat of apical plasma membrane.

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