FATTY ACID SYNTHASE IS A MAJOR POLYPEPTIDE CONSTITUENT OF CYTOSOLIC LIPOPROTEIN AND IS ASSOCIATED WITH COMPONENTS OF THE MILK LIPID SECRETORY PATHWAY

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(ABSTRACT)

Most of the lipid present in lactating mammary gland cytosol was associated with a high molecular weight aggregate isolated from cytosol by gel exclusion chromatography or by density gradient centrifugation. The major polypeptide constituent of this lipoprotein aggregate was the monomer of fatty acid synthase (FAS). The major milk lipid globule proteins, butyrophilin (Bu) and xanthine oxidase (XO), as well as the small GTP-binding protein ARF, also were present. This lipoprotein complex was abundant in cytosol from lactating but not from involuting mammary glands. HPTLC analysis of lipids extracted from the low density FAS (LDFAS) complex demonstrated the presence of the five major milk phospholipids as well as triacylglycerols, cholesterol, unesterified fatty acids, and diacylglycerols. ^{32}P-labeled phospholipids present in cytosol could be transferred to microlipid droplets (MLD) and endoplasmic reticulum (ER), *in vitro*, and could be precipitated along with FAS, and other polypeptide constituents of the LDFAS complex. Complexed FAS could be separated from noncomplexed FAS by density gradient centrifugation, native PAGE, and gel exclusion chromatography. A large amount of phospholipid

consistently was retained with the complexed form of FAS. These results suggest that FAS migrates to a low density fraction by virtue of its association to other proteins and lipids.

FAS was found to be associated with ER, intracellular lipid droplets, and the milk lipid globule membrane (MLGM). A similar complex to LDFAS was isolated from ER from liver and mammary gland homogenates following incubation in buffer containing ATP. Polypeptide constituents of this complex had similar electrophoretic patterns to LDFAS, but behaved differently from LDFAS constituents when fractionated with the detergent TX-114. While most of the polypeptides in LDFAS partitioned equally into the detergent and aqueous phases, a constituent with an approximate molecular weight of 70 kDa was enriched in the detergent phase. For the ER-derived FAS complex, most of the polypeptides remained in the aqueous phase but the detergent phase also was enriched with a polypeptide similar in size to the LDFAS detergent enriched constituent. Western blot analysis failed to detect Bu in the ER-derived complex. However, protein disulfide isomerase (PDI) was detected in this complex as well as a polypeptide with approximate molecular weight 50 kDa that cross-reacted with PDI antibody. Extraction of lipids from this ER-derived complex demonstrated the presence of large quantities of unesterified fatty acids, with relatively low amounts of complex lipids.

In studies using ^{125}I labeled LDFAS, labeled polypeptides were shown to associate with ER and intracellular lipid droplets and their dissociation was

stimulated by ATP. Immunocytochemistry using antibody to rat liver FAS revealed distribution of FAS at localized regions of the cytoplasmic surface of rough endoplasmic reticulum and on surfaces of intracellular lipid droplets. Electron micrographs of the LDFAS complex showed a homogeneous morphology of granular, symmetrical particles ranging in size from 40 nm to 170 nm in diameter. These particles resembled low density lipoprotein (LDL) in morphology.

From the available data, the following model was proposed for a possible involvement of FAS in lipid droplet secretion in the mammary gland. During active lipogenesis, FAS is targeted to ER membrane by association with a signal or targeting peptide(s) in the cytosol. The signal peptide then binds to selected regions of ER where signal receptors reside. Binding of FAS may initiate synthesis and accumulation of triacylglycerol between ER membrane bilayers. Upon the achievement of a "critical mass", the lipid core may be released into the cytoplasm in an ATP-dependent manner, surrounded by the membrane components that provided the hydrophobic pocket for lipid accumulation. Butyrophilin and the 70 kDa detergent-extractable constituent released from the ER and present in LDFAS are possible sources of such a function. Polypeptides from the cytosolic leaflet of the ER, and proteins peripherally associated with the leaflet then would comprise the polypeptide constituents of the lipid particle.

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DEDICATION

This dissertation is dedicated to my parents for their unconditional love and support and for the sacrifices they have made and continue to make for their children. I also dedicate this dissertation to Paul, for growing with me and for our loving and shared relationship. I further dedicate this final work for my Ph.D. degree to my mentor, Tom, for showing me by example, how to do science and to enjoy doing science.

Finally I dedicate my work to my family, and to special people who are watching over me; Omma and Oppa, Nana and Grandpa, John, Uncle Bob and Aunt Marie.

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AMP-PNP	5'adenylylimidodiphosphate
Apo B	apoprotein B
ARF	ADP ribosylation factor
CLD	cytoplasmic lipid droplets
cyt KBR	high salt-treated cytosol
DMS	dimethyl suberimidate
DPM	disintegrations per minute
DTT	dithiothreitol
ER	endoplasmic reticulum
FAS	fatty acid synthase
FPLC	fast protein liquid chromatography
G3P	glycerol-3-phosphate
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid
HKM	HEPES buffered KCl and magnesium acetate
HPTLC	high performance thin layer chromatography
IgG	immunoglobulin G
kDa	kilodaltons
LDFAS or LDF	low density fatty acid synthase
LDL	low density lipoprotein
MLD	micro lipid droplet
MLG	milk lipid globule
MLGM	milk lipid globule membrane
NSF	N-ethylmaleaimide sensitive factor
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
PVDF	polyvinylidene diflouride
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
SPM	sphingomyelin
TLC	thin layer chromatography
TX114	Triton X-114
VLDL	very low density lipoprotein

I

LITERATURE REVIEW

Introduction

The function of mammary epithelial cells is to synthesize milk constituents for the provision of nutrients for the young. Protein and carbohydrate synthesis and secretion in mammary cells occur by a typical endomembrane secretory pathway (Franke et al., 1976; Keenan et al., 1978; Keenan et al., 1979). Although lipid synthesis is similar to that in other cell types, the packaging and secretion of lipid by mammary epithelial cells occurs by a unique process (Bargman and Knoop, 1959; Franke et al., 1976; Peixoto de Menezes and Pinto de Silva, 1978). In most cell types triacylglycerols are the principal form for storage and transport of fatty acids. The insolubility of triacylglycerols in an aqueous environment necessitates their association with proteins as well as other lipid forms. In plasma, lipids are stabilized by association with proteins in a complex referred to as the lipoprotein complex. The surface proteins (apoproteins) stabilize the lipid and provide markers that mediate interactions of lipoprotein particles with cell-surface receptors and enzymes. The specificity of apolipoproteins for their receptors provides specific targeting of lipid to cells requiring fatty acid as a substrate for β-

oxidation or as a precursor for lipid synthesis (Davis, 1991). In mammary epithelial cells, fatty acids are incorporated into triacylglycerols that are packaged, transported and secreted as milk lipid droplets (review, Keenan and Dylewski, 1993). During the assembly and growth of droplets prior to secretion, proteins and polar lipids associate with the lipid core, forming a surface that stabilizes the triacylglycerol in the aqueous environment of the cell. The secreted product is stabilized in milk by a tripartite membrane structure resulting from the envelopment of the intracellular droplet by differentiated regions of the apical plasma membrane upon its secretion from the cell.

The Milk Lipid Globule

The milk lipid globule (MLG) is comprised of a triacylglycerol-rich core surrounded by a tripartite membrane structure (Keenan and Patton, 1993). The membrane, referred to as the milk lipid globule membrane (MLGM), originates from the endoplasmic reticulum (ER), differentiated regions of the apical plasma membrane, and possibly other intracellular contributors yet undetermined. The polar lipid and protein monolayer that encases the lipid core prior to secretion originates, at least in part, from the ER (Dylewski et al., 1984; Deeney et al., 1985). The primary unit membrane bilayer is derived

from localized differentiated regions of the apical plasma membrane that envelop the droplet as it is extruded from the cell. These differentiated regions of the apical plasma membrane are characterized by a dense proteinacious coat material associated with the cytoplasmic surface of the bilayer. The monolayer surrounding the intracellular lipid droplets serves to stabilize the lipid and prevent aggregation as it traverses the aqueous environment of the cell. Additionally, it is the surface coat that mediates any interactions of lipid droplets with intracellular components. The fusion events that result in growth of lipid droplets likely involve interactions between surface components of lipid droplets (Valivullah et al., 1988). If cytoskeletal elements are involved in intracellular transport of lipid droplets, it is likely by interaction with components of the surface monolayer. Prior to extrusion from the cell, lipid droplets come in contact with differentiated regions of the apical plasma membrane. It is has been suggested that it is the droplet surface material that interacts directly or indirectly with the cytoplasmic coat material at the plasma membrane (review, Keenan and Patton, 1993; Keenan and Dylewski, 1993).

The intracellular origin of the MLG is the endoplasmic reticulum (review, Keenan and Dylewski, 1993). The earliest precursor lipid droplets that have been observed by electron microscopy are less than 0.5 micrometers in diameter and have been termed microlipid droplets (MLD; Dylewksi et al., 1984). Precursor droplets appear in micrographs as lipid-rich cores

surrounded by an amorphous granular coat material lacking unit membrane structure. These droplets grow in size as they traverse the cell in the apical direction towards differentiated sites of secretion at the plasma membrane. The larger droplets are referred to as cytoplasmic lipid droplets (CLD) and are classified as those droplets with diameters greater than 1.0 micrometer. Identification of intracellular milk lipid globule precursors prompted studies of spatial and temporal events within the mammary epithelial cell that result in the secretion of the milk lipid globule.

Intracellular Origin of Milk Lipid Globules

Composition of Milk Fat

The amount of fat present in milk varies from species to species and appears to reflect environmental challenges to the survival of the neonate (Jenness, 1974). The amount of lipid by weight in milk from humans and cows is approximately 4%. In rats and other laboratory animals, lipid accounts for 5 - 10% by weight, and in blue whales and other marine mammals lipid can account for up to 50% by weight of milk. Although quantitatively fat content varies between species, qualitatively lipid content is remarkably constant. By mass, 90% or more of milk fat is triacylglycerol (Christie, 1983). The

remaining lipid mass is accounted for largely by free and esterified sterols, partial glycerides, and phospholipids that comprise the membrane that encases and stabilizes the triacylglycerol droplet. Between species milk fat can be differentiated by variations in the extent of desaturation of long chain esterified fatty acids and by chain length. Ruminant milk is characterized by triacylglycerols possessing a high percentage of short chain fatty acids ($C_{4:0}$ and $C_{6:0}$), while that of non-ruminants contains a high percentage of medium chain fatty acids ($C_{8:0}$ - $C_{12:0}$). The occurrence of medium and short chain fatty acids is a unique feature of milk triacylglycerols and reflects a tissue-specific synthesis by differentiated mammary epithelial cells (Smith and Abraham, 1975; Christie, 1983).

Triacylglycerol Synthesis

Triacylglycerols are synthesized by esterification of activated fatty acids to glycerol (Brindley, 1991). The fatty acids incorporated into triacylglycerol in mammary epithelial cells are derived from two sources, uptake from plasma triacylglycerols and de novo synthesis. Long chain fatty acids, released from plasma lipoproteins, are taken up by the mammary epithelial cells at the basal membrane. Low density lipoprotein (LDL; d 1.005 - 1.063) and chylomicrons ($d < 1.00$) are the major lipoprotein contributors of triacylglycerols to the

mammary gland. Mammary gland does not take up cholesterol and phospholipid from these lipoprotein fractions, indicating that in mammary gland the role of the lipoproteins is to provide triacylglycerols to the mammary epithelial cell during lactation (Gooden and Lascelles, 1991).

Circulating chylomicrons and very low density lipoproteins (VLDL) interact with lipoprotein lipases at the luminal surface of capillary endothelial cells (Glauman et al., 1975; Fielding and Fielding, 1991). The lipases hydrolyze triacylglycerol and release unesterified fatty acids and monoacylglycerols that are then taken up by the endothelial cells and transported to the interstitial space for uptake by adjacent epithelial cells. The unesterified fatty acids bind to albumin in the serum to form stable complexes for transport. The low density lipoprotein (LDL), resulting from lipolytic extraction of triacylglycerol from VLDL, is further metabolized by receptor-mediated endocytosis via the LDL receptor at the basal membrane of the cell. The mechanisms of transport of unesterified fatty acids to sites of utilization within the cell still are unclear. Fatty acid binding proteins of low molecular weight (14-17 kDa) have been isolated from the cytosol of many cell types and their characterization has been extensive. The high affinity of these peptides for fatty acids has led to speculations about an *in vivo* role in the transport of fatty acids, however, conclusive data are lacking (Veerkamp et al., 1991).

Medium chain fatty acids are unique to milk lipids and are synthesized

de novo in mammary epithelial cells. The first committed step in the biosynthesis of fatty acids is the conversion of acetyl coenzyme A (acetyl-CoA) to malonyl coenzyme A (malonyl-CoA) in the cytosol by acetyl-CoA carboxylase (Goodridge, 1991). The carboxylase is the rate-limiting enzyme in lipogenesis and its activity is correlated with the lipogenic capacity of mammary tissue during pregnancy and lactation (Mackall and Lane, 1977; McNeillie and Zammit, 1982). The enzyme is regulated both by covalent modification and binding of the allosteric regulator citrate. The dephosphorylated form of the enzyme is active and allosteric regulation by citrate amplifies its activity.

In mammals, the conversion of acetyl-CoA and malonyl-CoA to long chain fatty acids occurs through a series of sequential reactions catalyzed by the soluble multifunctional enzyme, fatty acid synthase (FAS: Goodridge et al., 1991). The reaction is summarized below:

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ (\text{NADPH}+\text{H}^+) \rightarrow$$
$$\text{Palmitate}\ (C_{16:0}) + 7\ CO_2 + 14\ \text{NADP}^+ + 8\ \text{CoASH} + 6H_2O$$

Fatty acid synthase has been isolated from rat mammary gland cytosol and found to be identical to FAS from rat liver (Smith and Abraham, 1970). The latter has been cloned and sequenced (Amy et al., 1989). The enzyme is a

homodimer with a molecular weight of 478 kDa. Each polypeptide chain contains an acyl carrier domain and six distinct catalytic domains, arranged from amino terminal to carboxyl terminal as follows: Beta-ketoacyl synthase, acyl/malonyl-CoA transferase, dehydrase, enoyl reductase, acyl carrier protein (containing the 4'phosphopantethiene moiety), and thioesterase I. The relatively long stretch of residues between the transferase and the dehydrase has not been assigned a function (Goodridge, 1991; Witkowski et al., 1991). Each FAS monomer contains a distinct active site for each enzyme activity. The enzyme is only functional in the dimeric form, where the subunits are arranged in a head-to-tail orientation.

Synthesis of fatty acids occurs by the sequential addition of two-carbon units derived from malonyl-CoA to a growing acyl chain of acetyl-CoA origin. During elongation, the acyl chain is covalently attached to the 4'phosphopantethiene moiety of the acyl carrier peptide. The typical end-product of fatty acid synthase is palmitate ($C_{16:0}$). Release of the free fatty acid from the enzyme occurs via hydrolysis of the thioester bond catalyzed by thioesterase I, an enzyme that specifically recognizes long chain acyl moieties of greater or equal to 16 carbon units. However, in mammary gland of lactating animals, the predominent fatty acids produced possess short to medium chain lengths. This results from mammary gland-specific expression of thioesterase II, which hydrolyzes the thioester bond of nascent fatty acids of

much shorter chain length than thioesterase I (Libertini and Smith, 1979; Smith and Ryan, 1979). Thioesterase II is not part of the FAS complex but rather is found as a soluble cytosolic protein with molecular mass of 32 kDa (Knudson et al., 1977; Libertini and Smith, 1978; Rangan et al., 1991).

The activation of fatty acids by thio-esterification to coenzyme A, carried out by ATP-dependent coenzyme A synthetases, is the first step in triacylglycerol synthesis (Brindley, 1991). Three different pathways exist for triacylglycerol synthesis from activated fatty acids (Moore and Christie, 1979). The principal pathway of synthesis in mammary gland is the *sn*-glycerol-3-phosphate (G3P) pathway. Glycerol-3-phosphate is the main acceptor of activated fatty acids. Glycerol-3-phosphate is acylated first to 1-monoacylglycerol-3-phosphate (lysophosphatidate) by the enzyme glycerolphosphate acyltransferase, located at the cytoplasmic face of smooth and rough endoplasmic reticulum. A second distinct acyltransferase (monoacylglycerol acyltransferase), selective for unsaturated fatty acids, catalyzes the esterification at position-2 of lysophosphatidate, forming phosphatidate. Phosphatidate is converted then to diacylglycerol by phosphatidate phosphorylase, whose activity has been reported in several cell fractions including ER and cytosol. Finally, triacylglycerols are formed by esterification at position-3 of diacylglycerol by diacylglycerol acyltransferase at the ER (Brindley, 1991). In mammary tissue of certain species, the

esterification at the 3-position appears to be specific for short chain fatty acids. Approximately 95% of the short chain fatty acids of these species in milk triacylglycerol were localized to the 3-position of the glycerol molecule (Bauman and Davis, 1974).

Formation of Intracellular Lipid Droplets at the Endoplasmic Reticulum

Localization of triacylglycerol synthetic activity to ER-enriched microsomes indicated that precursor lipid droplets originated from the endoplasmic reticulum (Cooper and Grigor, 1980). Electron microscopy combined with autoradiography demonstrated initial concentration of labeled fatty acids over the endoplasmic reticulum (Stein and Stein, 1967; Daudet et al., 1981). Biochemical analysis further showed that radiolabel from fatty acid was incorporated into acyl lipids (Stein and Stein, 1967). Morphological studies have demonstrated the presence of precursor lipid droplets that appear to bleb from focal points along the rough endoplasmic reticulum (Dylewski et al., 1984a; Zaczek and Keenan, 1990). Triacylglycerol accumulation appears to occur at focal points scattered along the reticular network, either at or within the ER membrane. The mechanism for accumulation yet is undetermined, however, possible mechanisms have been suggested. Long and Patton (1978) have suggested that triacylglycerols accumulate between leaves of the ER

membrane bilayer and become encased by the cytoplasmic leaflet as the droplet is released into the cytosol. Although some morphological data support such a mechanism (Patton and Keenan, 1975; Zaczek and Keenan, 1990), supporting biochemical evidence is lacking due to the lack of an appropriate assay system. The manner in which the triacylglycerol accumulates at these focal points between the bilayer halves also remains unaddressed.

Freeze fracture evidence obtained by Peixoto de Menezes and Pinto de Silva (1979) showed association of lipid droplets with the cytoplasmic face of both the ER and Golgi complex. The micrographs were interpreted as suggesting a progressive insertion of lipids into the droplets whose precursors were membrane components. The researchers suggested that membrane phospholipid may be converted to triacylglycerols that flow into the lipid droplet.

Recently, a cell-free system has been developed that reconstituted formation of intracellular lipid droplets by incubation of endoplasmic reticulum in a cell-free incubation media in the presence of cytosol (Keenan et al., 1992). Droplets that were released from isolated ER resembled *in situ* isolated lipid droplets in composition and morphology. The requirement for a mammary cytosol fraction was suggestive of involvement of a cytosolic component in lipid droplet formation *in vivo*.

Intracellular Transport and Growth of Lipid Droplets

Morphological evidence repeatedly has confirmed the pathway of secretion of lipid droplets. Lipid droplets originate from the endoplasmic reticulum region and are targeted to selected sites at the apical plasma membrane (review, Keenan and Dylewski, 1993). Droplets appear to grow in volume as they traverse the cell unidirectionally (Stemberger and Patton, 1981; Vallivulah, 1988). While a greater number of smaller droplets are concentrated towards the basal region of the cell, an increasing number of larger droplets appear in the apical region (Stemberger and Patton, 1981). In electron micrographs, several investigators have observed what appear to be droplets fusing with one another (Wooding, 1971; Stemberger and Patton, 1981; Dylewski et al., 1984). In all cases, observations were of MLD fusing with other MLD or with larger CLD. No investigators have been able to observe CLD fusing with CLD. Fusion events have been reconstituted in a cell-free system (Vallivulah et al., 1988). Fusion was insensitive to N-ethylmaleimide, required a high molecular weight fraction from mammary gland cytosol, and was promoted by concentrations of calcium in the range of 10^{-6} to 10^{-3} M . There is evidence also for an involvement of gangliosides in lipid droplet surfaces in fusion events.

Fusion events are one means of droplet growth. Other mechanisms of growth are likely, but have not been sufficiently explored. Patton (1973) suggested a role for lipid transfer proteins in delivering triacylglycerols from their sites of synthesis to growing lipid droplets. Lipid transfer proteins have been identified and characterized in other cell types (review, Voelker, 1991), but little is known concerning their occurrence in mammary epithelial cells. This is a surprising void in the literature considering the prodigious rate of lipid synthesis and turnover that takes place in mammary epithelial cells during lactation.

The secretory pathway of milk lipid droplets is distinct from those for the assembly and secretion of triacylglycerol in other cell types. In non-mammary cells, triacylglycerol is incorporated into lipoprotein particles within the cisternae of the ER and Golgi complex and secreted by exocytosis from Golgi-derived secretory vesicles at cell surfaces (Glauman et al., 1975; Pagano and Sleight, 1985). In contrast, lipid droplets in mammary epithelial cells traverse the cell from their sites of origin at the ER to apical regions through the cytoplasm (review, Keenan and Dylewski, 1993). The mechanism by which these intracellular droplets traverse the cytoplasm is yet to be determined. A role for cytoskeletal elements in guiding the lipid droplets towards the apical plasma membrane has been suggested and investigated. Results remain inconclusive and equivocal (Mather and Keenan, 1983). Studies using

inhibitors that disrupt or inhibit assembly of microtubules and microfilaments have proved troublesome due to inhibition of secretion of all milk constituents and due to non-specific secondary effects within the cell. However, circumstantial evidence and the known role(s) for microtubules in organelle transport does support the hypothesis that cytoskeletal elements may be involved in milk lipid trafficking. Cytoplasmic microtubules are numerous in milk secreting epithelial cells and the amount of tubulin present in mammary epithelial cells increases markedly with the onset of milk secretion (Nickerson and Keenan, 1979; Guerin and Loizzi, 1980).

Intermediate filaments also may play a role in lipid droplet formation, transit, and/or secretion. Prekeratin intermediate filaments are abundant in mammary epithelial cells (Franke et al., 1978), and keratins bind lipid tightly (Asch et al., 1990). Cytokeratins have been found in association with intracellular lipid droplets isolated from lactating gland. However, their role is as yet unclear (Keenan, unpublished). Storage lipid droplets in adipocytes (Franke et al., 1987) and adrenal steroidogenic cells (Allmahbobi and Hall, 1990) appear to be encased in an intermediate filament cage during growth.

Characterization of Intracellular Lipid Droplets

Intracellular lipid droplet precursors have been described numerous times since their initial observation by Bargman and Knoop (1959). The lipid droplets appear in the cytoplasm as triacylglycerol-rich cores surrounded by a granular coat material that is lacking unit membrane structure and is thickened in parts with apparent tripartite structure (Dylewski et al., 1984; Deeney et al., 1985). Morphological data resulted in the categorization of two classes of intracellular droplets, MLD and CLD, that differed in size and density. The MLD were the earliest observable droplets released from the endoplasmic reticulum into the cytoplasm (Deeney et al., 1985). The larger CLD, which were more frequent in occurrence towards the apical region of the cell (Stemberger and Patton, 1975), were the result of growth of MLD by fusion and possibly other growth mechanisms (Vallivulah et al., 1988: see section on transport and growth). The two classes of droplets could be separated from each other and components of the endomembrane system by exploitation of density differences. Isolation of droplets by sucrose density gradient centrifugation facilitated biochemical characterization of intracellular droplets.

Lipid composition: Triacylglycerols account for the bulk of the lipid in milk and comprise the core of all sizes of lipid droplets. A heterogeneous population of

lipids interact with protein to constitute the protective monolayer surrounding the droplets. The major lipids found in the surface material include the phosphoglycerides of serine, choline, inositol, and ethanolamine, sphingomyelin, cholesterol, monohexosylceramide, dihexosylceramide, and gangliosides. Although MLD and CLD contain the same classes of lipids, differences have been noted in relative amounts of certain lipid classes (Dylewski et al., 1984; Vallivulah et al., 1988). The surface coat of MLD is diminished in the amounts of gangliosides and total phospholipid content in comparison with CLD surface coat material. Other differences in lipid composition are attributed to overall variations in total lipid to protein ratios, consistent with size and density of the droplets. When compared to lipids comprising the ER membrane, intracellular lipid droplets of both density classes contained a higher amount of sphingomyelin (SPM) and a lower amount of phosphatidylcholine (PC). The presence of SPM in lipid droplets raises questions about its origin in droplets that are purported to exclude processing through the Golgi apparatus from their secretory pathway. Sphingomyelin synthesis in liver, by transfer of phosphocholine from PC to ceramide, was found to be localized in Golgi apparatus (Futerman et al., 1990; Jeckel et al., 1990) or plasma membrane (Voelker and Kennedy, 1982).

Protein composition: SDS-PAGE analysis of the surface monolayer from MLD and CLD reveal complex peptide patterns with no discernable differences

between the two droplet classes (Deeney et al., 1985). Peptides with similar migration patterns to those of intracellular lipid droplets have been observed in SDS-PAGE profiles of MLGM. Deeney et al. (1985) identified at least three peptides that were common to ER, MLD, CLD, MLGM, and the proteinaceous submembrane coat material of MLG. Antibodies raised against CLD surface coat material cross-reacted with a doublet pattern that had similar mobility in all fractions (approximate mass range of 45 - 55 kDa). Antibody against MLGM cross-reacted with peptides having similar mobility to the doublet pattern from CLD antiserum, in all fractions except ER. The MLGM antiserum also cross-reacted with a peptide that comigrated in all fractions with the major MLGM protein, butyrophillin (Heid et al., 1982). Antiserum to purified butyrophillin recognized a peptide that migrated to the same position as butyrophillin for all fractions.

Secretion of the Milk Lipid Globule

The mechanism for lipid droplet secretion has been described repeatedly since initial observations in electron micrographs by Bargman and Knoop (1959). The intracellular droplets appear to become enveloped by differentiated regions of apical plasma membrane as they are secreted from the cell. Wooding (1977) obtained electron micrographs suggesting that

secretory vesicles fused with one another and the plasma membrane, effectively surrounding lipid droplets that had come in contact with the plasma membrane. The lipid droplet would then be secreted partially surrounded by plasma membrane and partially by secretory vesicle membrane. These observations, however, have not been made by other researchers and the available biochemical evidence supports the original mechanism described by Bargmann and Knoop (1959).

Prior to envelopment by the plasma membrane, intracellular droplets interact with the proteinaceous coat observed as an electron-dense region of apical plasma membrane in electron micrographs (Keenan et al., 1971; Wooding, 1977; Freudenstein et al., 1979). It is with the protein coat that droplets interact, rather than directly with the plasma membrane. Although the composition of the coat material has been investigated, the identity(s) of the specific components that interact with and/or recognize surface constituents of intracellular lipid droplets is not known. The coat material was shown to be comprised mainly of proteins (Freudenstein et al., 1979).

The major proteins found to comprise the coat material were the two prominent milk lipid globule membrane proteins butyrophillin and xanthine oxidase (Freudenstein et al., 1979; Franke et al., 1981; Jarasch et al., 1981; Mather and Keenan, 1983; Niera and Mather, 1990). Immunocytochemical studies have shown butyrophillin to be localized to the apical surface of milk

secreting cells. Xanthine oxidase is distributed throughout the cytoplasm, but is concentrated at the apical plasma membrane. These two polypeptides together can account for up to 60% of the mass of MLGM proteins, and they display tight association with one another (Keenan and Dylewski, 1993). Butyrophillin is a mammary specific, developmentally regulated glycoprotein. As the major milk lipid globule membrane protein, butyrophilin has been characterized extensively and the cow butyrophilin gene has been cloned and sequenced (Jack and Mather, 1990). The molecular weight as calculated from DNA-derived sequence data is 56,460 Da. The primary structure was consistent with an integral membrane protein having a single membrane-spanning domain with an exoplasmically exposed N-terminus and a cytoplasmically exposed C-terminus. Experiments showing selective vulnerability of the N-terminal domain to proteolytic degradation in the absence of detergent have confirmed this orientation (I.H. Mather, personal communication). The cytoplasmic orientation of the C-terminus suggested that any possible interaction between the submembrane coat material and intracellular lipid droplets would be with this domain. The primary sequence revealed no hydrophobic domains in this region, therefore any interaction with lipid droplets likely would be with proteins at the surface of the droplets or by way of protein containing intermediates.

Aims of this Research Project

Work in our laboratory has focused on the elucidation of the overall pathway for formation, transport, and secretion of the milk lipid globule and the mechanisms of specific steps therein. The majority of research in this area has dealt with the isolation and characterization of intracellular components of the secretion pathway as identified using electron micrographs. Recently, attention has turned towards the development of cell-free assay systems in which to test possible mechanisms leading to the secretion of the lipid globule into the alveolar lumen. Valivullah et al. (1988) demonstrated fusion of intracellular lipid droplets in a cell-free system and Keenan et al. (1992) were able to induce formation and release of precursor lipid droplets from ER. In both systems, a cytosol fraction from mammary gland tissue homogenates was necessary to promote fusion of lipid droplets and for formation of lipid droplets, respectively. The tissue-specific requirement for a mammary cytosol fraction provided the first evidence for a possible role of soluble cytoplasmic constituents in lipid droplet formation and secretion in *vivo*. The initial goal of this research project was to identify the cytosolic constituent(s) that promoted lipid droplet formation in the cell-free system. During the research project, as answers led to questions, the following set of objectives evolved:

1) To identify the cytosolic factor(s) that promote formation/release of lipid droplet precursors from mammary gland endoplasmic reticulum, *in vitro*.

2) To characterize the high molecular weight cytosolic lipoprotein aggregate that promoted the cell-free formation of lipid droplets, in terms of polypeptide and lipid composition.

3) To identify cytosolic polypeptides from mammary gland homogenates that were developmentally regulated.

4) To determine the association of the high molecular weight lipoprotein aggregate to other components of the milk lipid secretory pathway.

5) To propose a functional model for this aggregate in milk lipid globule formation and/or secretion.

II

MATERIALS AND METHODS

Materials

Primiparous Sprague-Dawley rats nursing 10 to 14 pups were from Dominion Laboratories, and were sacrificed between days 7 and 15 of lactation, or at 2 or 5 days after weaning of litters. DuPont/NEN supplied ^{32}P-orthophosphate, 8500-9120 Ci/mmol, and ^{125}I-protein G, 22 μCi/μg . Centricon-10 and -30 centrifugal concentrators, and YM-10 ultrafiltration membranes were from Amicon. Nitrocellulose sheets were from S&S Scientific. Boehringer-Mannheim supplied ATP, GTP, phosphoenolpyruvate, α-glycerophosphate, coenzyme A, and pyruvate kinase. Protein molecular weight standards for electrophoresis were from BioRad Laboratories. Scintillation fluids and a silver staining kit were from ICN. Crude IgG fraction of rabbit antiserum to rat fatty acid synthase and purified fatty acid synthase were from Dr. Stuart Smith, Children's Hospital, Oakland. Antiserum against cow FAS and purified FAS were from Dr. Craig Beatty, United States Department of Agriculture, Clay Center, NE. Affinity purified antiserum to cow xanthine oxidase was from Dr. Ian Mather, University of Maryland, College Park (Sullivan et al., 1982). Affinity purified antibody to cow butyrophillin was prepared as described (Freudenstein et al., 1979). Protein G-10 nm colloidal

gold conjugate was from Amersham. Insoluble Protein A and all other biochemicals were from Sigma.

Methods

Subcellular Fractionation:

Radioactive cell fractions were obtained from animals that had received an intraperitoneal injection of 2 to 5 mCi of ^{32}P-orthophosphate 10-14 hr before sacrifice. Animals were sacrificed by cervical dislocation, mammary glands were removed, minced thoroughly with scissors, and the minced tissue was washed repeatedly with cytosol buffer (10 mM Tris, 10 mM KCl, pH 7) containing 0.25 M sucrose until the wash solution remained clear. Cytosol was prepared by homogenization of tissue in cytosol buffer (4 ml/g wet weight) containg 0.25 M sucrose, filtration of the homogenate through a single and then double layer of cheesecloth, and centrifugation at 120,000 x g maximum for 2 to 4 hr at 2°C (Keenan et al., 1992). Following removal of floating lipids, cytosol was filtered through cheesecloth and centrifuged as above for 45 to 60 min. The clear supernatant was dialyzed overnight at 4°C against 2-3 changes, each of 20 volumes, of cytosol buffer. Cytosol was used as obtained, or was concentrated to a final concentration of 5-10 mg protein/ml by ultrafiltration or centrifugal concentration using 30 kDa cutoff membranes. For storage, concentrated cytosol was frozen in liquid nitrogen and stored at -70°C.

In some instances cytosol was prepared in parallel from livers obtained from donors of mammary tissue. Endoplasmic reticulum, that fraction of microlipid droplets floating through 0.8 M sucrose, and cytoplasmic lipid droplets were isolated from homogenates of minced and washed mammary tissue (Dylewski et al., 1984a; Keenan et al., 1992). These fractions were used immediately after isolation, or were suspended in 1 M sucrose, frozen in liquid nitrogen, and stored at -70°C. Endoplasmic reticulum was similarly isolated from homogenates of liver from donors of mammary tissue.

Milk fat globule membrane was obtained from the lipid phase of fresh cow's milk according to the procedure described by Mather (1978). The inner coat material of milk lipid globules was isolated as described by Freudenstein et al. (1979).

Potassium Bromide Density Gradient Centrifugation:

Cytosol was fractionated by KBr density gradient centrifugation. Solid KBr was dissolved in cytosol (33 g/100 ml), 22 ml of this solution was placed in a Quickseal centrifuge tube (Beckman Instruments), overlaid with 0.9% NaCl, and centrifuged in a VTi50 rotor at 45,000 RPM for 6-16 hr at 10°C. Fractions (2 ml) were collected from tube bottoms, dialyzed exhaustively against cytosol buffer, and assayed for protein and lipid content. Aliquots of KBr density gradient fractions were taken prior to dialysis for measurement of refractive

indices (RI). Densities were determined from tables that correlated RI of KBr solutions to densities. Alliquots from several fractions were weighed directly to confirm density values obtained from tables. To maximize recovery of the low density form of fatty acid synthase, fractions in which FAS comprised greater than 50% of the total coomassie stained protein (as determined by densitometry of SDS-PAGE gels) were collected as a single fraction with a syringe and needle inserted through the side of the tubes three cm below the upper horizontal seam of the tube. The crude low density fatty acid synthase (LDFAS) fraction was dialyzed exhaustively against 20 mM Tris buffer, pH 7.0, 250 mM NaCl, 0.02% sodium azide, and concentrated with Centriprep 30 concentrators. LDFAS was either kept on ice for several days or frozen in liquid nitrogen and stored at -70°C.

Lipid Analysis:

Lipids were extracted from LDFAS by a modification of the Bligh-Dyer procedure (Kates, 1972). Radioactivity in lipid extracts was measured in BetaMax scintillation fluid. Thin layer chromatography (TLC) was performed using Whatman HPTLC plates. Nonpolar lipids were separated by developing plates in hexane-diethyl ether-acetic acid (44:6:0.5, v/v). Polar lipids were separated in methyl acetate-isopropanol-chloroform-methanol-0.25% aqueous KCl (25:25:28:10:7, v/v) (Heape et al., 1985), or by development in two

dimensions; first in chloroform-methanol-water-28% ammonia (30:20:2:0.5, v/v) and then, in the perpendicular direction, in chloroform-acetone-methanol-acetic acid-water (30:5:5:5:1.5, v/v). Radioactive constituents were detected by exposure of plates to Kodak X-Omat film at -70°C. Separated lipids were made visible by dipping plates into 3% cupric acetate in 8% phosphoric acid and heating in an oven at 180°C for 15 min (Fewster et al., 1969).

Protein Analysis:

Protein in fractions was measured with bicinconinic acid (Pierce Chemical Co.) with samples in 2% SDS. Electrophoresis (SDS-PAGE) was according to Laemmli (1970), in 8% polyacrylamide gels or 7% to 15% gradient polyacrylamide gels. Native gel electrophoresis was by the same procedure except with 6% gels that were made and run in the absence of SDS and 2-mercaptoethanol. Following electrophoresis, a piece of stacking gel shown to contain the FAS complex by Western blot detection, was excised from the gel entrance to approximately two cm into the gel and minced with a razor blade. Protein and lipid were eluted from the native gel by incubating the minced gel overnight at room temperature in elution buffer (20 mM Tris, pH 8.8, containing 2% (v/v) 2-mercaptoethanol and 1% (w/v) SDS). Gel pieces then were heated in boiling water for 15 minutes and centrifuged to recover the supernatant. Glycerol was added to the supernatants to 10% (v/v), samples were heated in

boiling water for 5 to 10 min, and stored at -25°C for SDS-PAGE analysis. Gels were stained with coomassie blue or with silver (Merril et al., 1981). For silver staining gels were fixed in a solution containing 50% methanol, 12% acetic acid, and 0.5 ml/L 37% formaldehyde, for at least one hour. Gels then were washed 3 times for 20 min each in 50% ethanol. Washed gels were pretreated for one min in sodium thiosulfate solution (0.2g/L) and rinsed 3 times for 20 sec each with water. Gels were then impregnated with silver nitrate solution (2g/L silver nitrate, 0.75 ml/L 37% formaldehyde) for 10 min followed by 3 rinses for 20 sec each with water. Images were developed in sodium carbonate solution (60 g/L sodium carbonate, 0.5 ml/L 37% formaldehyde), and development was terminated by rinsing gels with water and incubating in 50% methanol, 12% acetic acid for 10 min, followed by a final wash in 50% methanol.

In some cases proteins separated by electrophoresis were transblotted onto nitrocellulose sheets for Western blot analysis (see immunotechniques). Proteins separated by electrophoresis and recovered by transblotting onto PVDF membranes or by electroelution were used for amino-terminal sequencing. Proteins transblotted to nitrocellulose were used for internal sequencing of peptide digests, and acid hydrolysates were analyzed for amino acid composition at the Microsequencing Lab, Harvard University, Boston, Mass. In some cases, stained gels were scanned with a Shimadzu CS 9000

flying spot densitometer operated in the reflectance mode at 580 nm.

In experiments where ^{32}P-labeled or ^{125}I-labeled samples were separated by native or SDS-PAGE, proteins and lipids either were transblotted to nitrocellulose and exposed to Kodak X-Omat film at -70°C, or gels were dried under a vacuum and exposed directly.

Immunotechniques and Electron Microscopy:

Proteins separated by electrophoresis were transblotted onto nitrocellulose sheets, which then were incubated with antiserum against rat FAS, bovine FAS, bovine butyrophillin, bovine xanthine oxidase, or bovine protein disulfide isomerase (PDI) (Towbin et al., 1979). Detection was by ECL Western blot detection reagents (Amersham) with horseradish peroxidase-conjugated second antibody, followed by autoradiography. For immunocytochemistry, aliquots of rat LDFAS and cell fractions were embedded in Lowicryl K4M (Roth, 1982) after fixation in glutaraldehyde and dehydration as described (Deeney et al., 1985). Sections were incubated in serial dilutions of anti-rat FAS antiserum and visualization of antibody was by reaction with 10 nm colloidal gold-conjugated Protein G. Controls were sections treated as above but incubated with nonimmune rabbit IgG.

To study morphology of LDFAS, samples were fixed sequentially with 2.5% glutaraldehyde on ice for 90 min and then with 1% osmium tetroxide

overnight at 4°C and prepared for electron microscopic examination as described (Dylewski et al., 1984).

Chromatography:

Cytosol was fractionated by gel permeation in a 2.5 x 90 cm column of Sephadex G-100 equilibrated and eluted with cytosol buffer at 4°C (Nowack et al., 1987) or in a Superose-6 FPLC column eluted with low (cytosol buffer) or high (10 mM Tris, 250 mM NaCl, pH 7) ionic strength buffer. Fractions collected were assayed for protein, lipids, and radioactivity.

Triton X114 Phase Separation Fractionation:

Protein solutions were brought to a final concentration of 0.6% Triton X114 (TX114: 0.01M;cmc 0.17mM) by the addition of ice cold 6% TX114 (v/v) in LDFAS buffer (20 mM Tris, pH 7.0, 250 mM NaCl, 0.02% sodium azide). Protein samples were overlaid onto a 6% sucrose cushion containing 0.06% TX114 (v/v) on ice. Phase separation was produced by incubation for 3 to 4 min at 37°C. Phases then were separated by centrifugation at 2,000 rpm for 4 min at room temperature.

The upper, aqueous phase was withdrawn and fresh, ice cold TX114 was added to a final concentration of 0.6%. The aqueous phase was overlaid back onto the original sucrose cushion, incubated and centrifuged again as

before. The supernatant finally was recovered as the aqueous phase and concentrated as necessary. The resultant oily pellet was recovered as the detergent-rich phase, this phase has been reported to contain lipids and hydrophobic protein constituents (Pryde, 1988). The pellet was washed by overlaying with fresh sucrose solution and agitating gently to rinse the sides of the tube and the surface of the pellet. The oily pellet was resuspended in distilled water, concentrated, and resuspended to dilute the detergent before analysis by SDS-PAGE.

Cell-Free Assays:

Formation and release of precursor lipid droplets by endoplasmic reticulum:

Lipid droplets were released from ER that had been immobilized onto nitrocellulose and incubated at 37°C in a complete assay mixture as described (Keenan et al., 1992). The complete assay mixture contained, in HKM buffer (25 mM HEPES, pH 7, 25 mM KCL, and 2.5 mM magnesium acetate), 2 mg cytosolic protein, ATP plus an ATP regenerating system (1.3 mM ATP, 10 mM phosphoenolpyruvate, 6 U pyruvate kinase), 20 μM GTP, 50 μM coenzyme A, 0.5 mM α-glycerophosphate, 0.1 mM dithiothreitol, and 0.2% serum albumin containing fatty acids in a final volume of 1 ml. Formation of MLD was measured by using ^{32}P-labeled ER and measuring amount of phospholipids

released. To measure transfer of phospholipids, unlabeled ER or MLD immobilized on nitrocellulose were incubated as above with ^{32}P-labeled cytosol or cytosolic fractions. Following incubation, nitrocellulose strips were recovered, washed through 4 changes of 0.25 M sucrose in HKM buffer, and lipids were extracted.

ATP-dependent release of peripheral membrane proteins from endoplasmic reticulum:

Proteins and lipoprotein complexes were released from endoplasmic reticulum preparations by incubation in a cell-free incubation medium modified from Keenan et al. (1992). The final mixture contained HKM buffer (25 mM HEPES, pH 7.0; 25 mM KCl; 2.5 mM magnesium acetate), ER (between 2 to 5 mg/ml), 0.25 M sucrose, 2 mM dithiothreitol, 1 mM GTP, and an ATP regenerating system (1.3 mM ATP, 1.3 mM phosphoenolpyruvate, and 1 IU pyruvate kinase) in a final volume of 1 ml. Incubations were at 37°C or on ice for 30 minutes. At the end of the 37°C incubation period samples were cooled on ice for 10 min, and then all samples were overlaid onto a 0.8 M sucrose cushion in microfuge tubes and centrifuged at 16,000 x g for 30 min at 4°C. Pellets were recovered and washed three times in buffer containing 0.25 M sucrose. The upper phase was recovered as the soluble fraction containing released proteins. Samples either were fixed for SDS-PAGE analysis, applied

to a KBr gradient, assayed for fatty acid synthase activity, or used in binding assays.

Cross-Linking:

A working solution of dimethyl suberimidate (DMS) was prepared by dissolving 20 mg DMS in 1 ml of 1 M triethanolamine HCl, pH 9.7. One part of the DMS solution was added to 10 parts protein solution (< 1.0 mg/ml ; Lad and Hammes, 1974) and the mixture was incubated for 5 to 120 min at room temperature. Reactions were terminated by adding sodium dodecylsulfate or ammonium acetate, or by addition of one volume of ice cold trichloroacetic acid (50%, w/v), followed by centrifugation and washing with acetone to recover the precipitate. Samples were fixed and analyzed by SDS-PAGE as described.

Fatty Acid Synthase Assay:

Fatty acid synthase activity was measured according to Smith and Abraham (1975). Protein samples were preincubated in 100 mM potassium phosphate buffer (pH 6.6), containing 10 mM dithiothreitol, for 30 min at 37°C. Samples then were added to an assay mixture that contained 100 mM potassium phosphate buffer, pH 6.6, 30 μM acetyl coenzyme A, and 150 μM NADPH. Samples were pre-incubated in the assay mixture for 5 min at 37°C

before addition of the labeled substrate, ^{14}C malonyl coenzyme A (60,000 dpm/assay tube). The final volume for each assay was 0.50 ml. The reaction was carried out for 45 min at 37°C. To terminate the reaction 100 μL of 0.5 N NaOH were added and samples were boiled for 15 min to saponify the fatty acids. Free fatty acids were recovered by the subsequent addition of 100 μL of 1N HCl, and were extracted three times with hexanes. Extracts were washed once with 1% (v/v) acetic acid. Samples in hexane were dried under nitrogen gas and radioactivity was determined in 10 ml Beta Max scintillation cocktail. Activity was reported as cpm per microgram of protein in the assay mixture.

III

RESULTS

1) Identification of a High Molecular Weight Cytosol Fraction Active in Promoting MLD Formation and Release in a Cell-Free System

Introduction

Although the overall pathway of lipid droplet secretion is well documented, information about the mechanisms regulating formation and growth of intracellular lipid droplets is lacking (review, Keenan and Dylewski, 1993). The recent development of cell-free systems that mimic these events *in vitro* provided an assay to test effects of various soluble components that may be acting *in vivo*. A high molecular weight cytosol fraction was necessary to promote formation and release of MLD (Keenan et al., 1992) as well as to promote fusion of droplets (Vallivulah et al., 1988). The effect was specific to cytosol isolated from mammary gland homogenates from lactating animals. These systems provided the first evidence for involvement of soluble cellular components in the events leading to secretion of milk lipid globules. To isolate the cytosolic factor(s) necessary in the promotion of the cell-free formation and release of lipid droplets, cytosol was fractionated and assayed for the ability to promote release of ^{32}P labeled MLD's from immobilized ER into the incubation

medium. Active fractions were further fractionated, using non-denaturing procedures, and characterized.

Immediately prior to the onset of lactation, lipogenic activity in mammary epithelial cells increases prodigiously and remains high throughout lactation. A developmentally regulated increase in expression of lipogenic enzymes parallels this activity (Baldwin and Milligan, 1966). It was hypothesized that cellular constituents involved in the differentiation process of lipid droplet formation and release also may demonstrate developmental regulation. To test this hypothesis, cytosolic polypeptide patterns from rats at different developmental stages were compared.

Results

A) *Protein and lipid profiles of the cytosolic fraction*:

Fractionation of cytosol from lactating mammary gland by gel exclusion chromatography demonstrated that the void volume of a Sephadex G-100 column was enriched in cell-free activity. The major protein peak eluted in the void volume, indicating a size greater than 100 kDa (Fig. 1). In a cell-free system for the formation and release of intracellular lipid droplets from ^{32}P labeled endoplasmic reticulum, this fraction increased the quantity of ^{32}P-

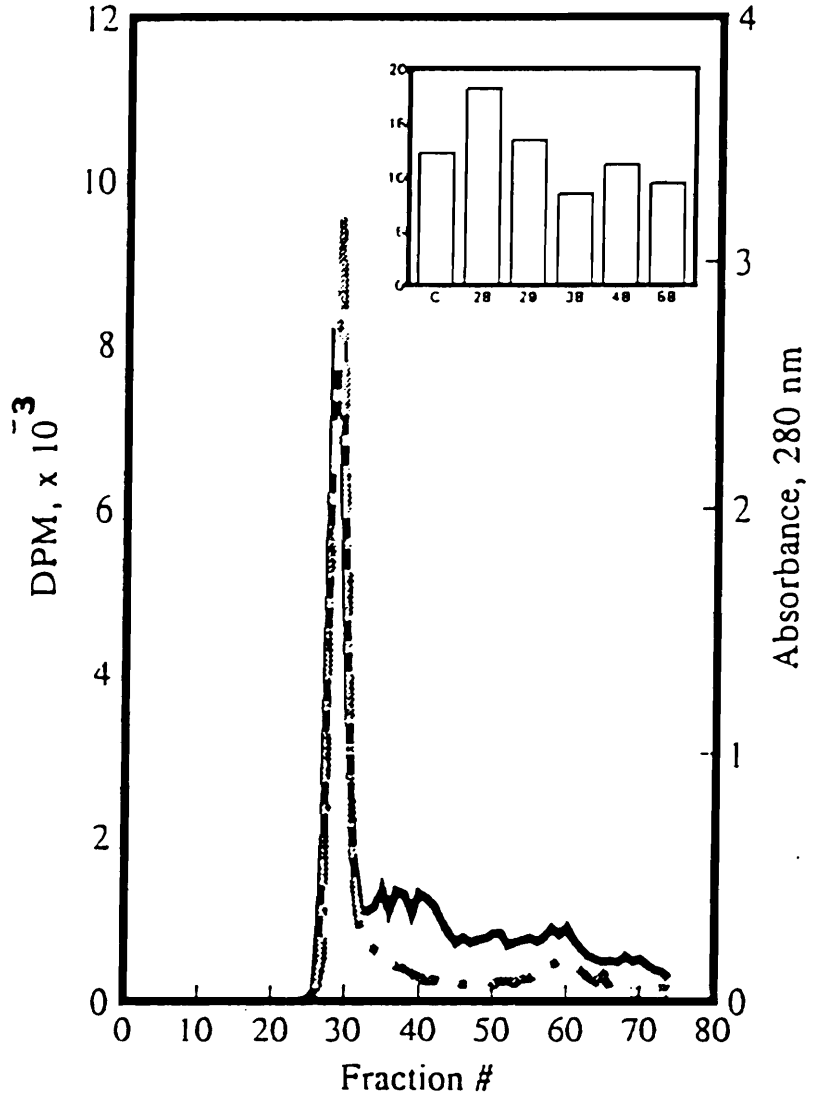

Figure 1: Gel filtration fractionation of cytosol from lactating mammary gland. Separation was in a Sephadex G-100 column equilibrated with 0.1 M Tris, 0.1 M KCl, pH 7.0. Solid line, absorbance at 280 nm, dashed line, total ^{32}P in fractions. Insert, the ability of unfractionated cytosol (C), and indicated column fractions to promote release of ^{32}P-labeled lipid droplets from endoplasmic reticulum. Identical amounts of protein were used for all samples. Data were presented as % release of ^{32}P associated with immobilized endoplasmic reticulum (ordinate) versus fraction number (abscissa).

labeled phospholipid released into the media by 50% as compared to unfractionated cytosol (Fig. 1, insert). Analysis by SDS-PAGE of the polypeptides comprising this fraction revealed the presence of several large polypeptides (Fig. 2). Particularly prominent were peptides migrating with apparent molecular masses of 220 kDa, 155 kDa, and 116 kDa. Several low molecular weight polypeptides also were present in the void volume fraction whether elution was with low or high ionic strength buffer. This may indicate that these low molecular weight polypeptides were subunits of some high molecular weight complex or were present as aggregates. Lipid droplet promoting activity was equal to or slightly less than that of unfractionated cytosol in other fractions that did not absorb strongly at 280 nm. Whether these fractions contained factors involved in the promotion of lipid droplets was not pursued in these experiments. Rather, the focus of the research turned to the existence of a high molecular weight protein fraction that contained the bulk of cytosolic lipid (see below) and that was active in promoting lipid droplet formation *in vitro*.

The possibility that the high molecular weight cytosol fraction served as a source of lipid during droplet formation was explored by studying the distribution of lipid in cytosol from mammary gland homogenates. Cytosol from lactating rat mammary gland contained approximately four percent of the total lipid in tissue homogenates. Of the radiolabel present in cytosol isolated from

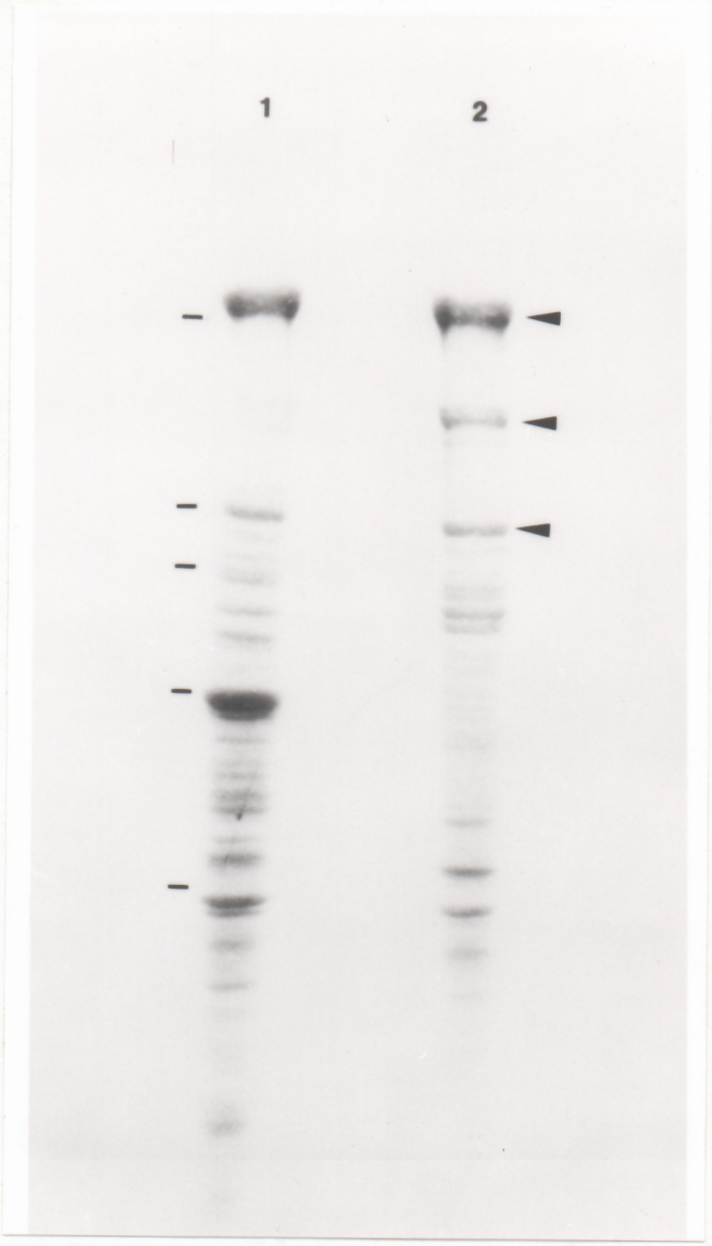

Figure 2: Electrophoretic separation of polypeptides from cytosol (lane 1), and the gel exclusion the void volume of a G-100 gel filtration column (lane 2). Separation was by SDS-PAGE in a 5 to 15% gradient gel which was stained with coomassie blue. Arrowheads denote positions of 220 kDa, 155 kDa, and 116 kDa polypeptides. Positions of standard proteins, marked by bars from top to bottom, were myosin (200 kDa), beta-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa), respectively.

mammary gland homogenates from rats injected with ^{32}P-orthophosphate, typically 20-25% was present in lipids. Typically 40% of the ^{32}P present in the G-100 void volume fraction was in phospholipipids. This ^{32}P-labeled lipid accounted for greater than 95% of the total radiolabeled lipids present in cytosol. Unfractionated cytosol and the void volume fraction had nearly identical patterns of nonpolar (Fig. 3a) and ^{32}P-phospholipids (Fig. 3b & 3c). The five major phospholipids were characteristic of the coat material of intracellular milk lipid droplets and the endoplasmic reticulum membrane; phosphatidylcholine (PC), phoshatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (SPM), as well as the neutral lipids cholesterol, triacylglycerols, fatty acids, and diacylglycerols. Unfractionated cytosol and the void volume fraction contained similar proportions of labeled lipid, although no lysophosphatidyl ethanolamine was detected in the void volume fraction.

Cytosolic lipid transfer proteins have been identified in several cell types, but have not been studied in mammary gland (review, Voelker, 1991). Phospholipids in unfractionated cytosol were transferred to MLD and to ER which had been immobilized onto nitrocellulose (Fig. 4). The ^{32}P-labeled phospholipids present in cytosol were transferred progressively with time to MLD and ER; at 37°C the MLD contained about 20% of the phospholipid originally present in cytosol, and ER contained about 12%, after 150 min

incubation. Rates of transfer to ER and MLD were much lower when incubation was at 4°C (Fig. 4). Labeled phospholipids in cytosol also became associated with MLD formed and released from immobilized ER in a cell-free incubation system. In a representative experiment, approximately 11% of the ^{32}P-phospholipid in cytosol became associated with MLD produced upon incubation of immobilized ER for 60 min under optimum conditions for MLD formation (not shown).

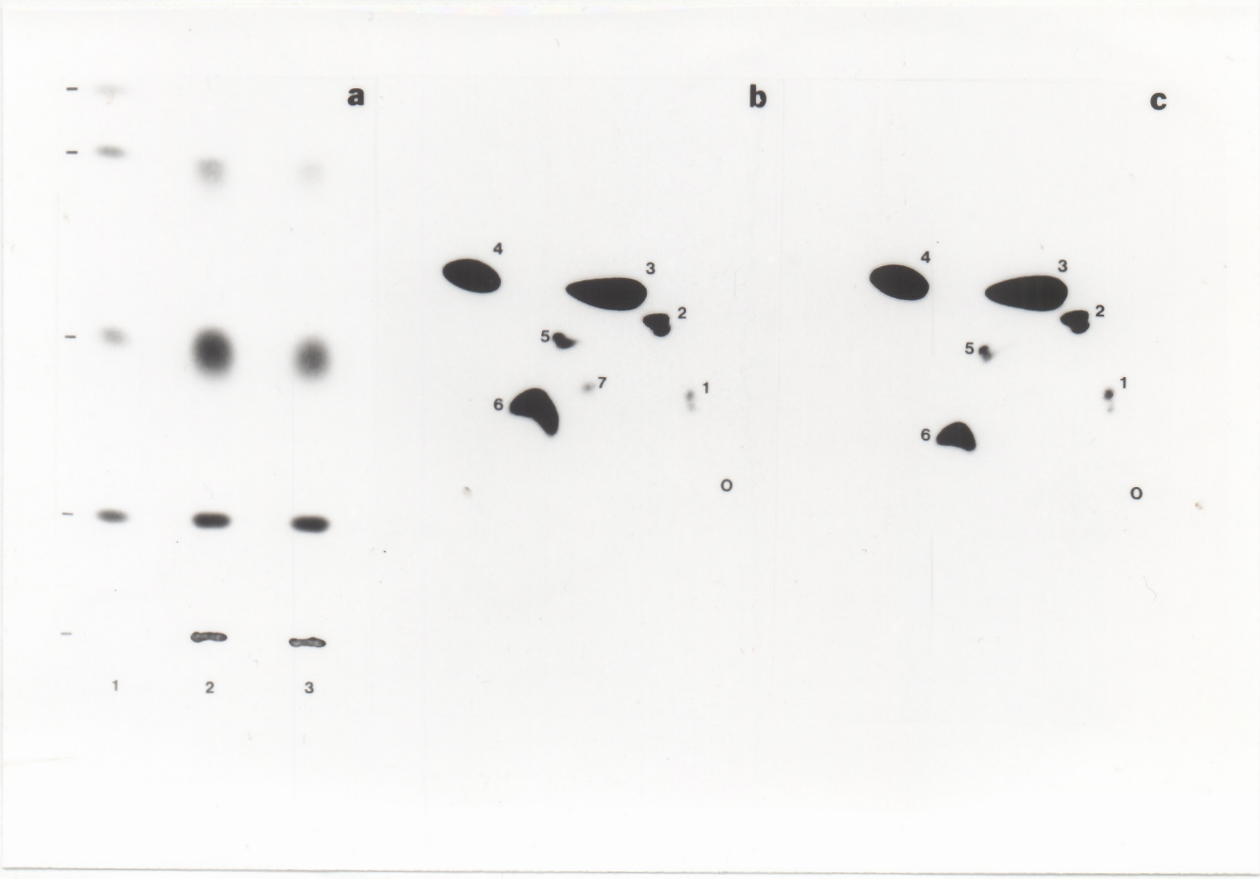

Figure 3: Lipid composition of unfractionated and gel filtration-fractionated cytosol. (a) Nonpolar lipids from unfractionated cytosol (lane 2), and the void volume fraction from cytosol (lane 3) were separated on a thin-layer plate and detected by charring. Positions of lipid standards (lane 1), marked by bars from top to bottom, were cholesterol esters plus methyl esters, triacylglycerols, unesterified fatty acids and cholesterol, respectively. The lower-most bar marks the origin. Plates were developed in hexane:diethyl ether:acetic acid (85:15:1) and charred with cupric acetate-phosphoric acid for visualization of lipids. Polar lipids of unfractionated cytosol (b) and the void volume fraction of cytosol (c) were separated by chromatography in two dimensions, and ^{32}P-labeled phospholipids were detected by autoradiography. Constituents tentatively identified by co-migration with standard lipids were (1) lysophosphatidyl choline, (2) sphingomyelin, (3) phosphatidyl choline, (4) phosphatidyl ethanolamine, (5) phosphatidyl serine, (6) phosphatidyl inositol, and (7) lysophosphatidyl ethanolamine. O denotes the origin.

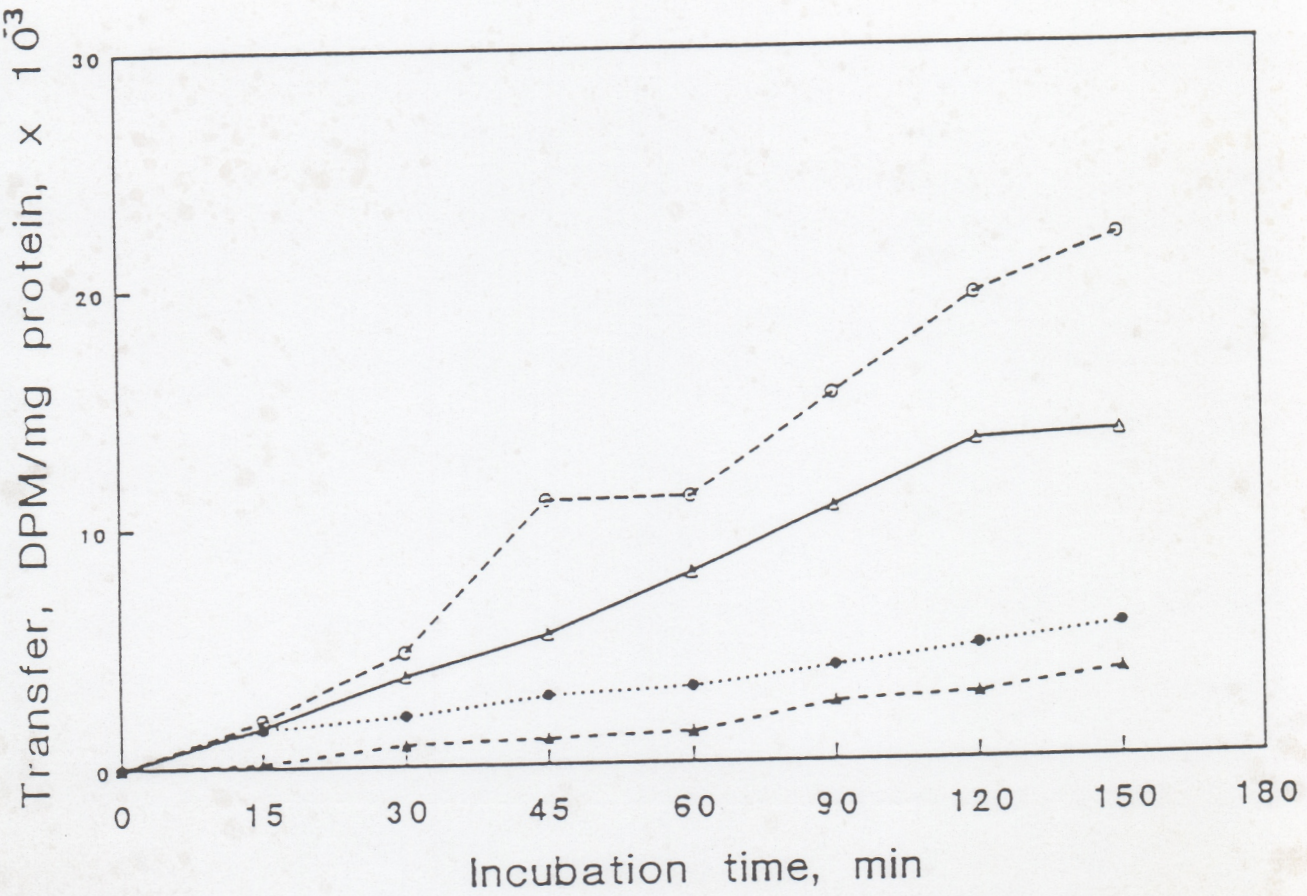

Figure 4: Transfer of phospholipids from cytosol to endoplasmic reticulum and to microlipid droplets from mammary gland. Microlipid droplet (circles) and endoplasmic reticulum (triangles) preparations from unlabeled animal were immobilized onto nitrocellulose circles and incubated with unfractionated cytosol from a ^{32}P-loaded rat. At the time points indicated, samples were recovered and the amount of ^{32}P-phospholipid transferred to the immobilized fractions was measured. Data were expressed on the basis of transfer per mg immobilized fraction protein. Nitrocellulose containing nonfat dry milk was used to determine background transfer. Incubations were at 37 degrees (open symbols) or at 4 degrees (filled symbols).

B) ***Developmental expression of major peptides comprising the G-100 exclusion volume fraction:***

The ability of the high molecular weight protein-lipid fraction to stimulate lipid droplet formation in cell-free systems and its ability to transfer lipid among intracellular components, was suggestive of a role for this complex as a lipid source during formation and growth of lipid droplets. If this were the case, one would anticipate developmental expression of the polypeptides comprising the complex. To determine differential expression of polypeptides, cytosol from mammary gland and liver of rats which were lactating, two days involuted, and five days involuted, was analyzed. Equivalent protein amounts of cytosol from liver and mammary tissue of the same animal were separated by SDS-PAGE (Fig. 5). Stained gels were scanned to quantify relative amounts of peptides (Table 1). Polypeptide profiles for liver cytosol were similar regardless of the animal's lactational status. Polypeptide profiles from mammary gland cytosol differed depending on the lactational status of the animal. In mammary gland, cytosol from lactating animals appeared to be enriched in several high molecular weight polypeptides as compared to cytosol from animals in the second and fifth days of involution (Fig. 5). The lactation-enriched polypeptides migrated with apparent molecular weights of 220 kDa, 116 kDa, and 100 kDa. The 220 kDa polypeptide accounted for 15 to 30% of the total

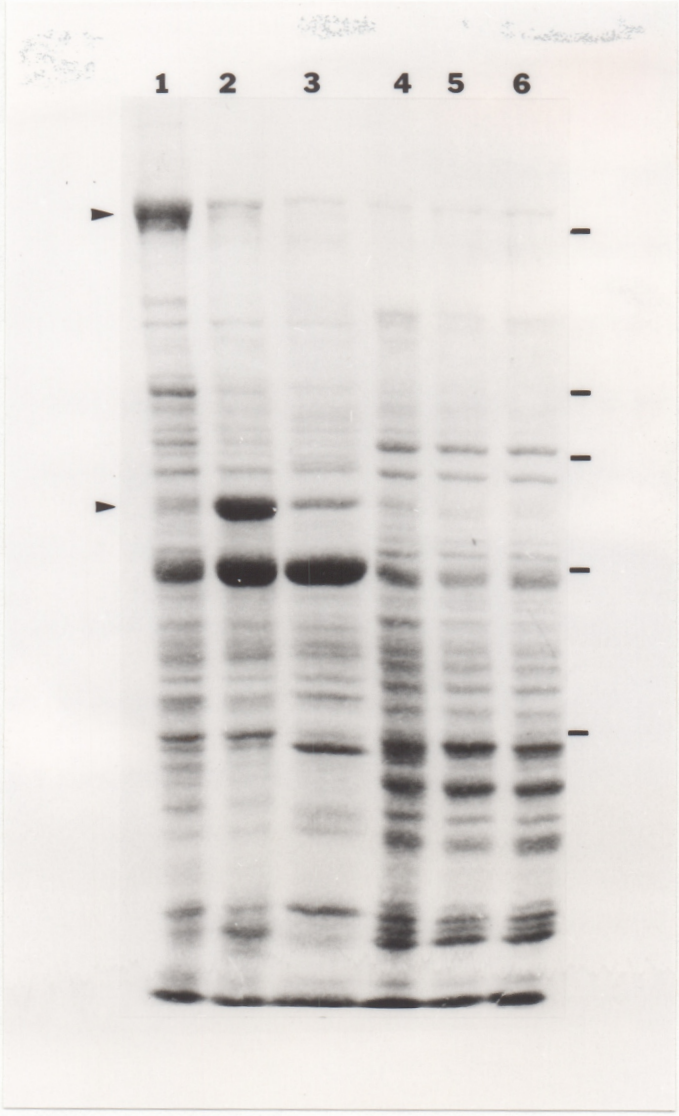

Figure 5: Polypeptide composition of mammary gland cytosol from lactating (lane 1), 2 day involuted (lane 2), and 5 day involuted (lane 3) rats. Lanes 4,5, and 6 are of liver cytosol from the same animals in 1,2, and 3, respectively. Separation was by SDS-PAGE, and the 10% acrylamide gel was stained with coomassie blue. Bars along lane 6 denote the positions of 200 kDa, 116 kDa, 97 kDa, 66 kDa and 45 kDa standards from top to bottom, respectively. The upper arrow signifies the lactation-enriched 220 kDa band and the lower arrow signifies the involution-enriched 80 kDa band identified as transferrin (see Appendix).

Table 1: Quantification of major polypeptides in mammary gland cytosol from animals that were lactating (L), two days involuted (2D), and five days involuted (5D), by densitometric scanning of electrophoretic gels represented in Fig. 5. Results are presented as percent of total coomassie blue stained protein scanned in each lane. Data are typical of gels scanned and consistent with ELISA measurements of similar samples (see Appendix).

	% Coomassie-stained protein		
Peptide MW	**L**	**2D**	**5D**
220 kDa	**19**	**1.7**	**0.2**
116 kDa	**3.9**	**0.4**	**0**
80 kDa	**2.6**	**16.8**	**3.2**
68 kDa	**16.4**	**29.7**	**31.7**

coomassie stained protein in cytosol from lactating animals; as compared to 1.7 to 6% on the second day of involution, and less than 3% on day 5 of involution. All three polypeptides were present in the high molecular weight lipid-protein fraction determined to be necessary for cell-free formation of intracellular lipid droplets from endoplasmic reticulum (see Fig. 2). In addition to reduced amounts of high molecular weight polypeptides, cytosol from glands that had been involuting for two days was enriched in polypeptides with apparent molecular masses of 68 kDa and 80 kDa. Similarly, cytosol from five day involuted glands also was enriched in the 68 kDa polypeptide, and contained the 80 kDa peptide, although in lesser amounts as compared to cytosol from two day involuted gland. The 80 kDa constituent was investigated further due to its transient and marked increase at the onset of involution (Keon and Keenan, 1993). These experiments resulted in the identification of a cytosolic form of mammary gland transferrin that may serve as a marker for cell involution (see Appendix A).

C) ***Potassium Bromide density gradient fractionation to isolate a low-density lipid-protein complex:***

In order to determine which of the polypeptides in the void volume of the G-100 column were physically associated with lipid, cytosol from lactating mammary gland was fractionated by KBr density gradient centrifugation.

Although present in fractions throughout the gradient, as determined by densitometric scanning, the 220 kDa and 116 kDa polypeptides were the major components in fractions migrating with densities less than 1.17 g/ml. Mammary gland cytosol from ^{32}P labeled rats also was separated on a KBr gradient, lipids were extracted from individual fractions and separated by HPTLC (Figs. 6 & 7). Polar (Fig. 6) and neutral (Fig. 7) lipids were distributed throughout the gradient, however, 91.4% of the total labeled phopholipid was found in fractions with densities less than 1.17 g/ml. This increase in phospholipid content correlated with the progressive enrichment of the high molecular weight proteins in these fractions (Table 2). In the two fractions with the highest phospholipid to protein ratios (calculated as ^{32}P-lipid dpm/mg protein), corresponding to densities of 1.071 and 1.102 g/ml, the 116 plus 220 kDa polypeptides accounted for about 79% and 66% of the total coomassie-stained protein, respectively. The 220 kDa polypeptide alone accounted for about 65 and 52%, respectively, of the total coomassie stained protein in these fractions. No selectivity in polar lipid distribution was observed, all fractions contained similar proportions of PC, PS, PE, PI, and SPM. Neutral lipid distribution, however, displayed some selectivity (Fig. 7). Fractions with densities of 1.23, 1.17, and 1.10 g/ml, were enriched in triacylglycerols.

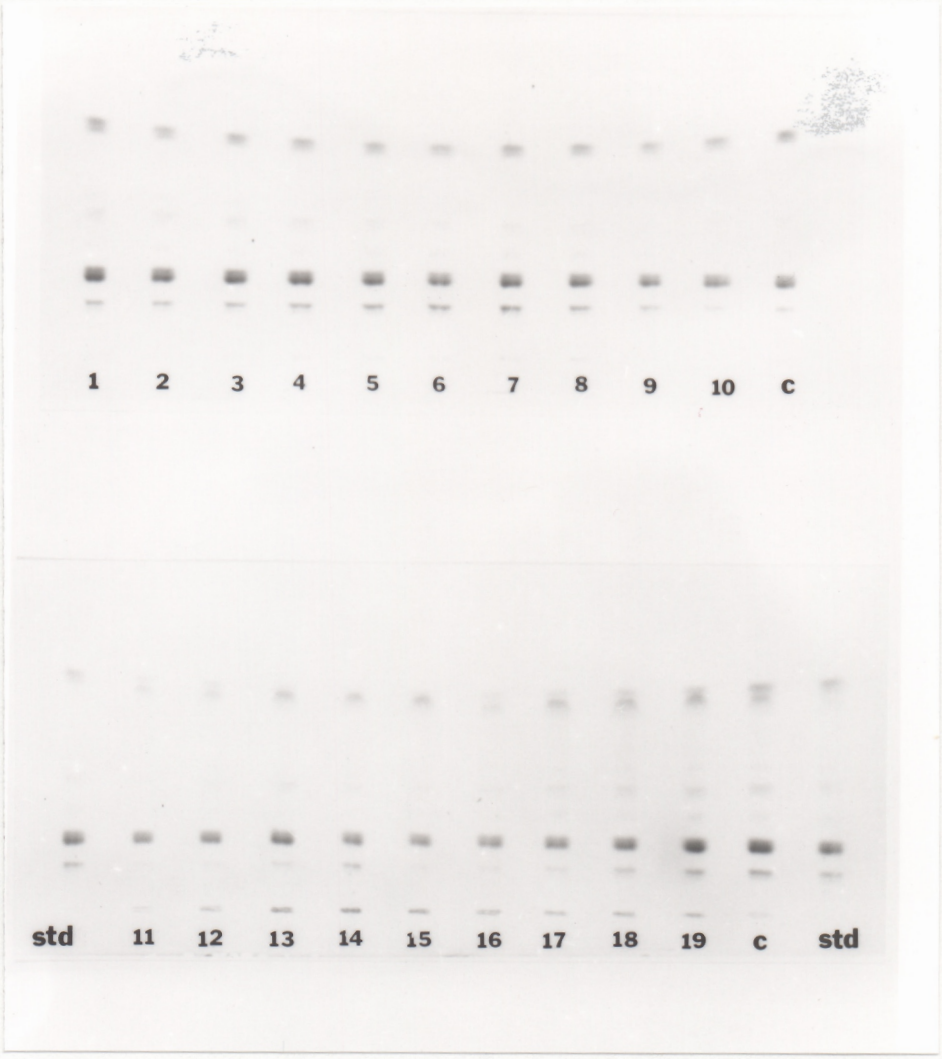

Figure 6: Mass distribution of polar lipids in cytosol following KBr density gradient centrifugation. Thin-layer plates were developed and charred. Lanes were numbered from highest (1) to lowest (19) density fractions from KBr density gradient centrifugation. For reference, lipids from unfractionated cytosol (C) were included on plates. Bars denote positions of phosphatidyl ethanolamine, phosphatidic acid plus dihexosylceramide, phosphatidyl serine, phosphatidyl choline, sphingomyelin, lysophosphatidyl choline, and the origin, respectively, from top to bottom. Equal amounts of lipid, based on ^{32}P-phospholipid content, were applied for each sample.

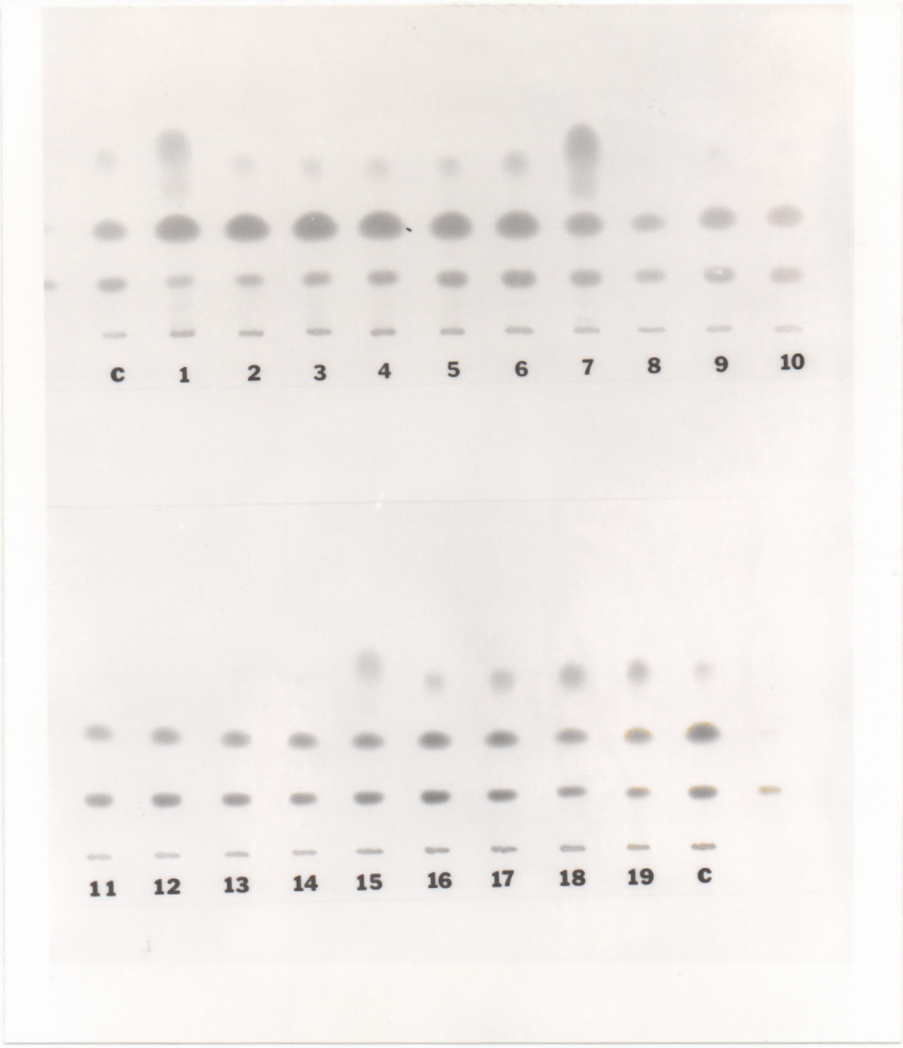

Figure 7: Mass distribution of nonpolar lipids in unfractionated cytosol and density gradient-separated fractions of cytosol. Thin-layer plates were developed and charred. Lanes were numbered as in figure 6. For reference, lipids from unfractionated cytosol (C) were included on plates. Bars denote positions of triacylglycerols, unesterified fatty acids, cholesterol, and the origin, respectively, from top to bottom. Amounts applied were the same as for polar lipid analysis (Fig. 6).

Table 2: Quantification of major polypeptides in density gradient fractions from lactating rat mammary gland cytosol by densitometric scanning of electrophoretic gels. Results are presented as percent of total coomassie blue stained protein scanned in each lane. Cytosol was labeled with ^{32}P and lipids were extracted from individual fractions. Results are presented as percent total DPM in a fraction recovered in lipid extracts (A) or DPM recovered in lipid extracts per mg of protein (B).

Percent Coomassie Stained Protein

A. Density (g/ml)	220 kDa	220+116 kDa	68 kDa	% DPM in lipid
1.230	4.1	4.1	55.3	3.3
1.193	9.2	10.6	45.7	7.8
1.174	16.8	21.0	32.9	14.6
1.156	15.4	18.8	21.4	27.8
1.138	15.1	18.4	16.9	47.1
1.119	17.1	21.0	12.1	60.3
1.095	30.2	36.7	11.5	74.7

B. Density (g/ml)	220 kDa	220 + 116 kDa	Lipid DPM/mg protein
1.169	26.2	30.8	3468
1.115	45.2	56.1	18244
1.102	52.7	65.8	30305
1.086	53.5	64.1	20606
1.079	56.4	67.0	17901
1.072	51.2	65.1	20633
1.071	64.9	79.3	28095

A role for the protein and lipid complex in lipid droplet formation and/or growth would presuppose existence of a similar complex in other mammalian species. Further, whether the major proteins associated with lipid droplets were present in the complex remained to be determined. Since antibodies against major milk proteins were available for cow, the presence of a similar complex in cow was investigated. KBr density gradient fractionated cytosol from lactating cow mammary gland displayed a similar lipid distribution and polypeptide enrichment pattern, although with slight differences in apparent masses of high molecular weight polypeptides (Fig. 8). The low density pool from cytosol isolated from both cow and rat contained the 220 kDa polypeptide as the major constituent. While the 116 kDa polypeptide was a prominent band in gels of rat fractions, there was no major polypeptide visible in this region in gels of cow fractions. Fractions from both species contained polypeptides migrating with molecular weights corresponding to 155 kDa, a triplet band pattern between 80 and 116 kDa, single bands at 70 kDa, 60 kDa and 45 kDa, as well as three bands below 30 kDa. Low density fractions from rat cytosol differed from cow fractions by the presence of an 80 kDa polypeptide in the former. These results indicated the presence of similar complexes in rat and cow, but with some species variability in polypeptide composition.

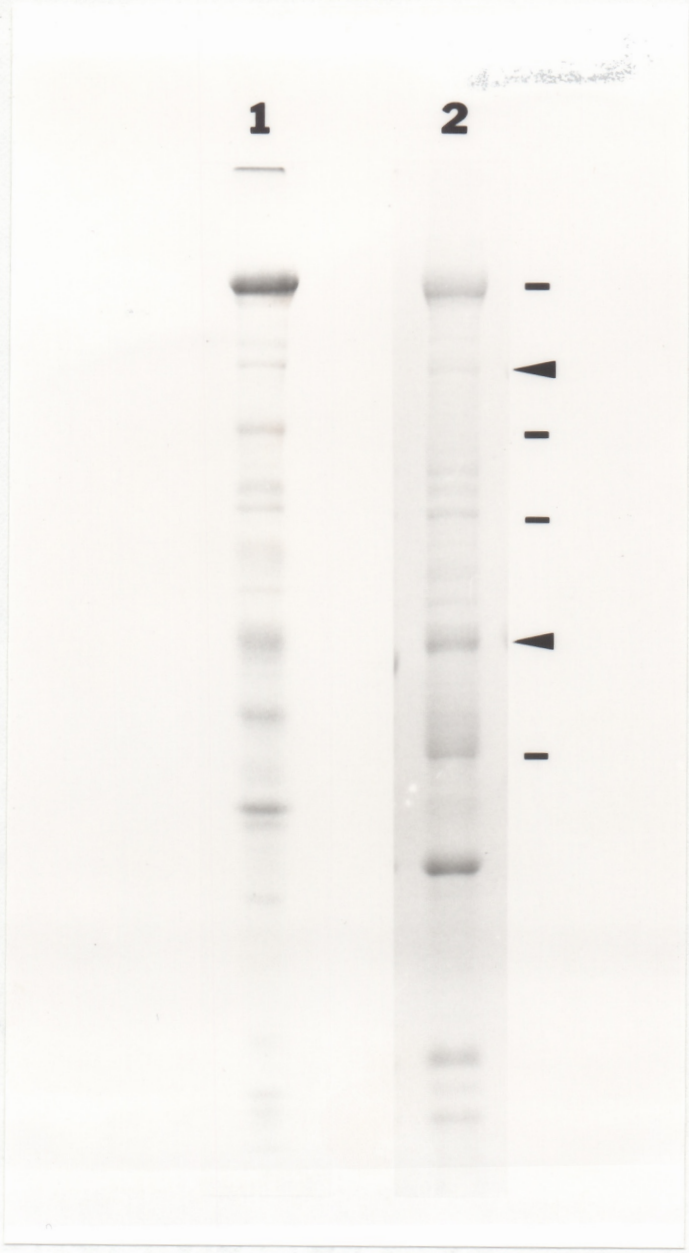

Figure 8: Comparison of SDS-PAGE polypeptide profiles from rat (lane 1) and cow (lane 2) low density fractions from KBr density gradient centrifugation of cytosol. Proteins were separated in 8 % gels by SDS-PAGE and visualization was by coomassie blue stain. Bars along lane 2 denote the positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards from top to bottom, respectively. The uppermost arrowhead indicates position of cow xanthine oxidase, lower arrowhead indicates position of cow butyrophilin.

2) Identification of the 220 kDa Peptide as the Monomer of Fatty Acid Synthase

Introduction

The 220 kDa and 116 kDa polypeptides that comprised the major protein components of the high molecular weight, lipid-rich fractions both from gel exclusion chromatography and from potassium bromide density gradient centrifugation were of similar mass to those peptides shown to be enriched in mammary gland cytosol during lactation. Studies by Baldwin and Milligan (1966) demonstrated enrichment of fatty acid synthase (FAS; a 478 kDa homodimer) in cytosol of mammary gland from animals that were lactating as compared to non-lactating animals. This begged the question of whether the 220 kDa constituent in the lipid rich fractions, that was found to be developmentally regulated in my work, might be the monomer unit of FAS. Attempts at N-terminal analysis of the polypeptides failed. This was consistent with the fact that the N-terminal of FAS is blocked in eukaryotic organisms (Smith and Abraham, 1970). Amino acid composition was similar to that reported for rat mammary FAS (Smith and Abraham, 1970). The 220 kDa polypeptide was confirmed to be FAS by immunodetection on Western blots, its migration in SDS-PAGE relative to purified FAS protein, and the ability of antiserum to FAS to specifically precipitate the protein.

Results

A) *SDS-PAGE and immunodetection of FAS:*

Unfractionated cytosol from lactating mammary gland; the high molecular weight, lipid droplet formation-promoting fraction of cytosol; the low density, lipid-rich KBr fraction; and a high molecular weight gel filtration fraction of the latter all contained a major polypeptide that co-electrophoresed with purified rat liver FAS (not shown). Amino acid composition of the 220 kDa protein (Table 3), determined following its elution from gels, was similar to the amino acid composition as was reported for fatty acid synthase from rat (Smith and Abraham, 1970). Attempts to determine the N-terminal sequence of this polypeptide failed, suggesting that the N-terminus was blocked, as is reported to be the case for FAS (Amy et al., 1989). The 220 kDa polypeptide, but not the 116 kDa one, was recognized by anti-fatty acid synthase antibodies on Western blots (Fig. 9). Nonimmune rabbit IgG failed to bind to either constituent. In the G-100 fraction, a second band of approximate molecular weight 150 kDa also was bound by anti-FAS antiserum. Since gel exclusion chromatography was done at room temperature, this peptide was likely a proteolytic product of FAS. The 155 kDa constituent of low density KBr fractions and unfractionated cytosol did not bind the antibody. Antiserum

Table 3: Amino acid composition of the 220 kDa polypeptide of the lipid-droplet-promoting fraction in comparison to compositional data for rat mammary gland FAS (Smith and Abraham, 1970). Amino acid analysis was performed at Washington University Protein Chemistry Laboratory, St. Louis. Results are reported as number of amino acids per dimer.

Amino Acid	Composition	
	FAS	220 kDa(x2)
ASP	296	336
THR	195	204
SER	285	316
GLU	405	486
PRO	227	152
GLY	301	486
ALA	324	270
CYS	56	44
VAL	267	204
MET	69	28
ILE	135	156
LEU	457	320
TYR	87	102
PHE	126	120
LYS	153	214
HIS	110	76
ARG	186	224

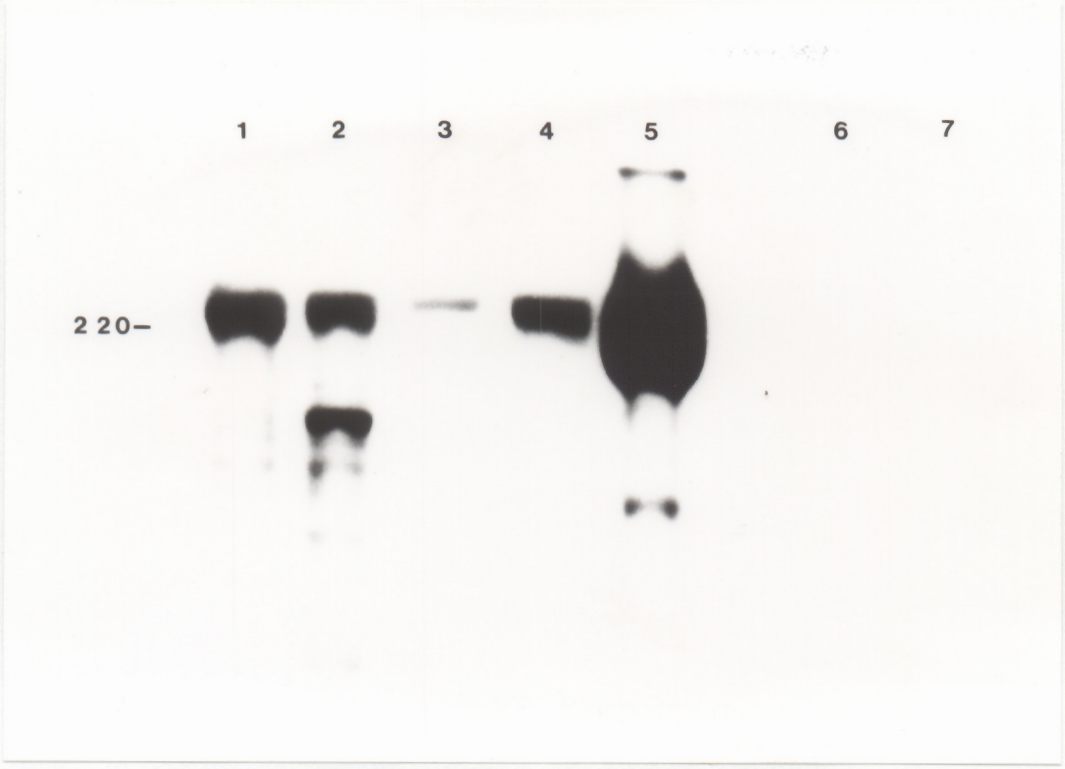

Figure 9: Western blot analysis of cytosol preparations with antiserum to rat fatty acid synthase. Lane 1, unfractionated cytosol; lane 2, Sephadex G-100 void volume fraction from cytosol; lane 3, low density, lipid-enriched fraction from a KBr density gradient separation of cytosol; lane 4, void volume fraction from Superose 6 FPLC separation of the gradient fraction of lane 3; lane 5, purified rat liver fatty acid synthase; lane 6, low density fractions from a KBr gradient separation of cow cytosol; and lane 7, unfractionated mammary gland cytosol from a 2 day involuted rat. Proteins were separated in an 8% acrylamide gel. Detection was by autoradiography after incubation of blot with ^{125}I-labeled Protein G.

against rat liver FAS selectively precipitated the 220 kDa polypeptide from unfractionated cytosol, while similar amounts of the non-specific IgG did not (Fig. 10). When the low density KBr fraction was used, the anti-FAS antibody selectively precipitated the 220 kDa polypeptide as well, along with a second polypeptide that comigrated with the 116 kDa standard, two polypeptides in the range of 140-160 kDa and one peptide with apparent mass of 50 kDa. In experiments in which ^{32}P labeled cytosol was separated by KBr density gradient centrifugation, approximately 65% of the label recovered in the low density (< 1.17 g/ml), 220 kDa polypeptide-enriched fraction was associated with lipid. The addition of increasing amounts of the FAS antiserum to the low density fraction resulted in increased precipitation of labeled lipid (Fig. 11) as well as the 220 kDa polypeptide identified as FAS. In contrast, addition of crude IgG from nonimmunized rabbits, in amounts equivalent to amounts of anti-FAS, did not specifically precipitate ^{32}P-labeled phospholipids.

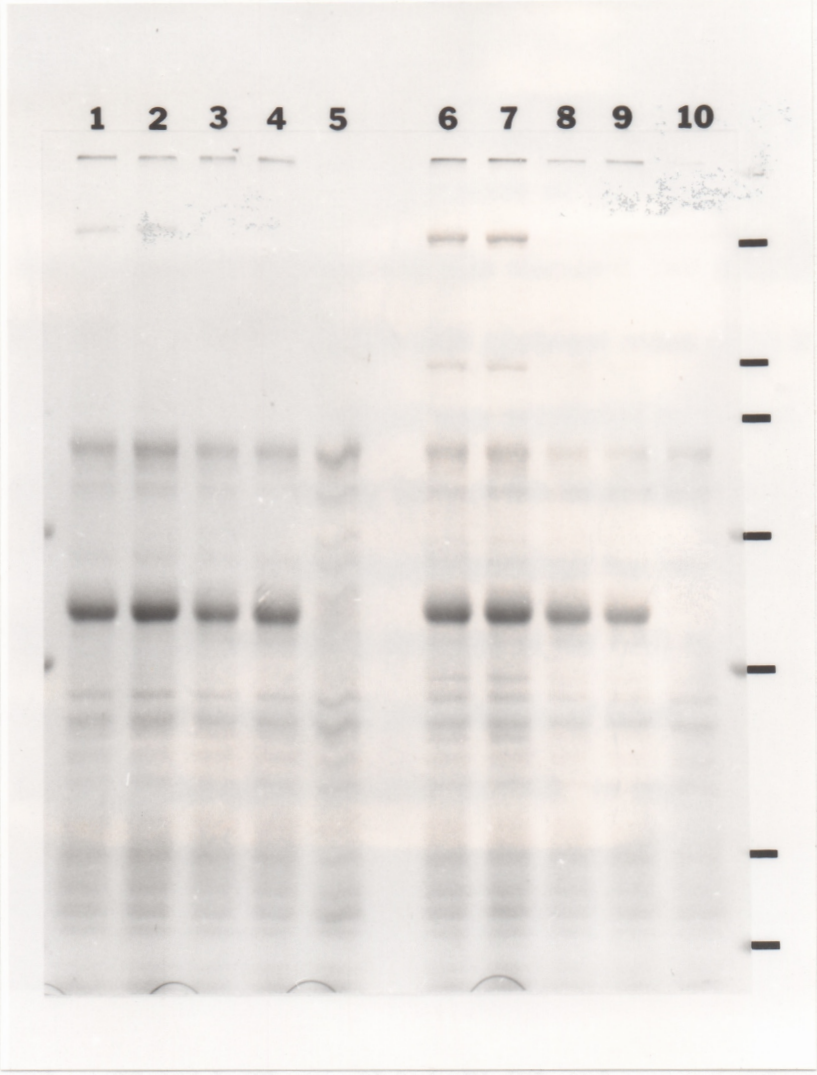

Figure 10: SDS-PAGE analysis of anti-FAS immunoprecipitated polypeptides from unfractionated cytosol (lanes 1 - 5) and the KBr low density fraction (lanes 6 - 10). Cytosol was incubated with anti-rat FAS antiserum (1 & 2, 6 & 7), or non-immune rabbit IgG (lanes 3 & 4, 8 & 9), and precipitated by incubation with immobilized protein A. Cytosol also was incubated with immobilized protein A directly to determine background binding (lanes 5 & 10). Visualization was by staining with coomassie blue. Bars denote the positions of 200 kDa, 116 kDa, 97 kDa, 80 kDa, 50 kDa, 24 kDa, and 14 kDa standards from top to bottom, respectively.

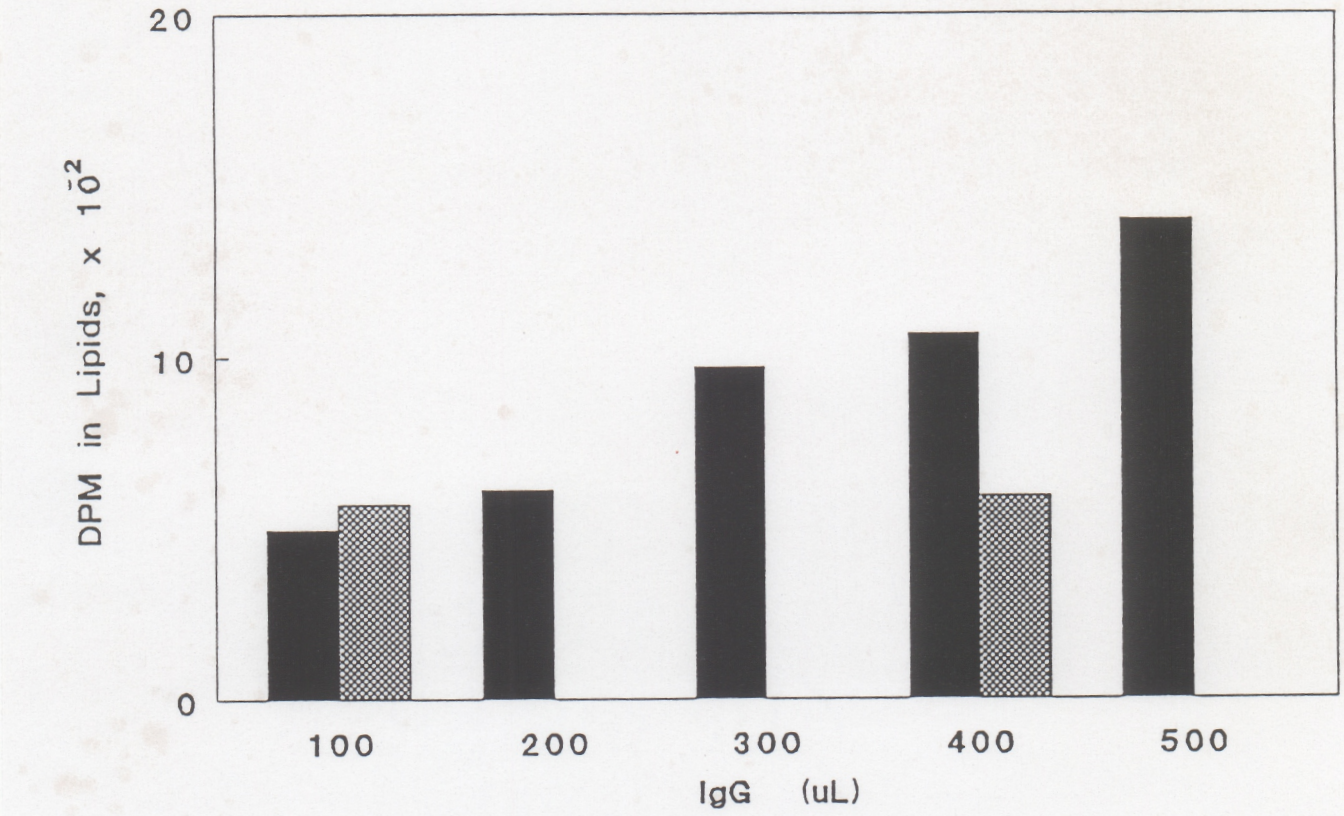

Figure 11: Immunoprecipitation of ^{32}P-labeled phospholipids with antiserum to fatty acid synthase. The low density, lipid-enriched fraction of cytosol from KBr density gradient separation was dialysed into cytosol buffer, and was incubated for 90 min at room temperature with indicated volumes of antiserum against fatty acid synthase. Insoluble Protein A was added, incubation continued for 60 min, and then precipitates were collected by centrifugation through 1 M sucrose in cytosol buffer. Pellets were washed twice by resuspension in 1 M sucrose and centrifugation, and lipids were extracted for measurement of the amount of ^{32}P-phospholipid precipitated. As a control in two cases where volumes of antiserum used were 100 and 400 μL, equivalent amounts of serum (based on absorbance at 280 nm) from nonimmunized animals (hatched bars) was substituted for anti-fatty acid synthase serum (solid bars).

B) ***Gel exclusion chromatography of free and complexed FAS:***

Uncomplexed FAS could be separated from LDFAS by gel exclusion chromatography (Fig. 12). Rat liver FAS was separated by FPLC on a Superose 6 column equilibrated with buffer containing 250 mM NaCl. A single peak containing FAS eluted within the included volume of the column. The calculated K_{av} was 0.30 and the corresponding molecular weight was 630 kDa. The LDFAS fraction, isolated by KBr density gradient centrifugation, was separated on the same column under similar conditions. A band that comigrated in SDS gels with a purified FAS standard and was recognized by anti-FAS antiserum, eluted with the void volume. The size exclusion limit of the Superose 6 column was 5×10^8 Da. It may be that the exclusion of the LDFAS complex from the matrix is a reflection of its configuration rather than its size. The interaction of protein and lipid likely results in a particle with a large Stoke's radius. The presence of low molecular weight polypeptides in the void volume suggests that these proteins are associated with a high molecular weight complex. FAS also was detected in a smaller peak that eluted within the included volume. This peak eluted with the same K_{av} as purified FAS. Protein recoveries following FPLC were low, apparently due to the interaction of the lipid-rich complex with the Sepharose matrix of the column. Stringent washing conditions were needed to clean the column after

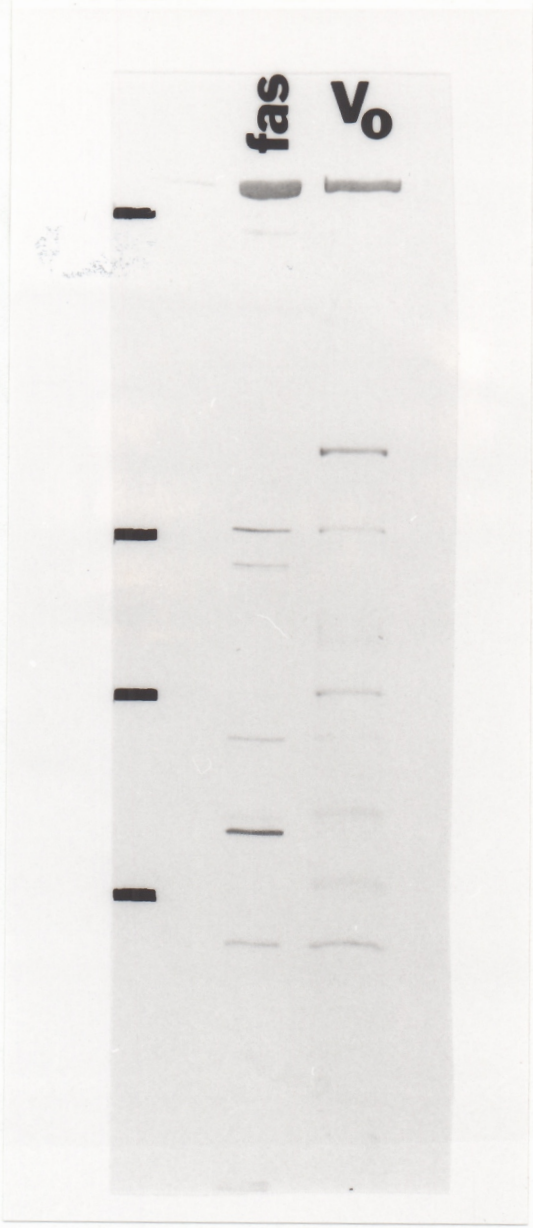

Figure 12: Electrophoretic separation of polypeptides from rat LDFAS following size exclusion chromatography on a Superose 6 column. Separation was by SDS-PAGE in an 8% gel which was stained with silver. Positions of standard proteins, marked by bars from top to bottom, were myosin (200 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa), respectively. The lane labeled **fas** indicates proteins from the LDFAS complex that eluted with a Kav similar to that for purified FAS. **Vo** indicates polypeptides recovered in the major protein peak with the void volume.

every application. To avoid this complication, native PAGE was attempted as a means to separate complexed FAS from noncomplexed polypeptides.

C) *Further purification of the low density fatty acid synthase (LDFAS) complex by native PAGE:*

Native PAGE separation of the rat LDFAS complex resulted in retention of the majority of protein as a high molecular weight complex at the entrance to a three percent stacking gel (Fig. 13a). Attempts were made to purify the complexed, lipid-associated form of FAS by PAGE in 6% gels polymerized and run in the absence of SDS and 2-mercaptoethanol. The majority of the coomassie stained protein remained at the entrance to the separating gel when the pH of the electrophoresis buffer was either 8.8 or 9.8. Western blot analysis, using anti-rat FAS antiserum, failed to detect FAS in nitrocellulose blots of separating gels. When proteins in the three percent stacking gels also were transferred to nitrocellulose, antibody reacted with constituents that were retarded at the entrance to the stacking gels, as well as constituents at the interface between the three percent and 6% gels (13b). ^{32}P-labeled rat LDFAS was separated by native PAGE as described above. When gels were dried and exposed to X-ray film immediately after electrophoresis, some of the label was detected at the gel front, while the majority of the label was retained at the

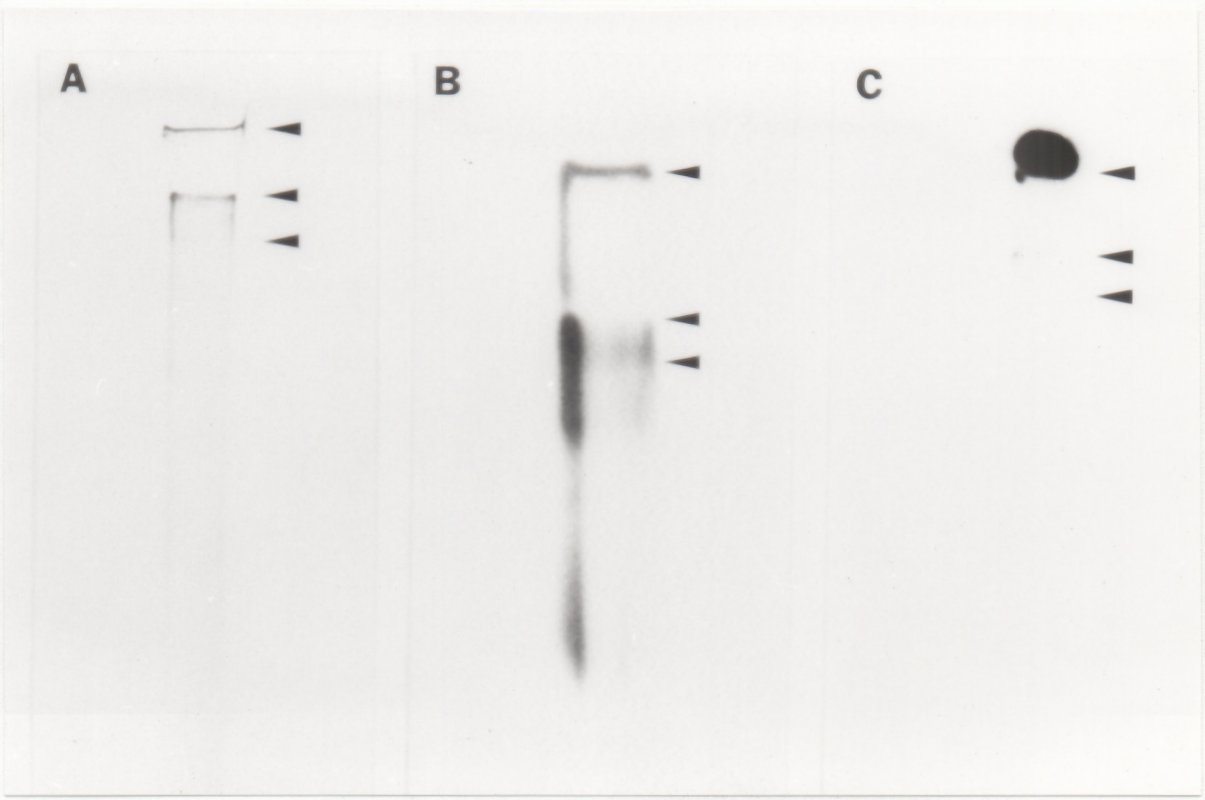

Figure 13: Native PAGE of ^{32}P labeled low density fraction from a KBr gradient. The top marker indicates position of the well base in a 3% stacking gel. The middle marker indicates the position of the interface of the stacking gel with the 6% separating gel. The bottom marker indicates the position of a band that entered the separating gel. Samples were in 10% glycerol and electrophoresis was performed in Tris-glycine buffer at pH 8.8. a) Visualization was by coomassie blue. b) Western blot using antiserum to rat FAS, detection was by ECL Western blot reagents with HRP-conjugated second antibody. c) Autoradiogram of separated ^{32}P labeled LDFAS following transfer to nitrocellulose.

entrance to the stacking gel (Fig. 13c). When gels first were stained and fixed prior to drying and exposure to X-ray film, radiolabel was detected only at the entrance to the 3% gel. Following separation of ^{32}P labeled LDFAS by SDS-PAGE, gels were stained, dried, and exposed to X-ray film to detect any phosphorylated proteins present. When samples containing 5,000 cpm of ^{32}P were loaded per lane and gels were exposed for 72 hrs, no radiolabel was detected within the gel or at the dye front, indicating that the radiolabel was not protein associated. In the presence of SDS, labeled free phospholipid migrated at the dye front and was washed away during fixing and staining procedures. Therefore, following separation in native gels, the label at the gel entrance likely was lipid associated with protein, while the label at the gel front likely was free phospholipid.

In similar experiments with native PAGE, purified FAS entered the 6% separating gel, with a relative migration of approximately 0.4, and was recognized by antibodies against FAS (Fig. 14). Treatment of the LDFAS complex with either 4M urea or 4M guanidine hydrochloride prior to electrophoresis did not facilitate the migration of complexed FAS into the gel under native conditions. Treatment with 1% Tween 20 or exposure to pH 12 did, however, facilitate entrance of FAS into the separating gel, indicating that the complex was disrupted in the presence of these agents. When exposed to low pH (pH 2.0) the LDFAS complex formed a flake-like white precipitate.

Figure 14: Western blot of purified liver FAS (lane 1), LDFAS (lane 2), and TX-114 aqueous phase LDFAS (lane 3). Polypeptides were separated by native PAGE, electroblotted to nitrocellulose and incubated with anti-rat liver FAS antiserum. Detection was by ECL Western blot detection reagents (Amersham) with horseradish peroxidase-conjugated second antibody, followed by autoradiography.

After adjusting the buffer to 33% KBr for density gradient centrifugation, the precipitate was observed to increase in quantity and could be recovered as a packed white floating layer similar in appearance to CLD isolated from mammary gland tissue homogenates. All polypeptides and lipids present in the LDFAS complex were recovered in this precipitate. Extraction of the LDFAS complex using the Triton X-114 phase separation technique resulted in the partition of most of the LDFAS proteins into the detergent phase (Fig. 15), suggesting that these polypeptides could interact with lipids.

The retention of the LDFAS complex at the entrance to the stacking gel provided a convenient method for removing polypeptides not associated with the LDFAS complex. The complex could be enriched by repeated application of sample to the gel, allowing noncomplexed constituents to migrate into the gel after each subsequent addition of sample. The proteins in this high molecular weight complex then were eluted from the gel and separated in a second dimension by SDS-PAGE (Fig. 16). The complex contained one major polypeptide of molecular weight 220 kDa (Fig. 16a) that was recognized by anti-FAS antibodies. A few other polypeptides, with molecular weights greater than about 50 kDa also were present, although in low quantities. Analysis of lipids from the eluted complex showed the presence of the five major phospholipids characteristic of milk lipid globule membranes (Fig. 16b) as well as triacylglycerols, cholesterol and fatty acids (not shown).

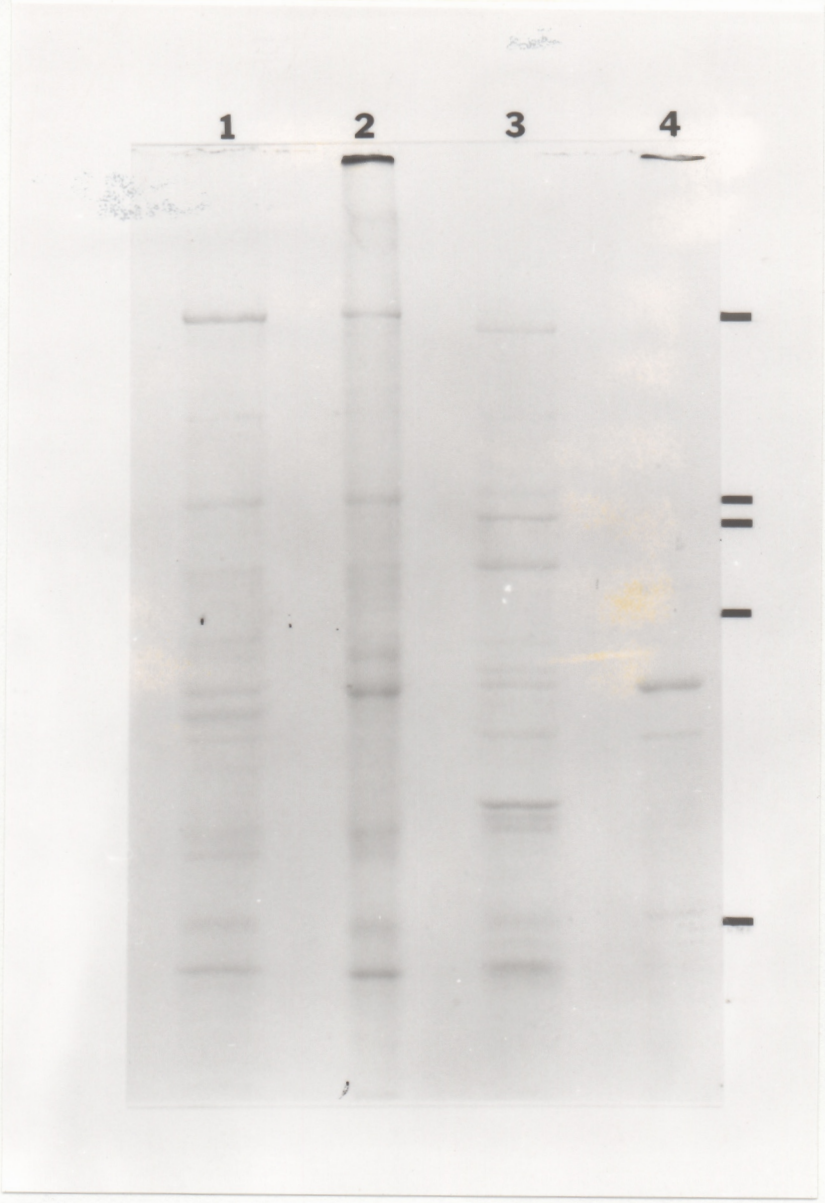

Figure 15: Electrophoretic patterns of polypeptides fractionated by Triton X-114 detergent phase separation. Proteins that partitioned into the detergent phase were recovered by precipitation with methanol and chloroform and separated by SDS-PAGE in 8 % gels. lane 1: unfractionated LDFAS, lane 2: LDFAS detergent phase proteins, lane 3: supernatant from + ATP incubations of ER, lane 4: detergent phase proteins from ER supernatant sample (experimentation and data for samples depicted in lanes 3 & 4 are discussed in section 3-B). Proteins were visualized by staining with coomassie blue. Bars denote positions of 200 kDa, 116 kDa, 97 kDa, 80 kDa, and 50 kDa.

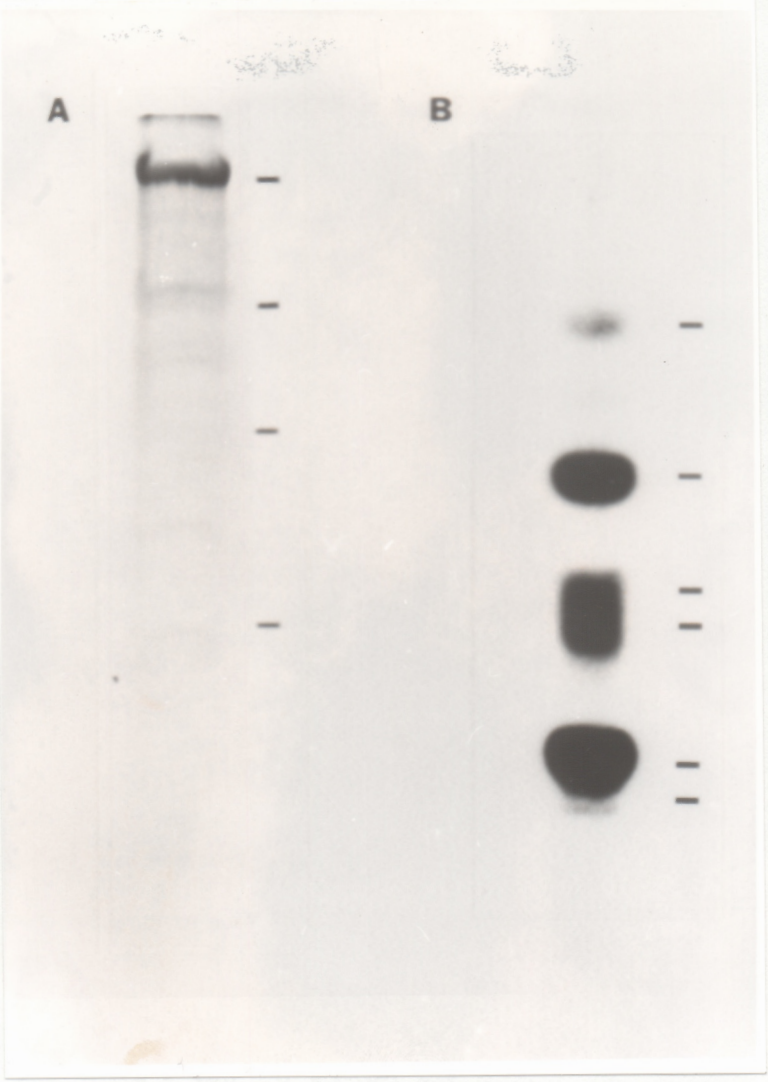

Figure 16: Protein and lipid analysis of sample eluted from the entrance to a 3% stacking gel following native PAGE. a) SDS-PAGE polypeptide composition of the low density complex eluted from the well base of a 3% stacking gel. The low density fraction was loaded into the well in a stop-run fashion so that peptides which entered the gel were repeatedly separated out. The high molecular weight complex was trapped in the well in an agarose plug (0.5% agarose), and electro-eluted for SDS-PAGE analysis. Bars denote the positions of 200 kDa, 116 kDa, 97 kDa, 66 kDa, and 45 kDa standards from top to bottom, respectively. b) Autoradiogram of ^{32}P labeled polar lipids extracted from the eluted complex. Bars denote positions of phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl inositol, phosphatidyl serine, phosphatidyl choline, and sphingomyelin, respectively, from top to bottom.

In comparable experiments in which cytosol from cow mammary gland was used, FAS also was the major polypeptide in a complex retained at the entrance to a three percent gel (Fig. 17a). Using cow cytosol preparations and available antibodies, it was possible to probe the complex for the two major proteins of milk lipid globule membranes, xanthine oxidase and butyrophillin (Figs. 17b & c, respectively). Both proteins were present in the LDFAS complex from the KBr gradient, and both remained associated with the complex following subsequent isolation by native PAGE. Ligand binding studies demonstrated that small GTP binding proteins were associated with cow LDFAS as well (Fig. 18). The G-proteins identified in this low density fraction were similar in size to those reported to be associated with cow MLGM (Ghosal et al., 1993).

D) *Cross-linking studies*:

In both gel exclusion chromatography and native PAGE experiments, LDFAS behaved as if it was a high molecular weight protein and lipid aggregate. In order to determine if these polypeptides were associated in a complex, cross-linking experiments were performed using dimethyl suberimidate (DMS). After exposure to DMS, the major polypeptides of the LDFAS complex were cross-linked with one another to form two large

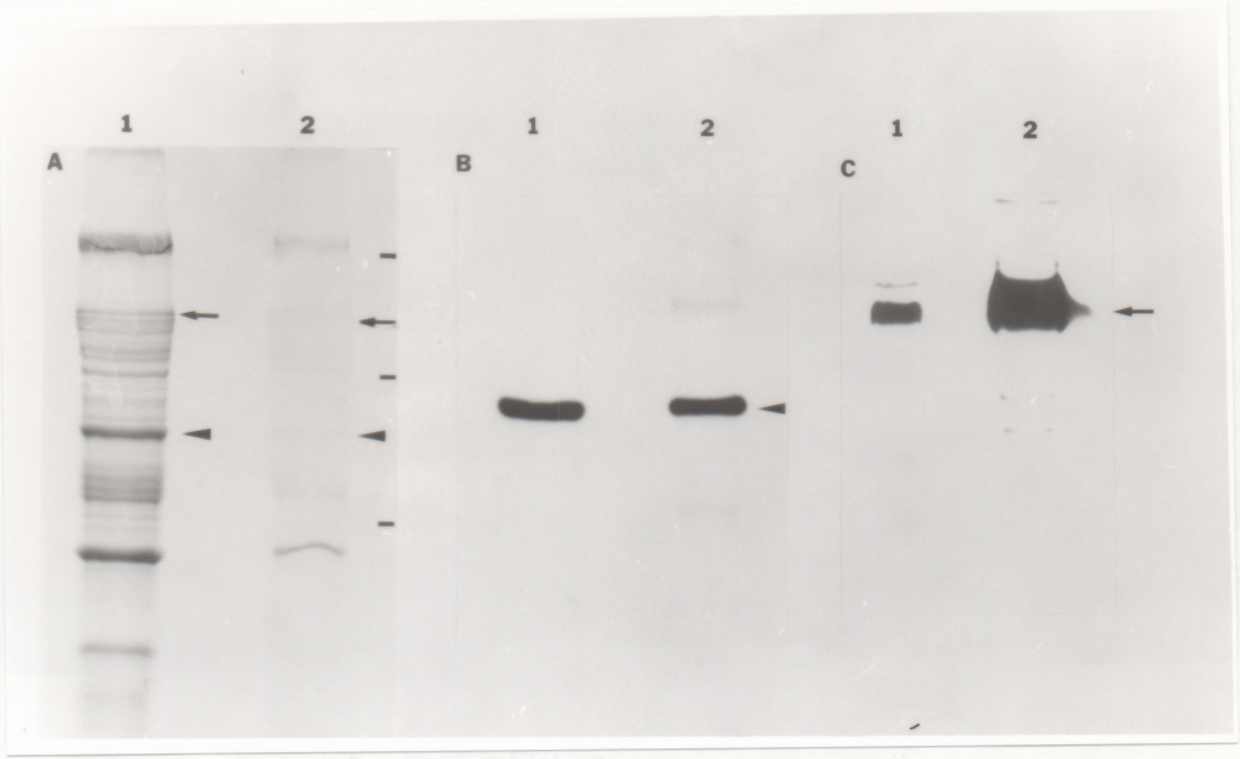

Figure 17: Polypeptide composition of cow LDFAS (lane 1) and the complex eluted from the entrance to the 3% gel following Native PAGE (lane 2). A) Visualization was by staining with coomassie blue. Bars denote the positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards from top to bottom, respectively. Arrows indicate positions of xanthine oxidase and arrowheads indicate positions of butyrophillin. B) Western blot analysis of samples in A) following transfer to nitrocellulose, using antiserum to cow butyrophillin or C) antiserum to cow xanthine oxidase.

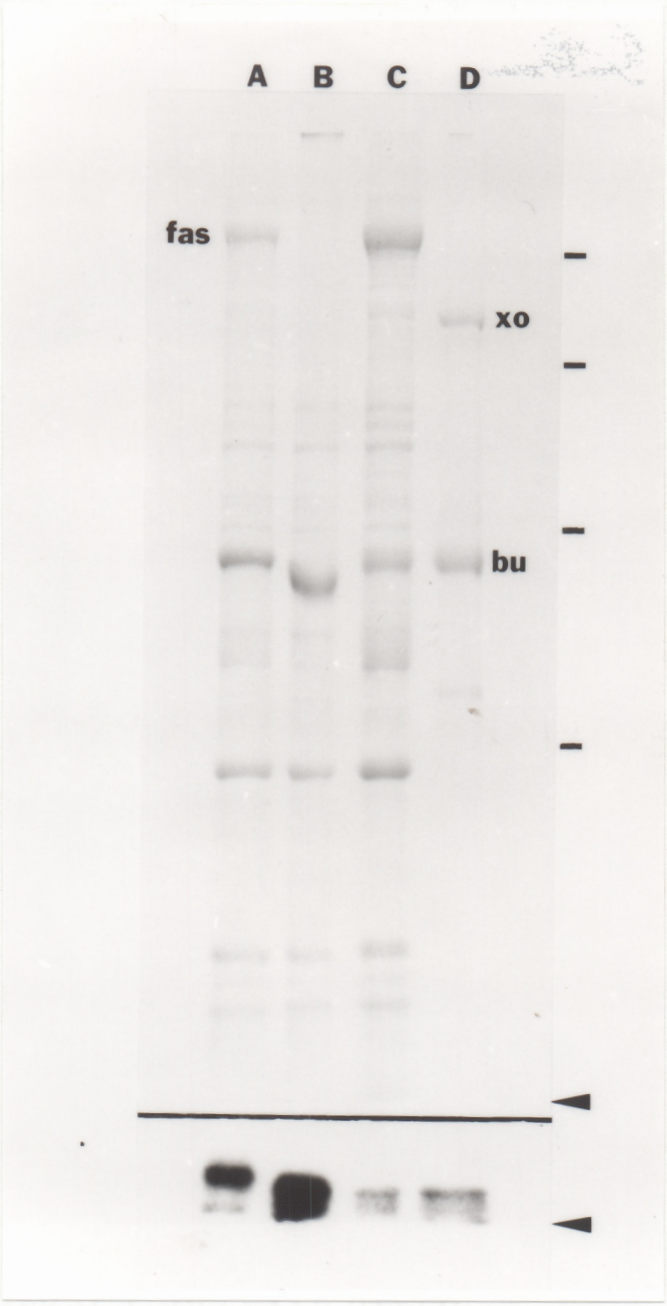

Figure 18: Electrophoretic pattern of cow cytosol density fractions and detection of small GTP-binding proteins. Unfractionated cow cytosol (lane A), high density fraction pool from KBr gradient (lane B), cow LDFAS (lane C) and cow milk lipid globule membrane (MLGM: lane D) were separated in 8 % gels by SDS-PAGE. In the panel below the line, the bottom portion of a duplicate gel was electroblotted to nitrocellulose and incubated with ^{32}P labeled GTP. Blots were exposed to X-Omat film for detection of GTP-binding proteins. Visualization of the gel was by coomassie blue stain. Bars denote the positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards from top to bottom, respectively. Positions of fatty acid synthase (fas), xanthine oxidase (xo) and butyrophillin (bu) are as indicated. Arrowheads mark the corresponding gel fronts.

complexes that were retarded at the entrance to a three percent stacking gel and at the interface between the stacking gel and an 8% separating gel, respectively, following SDS-PAGE (Fig. 19). All polypeptides that entered the separating gel were progressively diminished in amounts with increasing time of incubation with the DMS. Similar patterns of cross-linking were observed for both rat and cow LDFAS. Low concentrations (< 1 mg/ml) of protein were used in these studies to preclude nonspecific cross-linking events (Lad and Hammes, 1974).

The presence of milk lipids and MLGM proteins in the complex was consistent with the possibility that the LDFAS complex may be a previously unidentified precursor to intracellular lipid droplets or that it may serve as a lipid source for growing droplets. Since lipid synthesis occurs in the ER and lipid droplet precursors originate from the ER, one would anticipate association of the LDFAS complex with the ER, if either were the case. In an attempt to determine whether constituents of the LDFAS complex could associate with the ER, endoplasmic reticulum was incubated in a cell-free system with soluble LDFAS, or with serum albumin as control. Soluble and membrane associated proteins were separated by centrifugation and analyzed by SDS-PAGE (Fig. 20). Incubation resulted in release of several polypeptides from the endoplasmic reticulum when either LDFAS or BSA was present in the incubation medium (Fig. 20,bottom). Under either condition, there were no

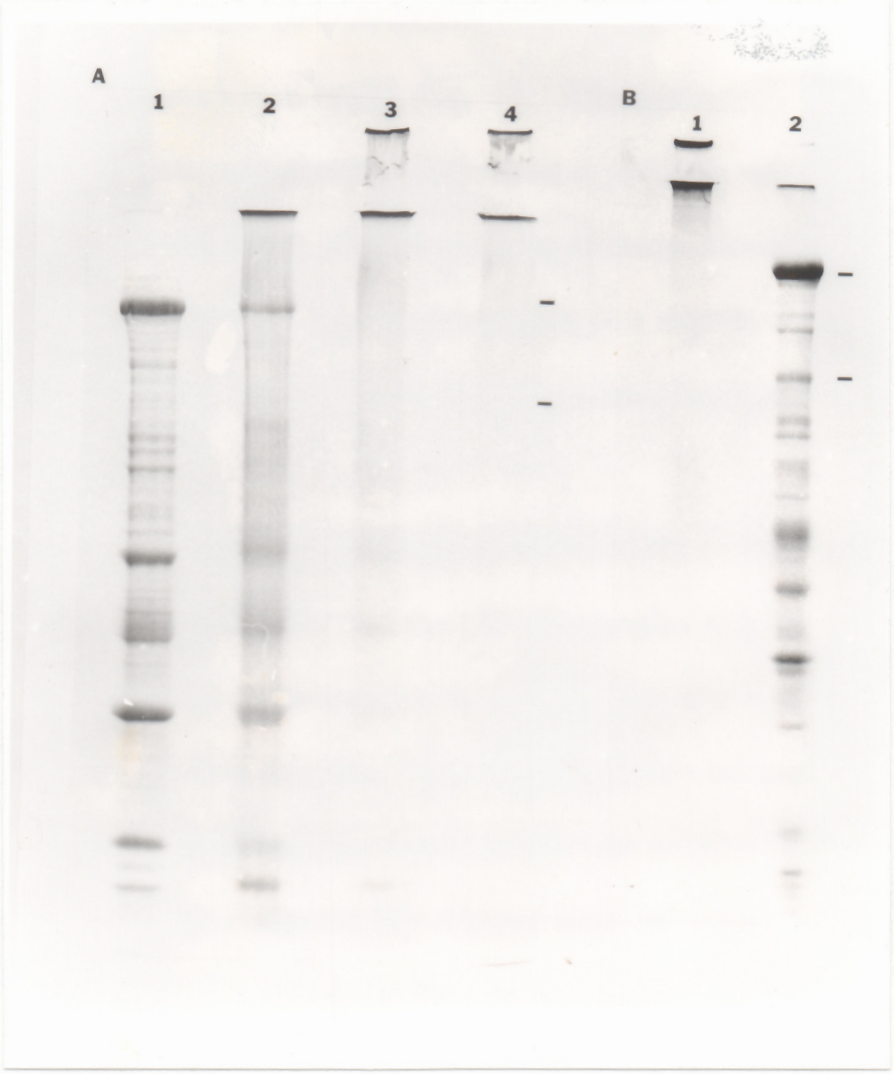

Figure 19: Polypeptide composition of LDFAS before and after incubation with the cross-linking reagent dimethyl suberimidate (DMS). Separation was by SDS-PAGE in 8% acrylamide gels. A) cow LDFAS at time zero (lane 1), 5 min. (lane 2), 15 min. (lane 3), and 30 min. (lane 4) incubation with cross-linker. B) rat LDFAS at time zero (lane 2) and 30 min. (lane 1) incubation with cross-linker. Visualization was by coomassie blue. Bars denote positions of 200 kDa and 116 kDa standards.

apparent differences in polypeptide patterns of post-incubation ER as compared to pre-incubation ER. However, the polypeptide patterns of the ER were complex and slight changes in these patterns likely were not detectable by coomassie staining. In both cases, where LDFAS was present in the incubation media as well as in controls where no LDFAS was present, polypeptides that were released from ER and recovered in the supernatant fractions had similar electrophoretic mobility to polypeptide constituents of the LDFAS complex. When DMS was added to the post-incubation soluble fraction, the major polypeptides formed cross-linked aggregates that were retarded at the entrance to a 3% stacking gel and 8% separating gel. BSA did not cross-link with the ER-released polypeptides. Western blot analysis demonstrated that FAS was present in the post-incubation supernatant whether or not LDFAS was present in the pre-incubation medium (Fig. 20, top). Fatty acid synthase also was detected in the cross-linked aggregates. The detection of FAS in supernatants from control samples where no exogenous FAS was included in the incubation media was consistent with dissociation of FAS from the ER. That FAS and other polypeptides released from the ER could be cross-linked, while exogenous bovine serum albumin was not, indicated that these proteins exist in complexed or aggregated forms.

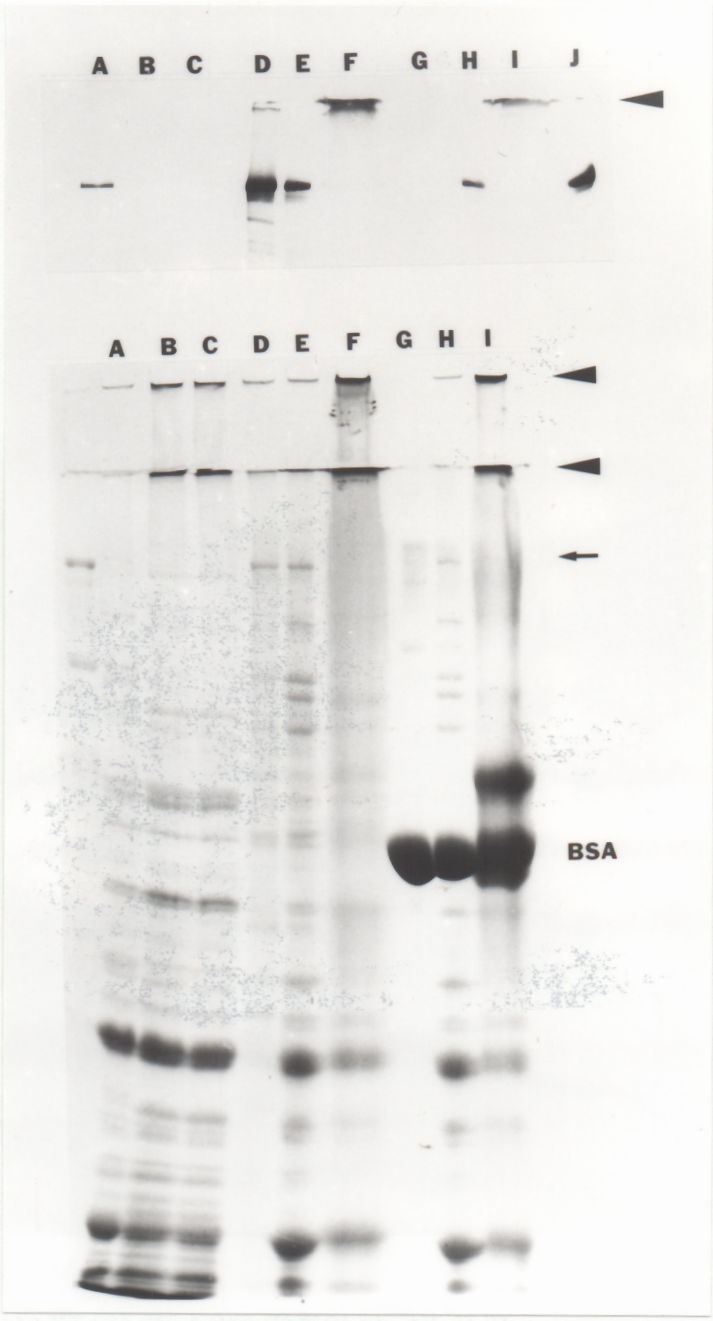

Figure 20: Bottom: Polypeptide composition of microsomal and supernatant fractions following incubations of LDFAS or BSA with endoplasmic reticulum in cell-free media. Microsomes were recovered by centrifugation and cross-linker was added to the supernatant. Separation was by SDS-PAGE in 8% acrylamide gels. Lane (A) was untreated ER control. Polypeptides were from pellets recovered following incubation of (B) LDFAS + ER, (C) BSA + ER, and from supernatants from the following experimental conditions: (D) LDFAS, (E) LDFAS + ER, (F) LDFAS + ER, cross-linked, (G) BSA, (H) BSA + ER and (I) BSA + ER, cross-linked. Visualization was by coomassie blue and bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively. Top: Autoradiogram of Western blot of fractions from above gel blotted to nitrocellulose and incubated with antibody solution against FAS. Lane (J) was purified rat liver FAS.

3) Association of FAS with Intracellular Components of the Milk Lipid Droplet Synthetic Pathway

Introduction

The following data are consistent with the possibility that LDFAS is a precursor lipid particle:

1) The cytosolic complex has been characterized thus far as a high molecular weight protein and lipid aggregate containing FAS as one of its major polypeptide constituents.

2) In cow LDFAS, the major milk lipid globule membrane proteins butyrophillin and xanthine oxidase, as well as small GTP-binding proteins, also were constituents of the complex.

3) The five major polar lipids found in milk, SPM, PC, PS, PE, and PI, triacylglycerols, cholesterol, and unesterified fatty acids were the major lipids in both the rat- and cow-derived FAS complexes.

If LDFAS was a precursor lipid particle, one should be able to find examples of this complex proximal to the site of lipid droplet release at the ER and/or in association with intracellular lipid droplets. The finding that FAS was released into the supernatant following incubation of ER in cell-free medium was

consistent with the association of FAS with ER. To further test this hypothesis, intracellular organelles and droplets were isolated by subcellular fractionation in order to test for the presence of FAS. FAS was detected by Western blot analysis using antiserum against liver FAS, and by assaying fractions for enzymatic activity by incorporation of radiolabelled malonyl-CoA into fatty acids. Distribution of FAS within the cell was determined by immunocytochemistry using antiserum to FAS. Affinity of the LDFAS complex for ER and intracellular lipid droplets was tested using ^{125}I labeled LDFAS polypeptides. Finally, the question of whether the protein-lipid complex resembled lipid droplet particles in morphology was resolved by electron microscopy.

Results

A) *Cell-fractionation studies:*

To determine the subcellular localization of the LDFAS complex, and in particular whether it associates with components involved in milk lipid globule formation, mammary gland homogenates were fractionated by differential and sucrose density gradient centrifugation. The association of FAS with intracellular components was determined by Western blot analysis of proteins

from cell fractions. Polyclonal antibodies against FAS recognized a polypeptide that comigrated with a purified rat liver FAS standard in rat and bovine mammary ER, intracellular lipid droplets, the secreted MLG coat material and MLGM fractions (Fig. 21). Fatty acid synthase could be partially dissociated from ER membranes upon treatment of microsomes with alkaline sodium carbonate buffer (Howell and Palade, 1982). Western blot analysis revealed that some FAS remained associated with the membrane fraction while a large amount of FAS was released into the supernatant. Similarly, lipid droplet-associated FAS was only partially dissociated by treatment with high pH sodium carbonate. Larger CLD's appeared to contain greater amounts of FAS, as a percent of total protein, as compared to precursor MLD's. A lipid-rich phase of milk, consisting primarily of the submembrane coat material, was extracted from milk fat globules (Freudenstein et al., 1979). FAS was identified as a constituent of this material by Western blot detection (Fig. 21). To confirm the presence of FAS in these cell fractions, enzymatic activity was measured by the incorporation of ^{14}C-malonylCoA into fatty acids. FAS activity was present in cytosol, endoplasmic reticulum, and intracellular lipid droplet fractions from mammary tissue homogenates (Table 4). No activity was detected, however, in LDFAS complexes isolated by KBr density gradient centrifugation or in MLGM. However, the LDFAS complex may have lost activity upon exposure to high salt concentrations during KBr density gradient

centrifugation, since unfractionated cytosol exposed transiently to KBr and then dialyzed against low salt buffer displayed an approximate 50% decrease in FAS activity. Failure to detect FAS enzymatic activity in MLGM may be due to inactivation over time after secretion from the cell. Following preincubation of ER in cell-free media, with or without ATP, greater than 80% of activity was lost from ER and was recovered in the supernatant fraction.

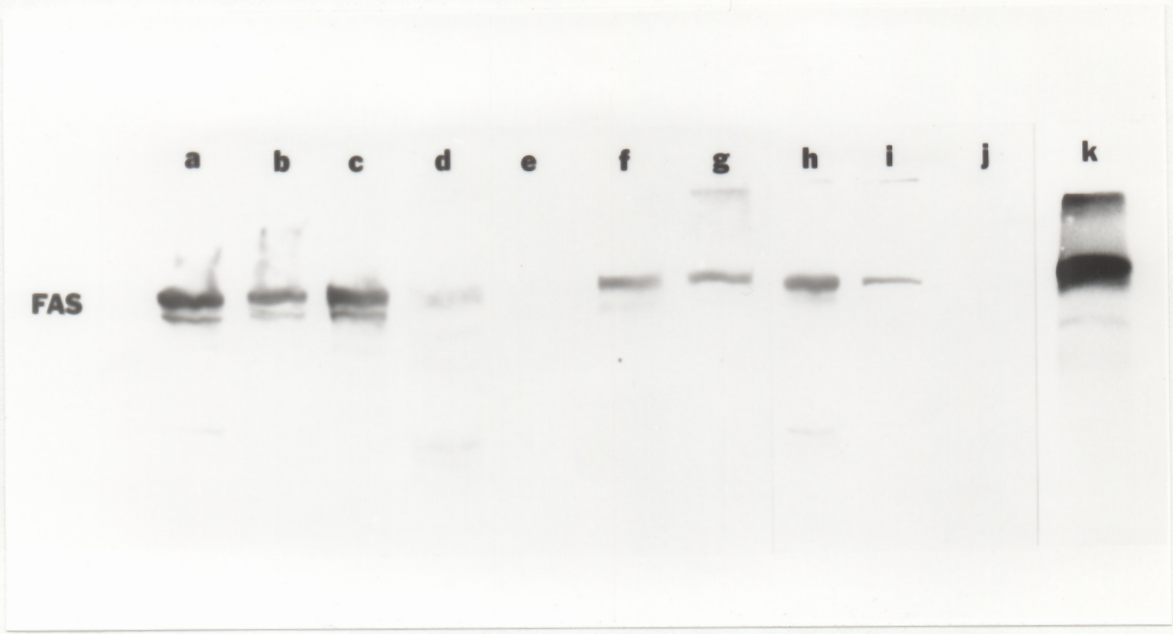

Figure 21: Western blot analysis of subcellular fractions from cow mammary gland homogenates and cow milk fat globule membrane separated by SDS-PAGE, with anti-serum to cow fatty acid synthase. Samples were (a) endoplasmic reticulum (ER), (b) ER following alkaline carbonate extraction, (c) soluble extract from (b), (d) microlipid droplets (MLD), (e) MLD following alkaline carbonate extraction, (f) cytoplasmic lipid droplets (CLD), (g) alkaline carbonate extracted CLD, (h) milk fat globule membrane (MFGM), (i) coat material from milk lipid droplets, (j) aqueous phase of MFGM, and (k) LDFAS.

Table 4: FAS enzyme assay of cell fractions from mammary gland tissue homogenates. Data are reported as averages of duplicate samples for each of three different sets of experiments. When samples were not assayed in a given experiment set, it is indicated by nd.

	cpm/mg protein/hr		
	1	2	3
cytosol	3500	1600	2150
cyt KBr	1640	nd	nd
LDF	0	0	0
MLD	nd	1600	1100
CLD	nd	3340	3600
MLGM	0	0	nd
ER	2440	2940	5400
ER +ATP	560	nd	nd
ER +ATP +LDF	560	nd	nd

B) ***ATP-dependent release of FAS from endoplasmic reticulum:***

Fatty acid biosynthesis and FAS itself occur in the cytosol. The occurrence of FAS in a membrane associated form has not been reported and raises the question as to the nature of its association. Some proteins exist in the cell both in soluble and membrane bound forms, and in some instances association-dissociation is regulated by ATP (Rothman and Orci, 1992). FAS initially was observed to be associated with endoplasmic reticulum during experiments to determine affinity of LDFAS for ER (see Fig. 20, section 2D). In these experiments, FAS was recovered in the supernatant of controls in which no exogenous FAS was present. This observation was consistent with dissociation of FAS from the ER. There were no exogenous nucleotides present in the incubation medium. To test whether nucleotides were effective in maintaining FAS in its membrane associated form, ER was incubated with nucleotides under various conditions. When ER was incubated in the presence of GTP or GTPγS, or in the presence of ATP at 4°C or 37°C in buffer containing 250 mM NaCl, the patterns of polypeptides released were similar to those seen in the absence of nucleotides (Fig. 22). A polypeptide with an electrophoretic mobility similar to that of FAS was released in all incubations. When buffer containing low salt concentrations (10mM KCl) was used, an ATP specific response was noted. A polypeptide that migrated with similar

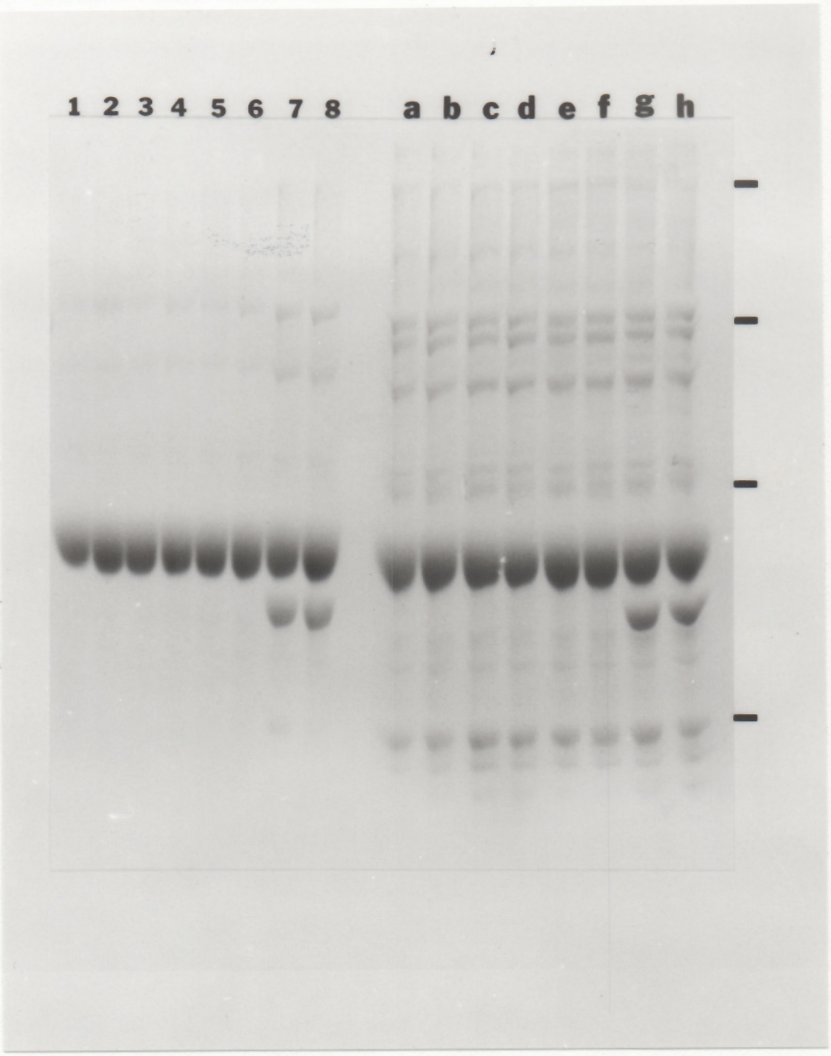

Figure 22: Electrophoretic patterns of polypeptides released into soluble supernatant fractions by incubating solutions of ER at 37°C (lanes 1,3,5,7 & a,c,e,g) or 4°C (lanes 2,4,6,8 & b,d,f,h) in low (numbers) and high (letters) salt buffers containing no nucleotide (1 & 2, a & b), GTP (3 & 4, c & d), GTPγS (5 & 6, e & f), or ATP (7 & 8, g & h). Following incubation, ER membranes were recovered by centrifugation and soluble supernatants were recovered and separated by SDS-PAGE in 8% gels. Visualization was by coomassie blue and bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively.

molecular weight to FAS was released (in amounts detectable by coomassie stain) only in the presence of ATP. Overall, low salt conditions decreased the amount and number of polypeptides released from the ER. This facilitated observation of the ATP-specific response. Polypeptides released from the ER in the presence of ATP had a similar electrophoretic profile to LDFAS polypeptides but behaved differently when fractionated by Triton X-114 detergent phase separation (see Fig. 15). A major peptide in the molecular weight range of 65-70 kDa partitioned into the detergent phase for both LDFAS and the ER complex. Contrary to LDFAS constituents, the higher molecular weight ER polypeptides did not partition into the detergent phase. This indicated differences in hydrophobicity of the respective proteins.

Experiments were performed to determine whether the ATP response was specific to mammary gland ER. Incubation of liver or mammary endoplasmic reticulum in cell-free media resulted in release of FAS into the supernatant when ATP was present in the media (Fig.23). These experiments differed from those experiments represented in Fig. 22 in that a reducing agent, DTT, was included in the incubation medium. To determine if the ATP-dependent response was driven by hydrolysis of ATP, the nonhydrolyzable analog, AMP-PNP was used (Fig. 24). Inclusion of ATP in the incubation medium stimulated release of FAS while the nonhydrolyzable form of ATP did not. ATP also stimulated release of several other proteins from ER that were

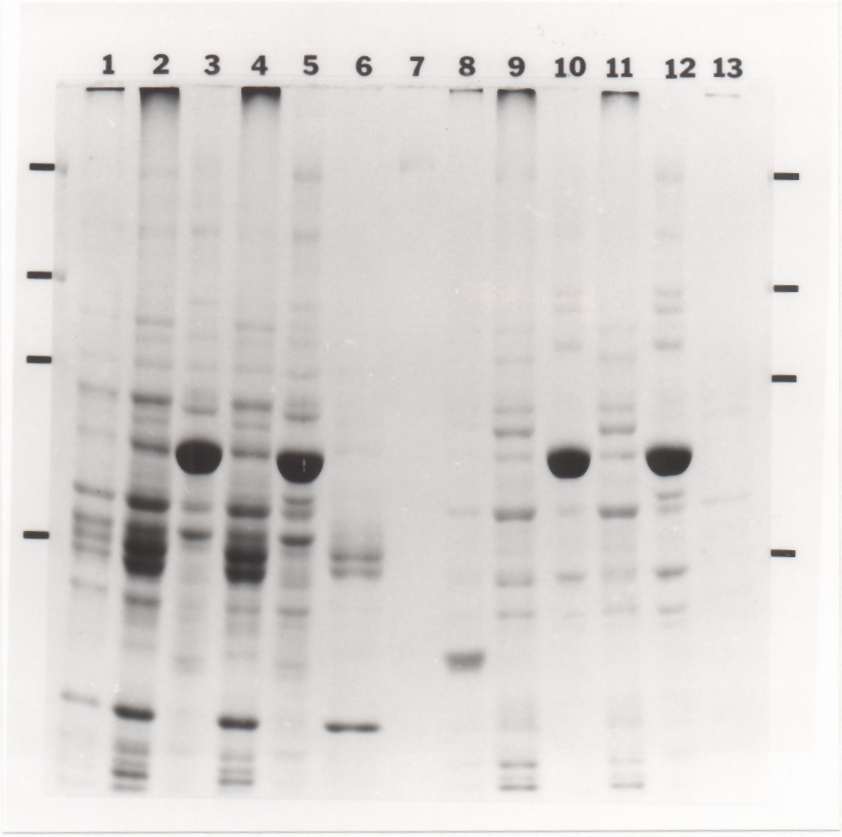

Figure 23: Electrophoretic patterns of ER polypeptides and polypeptides released from mammary and liver ER by incubation in cell-free media with or without ATP. lane 1: unincubated liver ER, lane 2: liver ER incubated without ATP, lane 3: supernatant from ER in lane 2, lane 4: liver ER incubated with ATP, lane 5: supernatant from ER in lane 4, lane 6: liver ER extracted with alkaline sodium carbonate, lane 7: liver FAS, lane 8: unincubated mammary ER, lane 9: mammary ER incubated without ATP, lane 10: supernatant from ER in lane 9, lane 11: mammary ER incubated with ATP, lane 12: supernatant from ER in lane 11, and lane 12: mammary ER extracted with alkaline sodium carbonate. Visualization was by coomassie blue and bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively.

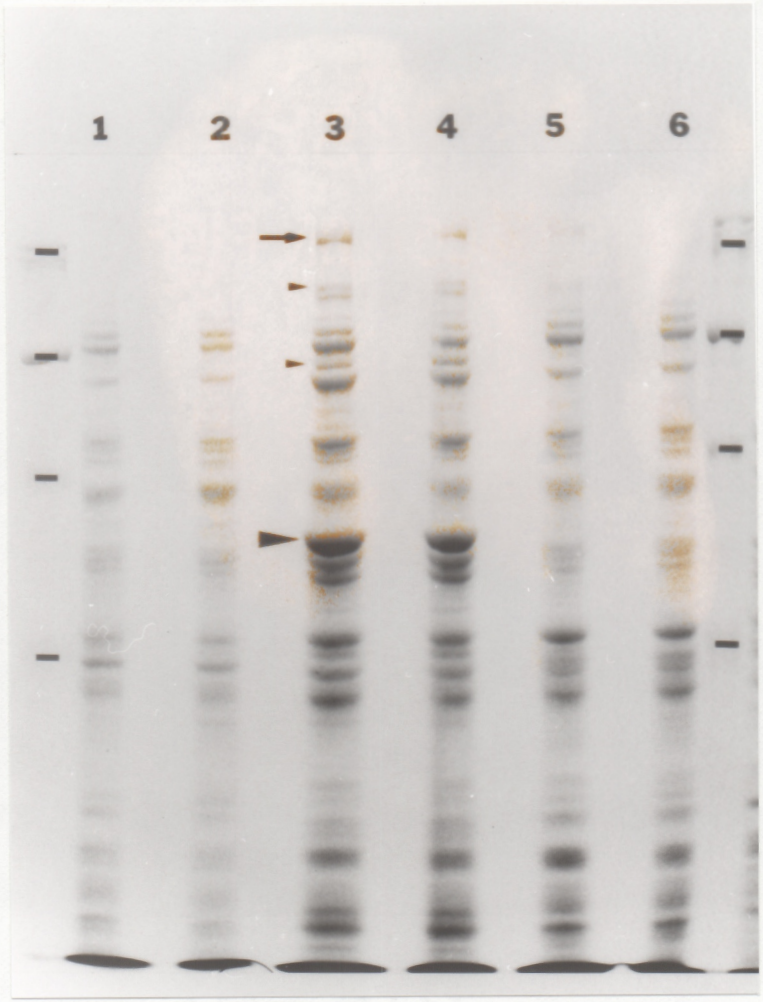

Figure 24: Electrophoretic patterns of polypeptides released from mammary gland ER in the absence (duplicate samples; lanes 1 & 2) and presence (lanes 3 & 4) of ATP, or in the presence of the non-hydrolyzable analog, AMP-PNP (lanes 5 & 6). Proteins were separated in 8 % acrylamide gels by SDS-PAGE. Visualization was by coomassie blue and bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively.

not released with the nonhydrolyzable analog. That release of polypeptides was stimulated by hydrolysis suggested possible regulation by phosphorylation. To determine whether polypeptides released in response to ATP hydrolysis were phosphorylated in their soluble forms, ER isolated from liver and mammary gland homogenates of lactating rats was incubated with γ-^{32}P-labeled ATP (Fig.25b). Ponceau staining of electroblots indicated that incubation of ER from both liver and mammary gland of lactating rats with radiolabeled ATP resulted in the ATP-stimulated release of FAS and other polypeptides. No radioactivity was detected in any of these polypeptides. Instead, the radioactivity was incorporated into membrane polypeptides that were not released in response to ATP. In liver ER, one band with an approximate molecular weight of 48 kDa became labeled with ^{32}P. In mammary ER, three membrane polypeptides with approximate molecular weights of 48 kDa, 45 kDa, and less than 30 kDa were labeled with ^{32}P. The two lower molecular weight polypeptides may be caseins, based on electrophoretic migration with M_r similar to those of caseins. The 48 kDa polypeptide, however, migrates with a higher molecular weight than would be expected for caseins. The 48 kDa phosphoprotein in liver ER is not a casein since liver does not express caseins. However, whether the 48 kDa phosphorylated polypeptide in liver ER and mammary ER are the same remains to be determined. A low amount of radioactivity was detected in three

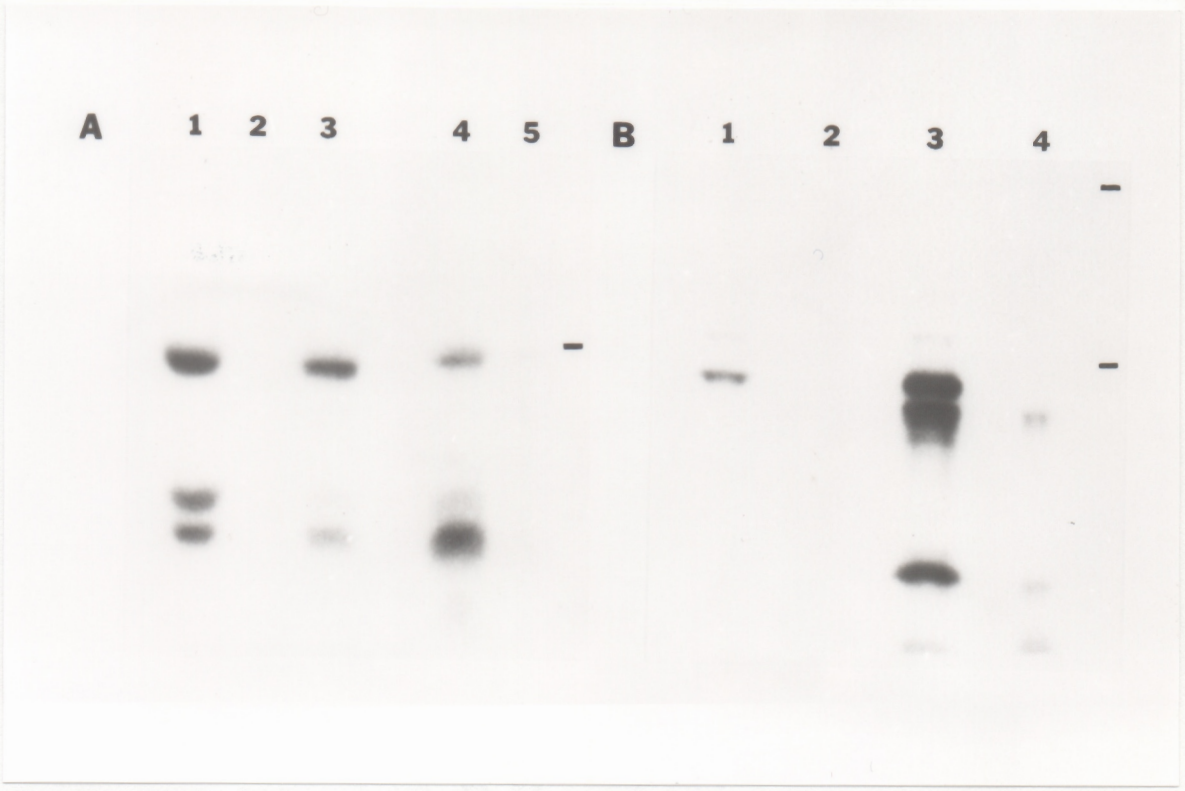

Figure 25: A) Autoradiogram of polypeptides from ^{32}P labeled ER and a supernatant fraction following incubations in cell-free media with or without ATP. ER was incubated as described, membranes and the supernatants were recovered by centrifugation and separated by SDS-PAGE in 8 % gels. Polypeptide were transblotted to nitrocellulose and exposed to X-ray film. lane 1: unincubated ER, lane 2: ER incubated + ATP, lane 3: ER incubated - ATP, lane 4: supernatant + ATP, lane 5: supernatant - ATP. B) Autoradiogram of ER and supernatant fraction from rat liver (lanes 1 & 2, respectively), and mammary tissue (lanes 3 & 4, respectively), following incubation in cell-free media containing ^{32}P labeled ATP. Polypeptides were separated as in A). Bars denote position of the 50 kDa marker.

soluble polypeptides from mammary gland ER and these may represent caseins released from damaged microsomes or secretory vesicles. No radioactivity was detected in the supernatant in the 48kDa region of the gel. From the above experiment it was concluded that the ATP-stimulated soluble forms of the ER polypeptides were not phosphorylated. To determine whether the membrane-associated forms of these polypeptides were phosphorylated, experiments were performed using mammary ER isolated from lactating rats injected with ^{32}P-orthophosphate. Radiolabel was incorporated into three ER polypeptides, corresponding in size to approximately 48 kDa and two bands less than 30 kDa. After incubation in cell-free medium containing ATP, no radiolabel was recovered in the ER membrane fraction. All three labeled polypeptides were recovered in the supernatant. When ER was incubated in the absence of ATP, the labeled polypeptides remained associated with the ER. These experiments demonstrated that in the presence of ATP, those ER polypeptides that were labeled *in vivo* were released from the membrane and remained phosphorylated in their soluble forms. In the absence of ATP the phosphorylated polypeptides remained associated with the membrane. These results indicated that the soluble and membrane bound forms of FAS and the other polypeptides were not regulated by phosphorylation. Certain observations from these experiments warrant further investigation but were not explored here: 1) The phosphorylation patterns of ER proteins differed for ER

that was labeled *in vivo* with ^{32}P-orthophosphate verses ER that was incubated *in vitro* with ^{32}P-ATP. This may be the result of tissue-specific kinases. 2) The presence of a phosphorylated polypeptide of estimated molecular weight 48 kDa in liver ER indicates that this protein is not a casein. Whether this is the same protein that is phosphorylated in mammary ER remains to be determined. 3) Finally, an inconsistency in release patterns needs to be addressed. The 48 kDa ER polypeptide labeled *in vivo* was released in its phosphorylated form by incubation with ATP, but the *in vitro* labeled ER polypeptide with similar molecular weight remained associated with the membrane when incubated in the presence of ^{32}P-labeled ATP. These may be distinct proteins that serve as substrates for two different kinases.

Experiments in which the LDFAS proteins were radiolabeled with ^{125}I revealed that the polypeptides present in the LDFAS complex were similar to proteins present in endoplasmic reticulum fractions (Fig.26). Radiolabeled polypeptides from the soluble LDFAS preparation associated with the ER *in vitro*, but association of the LDFAS proteins with the ER was enhanced in the absence of ATP or in the presence of the nonhydrolyzable analog, AMP-PNP (Fig. 27). When GTP was omitted from the incubation media, or when the nonhydrolyzable analog, GTPγS was used, association of the labeled complex with ER did not appear to be affected. Association of ^{125}I-labeled LDFAS components to ER in the absence of ATP was diminished when ER was

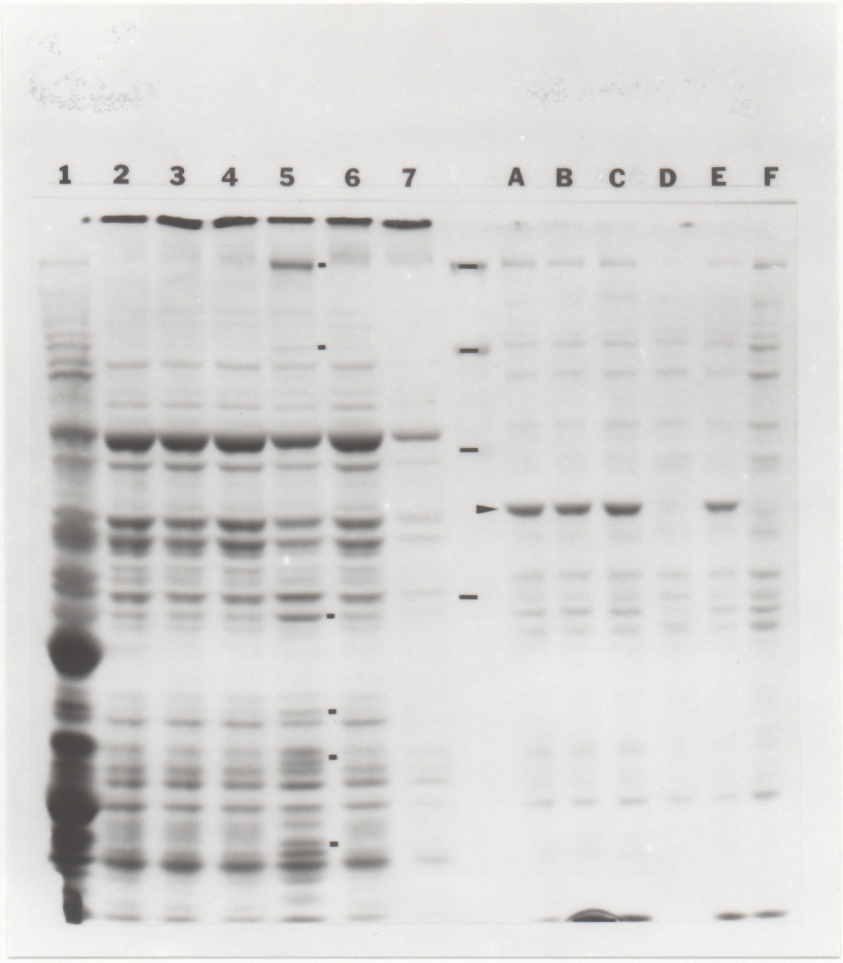

Figure 26: Electrophoretic patterns of ER and supernatant fractions after incubation of ER with ^{125}I-labeled LDFAS in cell-free media with or without ATP or GTP, or with corresponding non-hydrolyzable analogs, GTPγS or AMP-PNP, respectively. Numbered lanes are ER fractions: lane 1; unincubated ER, lane 2: - GTP, lane 3; + GTP, lane 4; GTPγS, lane 5; - ATP, lane 6; + ATP, lane 7; AMP-PNP. Letters indicate corresponding post-incubation supernatants from centrifugation of ER in lanes 2 through 7, respectively. Polypeptides were separated in 8% gels by SDS-PAGE and visualization was by coomassie blue stain. Bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively. Arrow indicates pyruvate kinase, a component of the ATP regenerating system. Visualization was by staining with coomassie blue. Markers along lane 5 indicate bands enriched in - ATP incubated ER relative to other conditions.

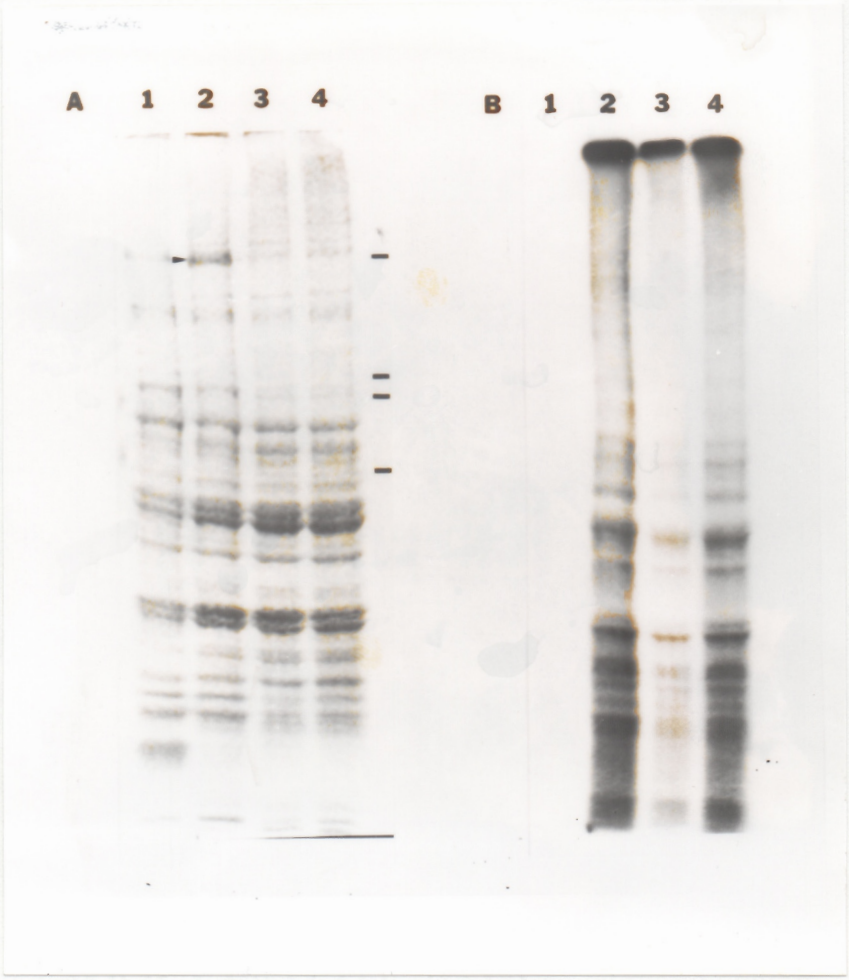

Figure 27: Electrophoretic patterns (A) and autoradiogram (B) of ^{125}I-labeled LDFAS proteins associated with ER following incubation in buffer containing GTP, DTT, and in the presence or absence of ATP, or containing AMP-PNP. Microsomes and associated ^{125}I LDFAS constituents were recovered by centrifugation through 1.2 M sucrose. Proteins were separated as described in Fig. 26, gels were dried under vacuum and exposed to X-ray film. Bars denote positions of 200 kDa, 116 kDa, 97 kDa and 80 kDa standards, from top to bottom, respectively. lane 1: unincubated ER, lane 2: - ATP, lane 3: + ATP, and lane 4: AMP-PNP.

preincubated in the presence of ATP as compared to ER that had been preincubated in the absence of ATP or to unincubated control ER (Fig. 28). These results were consistent with as ATP-hydrolysis driven dissociation of the LDFAS complex from the ER.

Peptides from the low density complex also associated with isolated lipid droplets of both density classes *in vitro* (Fig.29). When ATP was excluded from the incubation medium, association was enhanced. The amount of label that became associated was greater with MLD than with CLD on a protein basis. In cells that maintain mM concentrations of ATP, the effect seen here *in vitro* in the absence of ATP likely reflects an *in vivo* requirement for ATP to release the LDFAS complex from the membranes.

A polypeptide with approximate molecular weight 60 kDa that was released from ER membrane in response to ATP, has sequence homology at its N-terminal region to protein disulfide isomerase (PDI) from rat liver (Table 5). Proteins released from rat and cow ER in the presence and absence of ATP, cow liver PDI, and PDI isolated from rat mammary microsomes were separated in 7 to 15% gradient acrylamide gels by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes and incubated with polyclonal antibodies against cow liver PDI (Fig.30). The anti-PDI antibodies recognized a polypeptide in all fractions that co-electrophoresed with purified PDI standard. A lower molecular weight polypeptide (approximately 50 kDa)

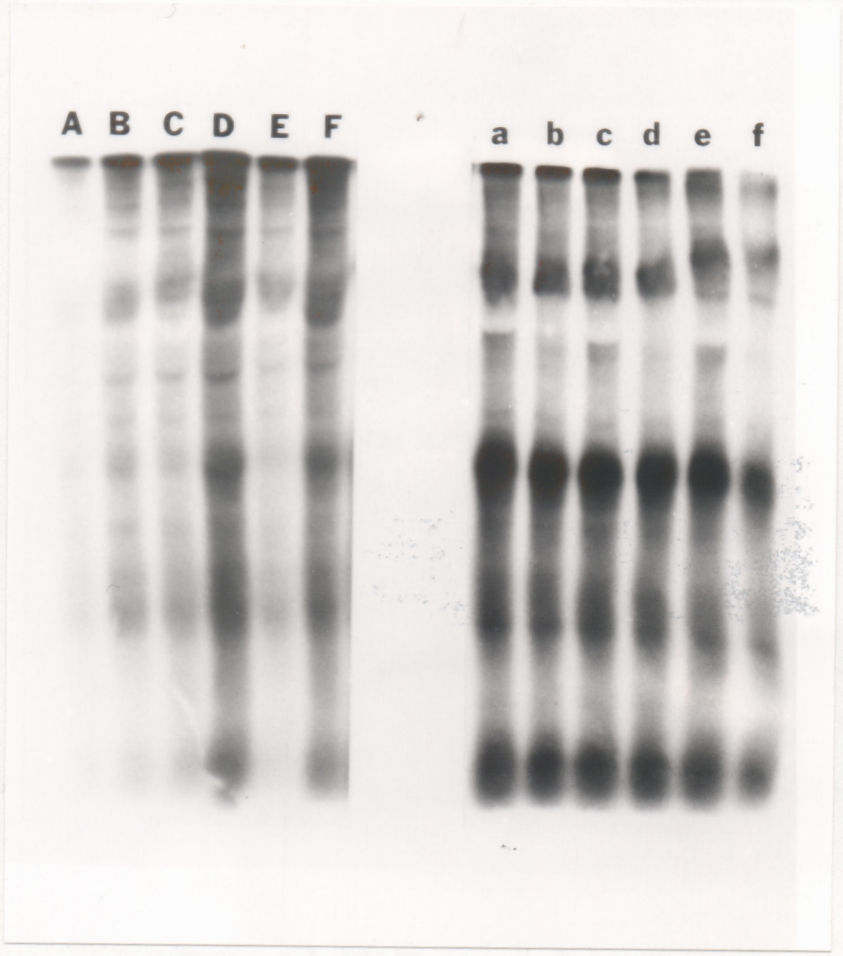

Figure 28: Autoradiogram of ^{125}I labeled polypeptides from LDFAS that associated with ER that had been preincubated in cell-free incubation mixture with or without ATP. ER was either not preincubated (E & F), preincubated without ATP (C & D) or preincubated with ATP (A & B) and recovered by centrifugation. Resuspended membranes then were incubated with ^{125}I labeled LDFAS in cell-free media containing no ATP (B, D, F) or containing ATP and a regenerating system (A, C, E). Lower case letters represent supernatant fractions from corresponding upper case-labeled lanes. Polypeptides were separated in 8 % gels by SDS-PAGE, gels were dried and directly exposed to X-Omat film for visulaization of labeled polypeptides.

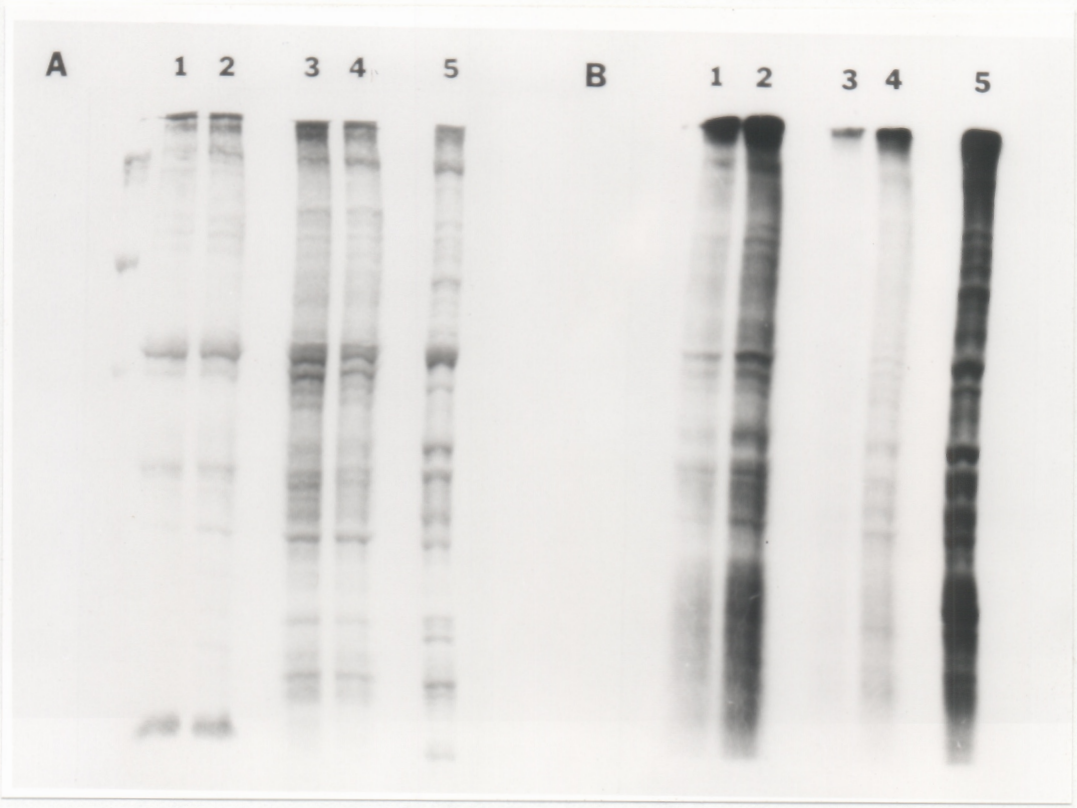

Figure 29: Association of ^{125}I labeled LDFAS to lipid droplets. MLD or CLD were incubated with ^{125}I labeled LDFAS in cell-free media with and without ATP. Lipid droplets were recovered by sucrose gradient centrifugation and washed. A) Electrophoretic pattern of post-incubation samples, ER incubated in the absence of ATP was used for comparison of polypeptide association to ER verses lipid droplets: MLD + ATP (lane 1), MLD - ATP (lane 2), CLD + ATP (lane 3), CLD - ATP (lane 4), ER - ATP (lane 5). polypeptides were separated in 8% gels by SDS-PAGE and visualization was by coomassie blue stain. B) Autoradiogram of the gel depicted in A). The gel was dried under vacuum and exposed to X-ray film. Lanes in B) correspond to lanes in A). Bars denote positions of 200 kDa, 116 kDa, 80 kDa, and 50 kDa standards, from top to bottom, respectively.

Table 5: Amino terminal sequence comparison of the 60 kDa protein from rat ER with rat liver protein disulfide isomerase (PDI) and PDI precursor polypeptide. Amino acid sequence (single letter abbreviation) was by solid-phase Edman degradation. A = high probability of correct identification, [A] = probable/reasonable, (A) = possible/low. PDI and PDI precursor sequences were from the protein sequence data bank.

Protein		Sequence
60 kDa:	1°	D A L E E E D N V L V L
	2°	L E V (G) D N V L [V] [L] K K
PDI		D A L E E E D N V L V L
PDI PRECURSOR		L E E E D N V L V L K K

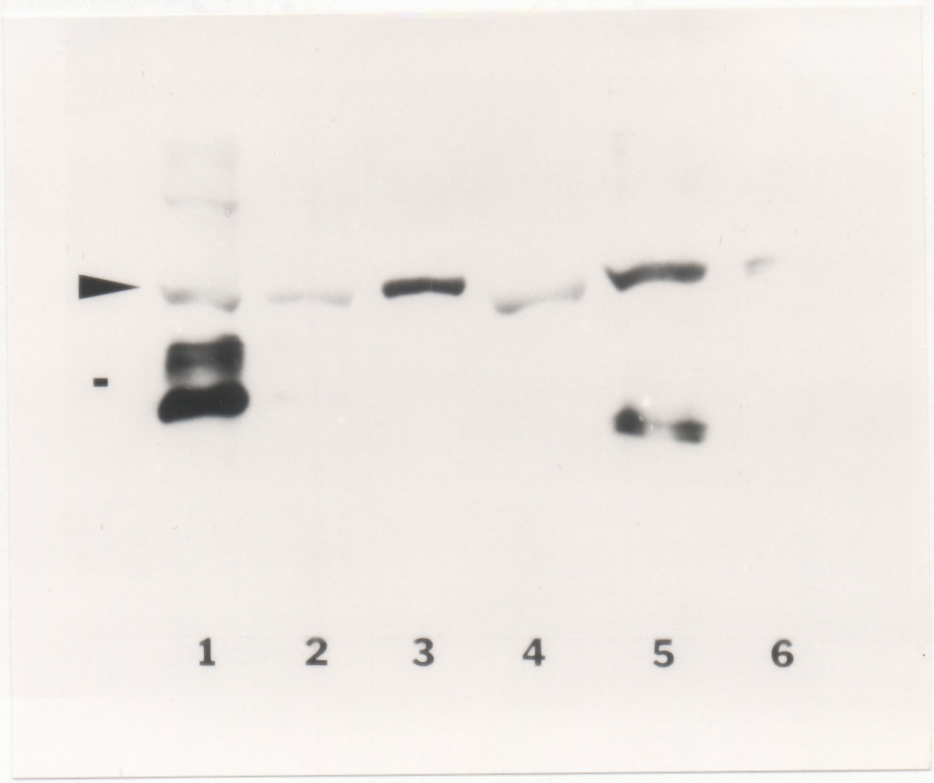

Figure 30: Western blot of a supernatant fraction from rat mammary ER incubated with (lane 1) or without (lane 2) ATP, commercial cow PDI (lane 3), partially purified rat liver PDI (lane 4), and supernatant fractions from cow mammary ER incubated with (lane 5) or without (lane 6) ATP. Polypeptides were separated by SDS-PAGE in 7 to 15 % gradient gels, electroblotted to nitrocellulose and incubated with anti-cow liver PDI antiserum. Detection was by ECL Western blot detection reagents (Amersham) with horseradish peroxidase-conjugated second antibody, followed by autoradiography.

than PDI was recognized by anti-PDI antibody only in fractions released from ER in the presence of ATP for both rat and cow. At lower concentrations of anti-PDI antiserum, the 50 kDa polypeptide was the only constituent that cross-reacted with the antibody, and was present in fractions released from the ER in the presence of either ATP or, to a lesser extent, the nonhydrolyzable analog, AMP-PNP. When GTP was left out of the incubation medium, but ATP was present, the antibody did not bind to a constituent in this region.

To determine whether the FAS complex released from the ER by ATP was similar to the LDFAS complex isolated from cytosol, proteins that had been released from the ER were fractionated by KBr density gradient centrifugation. Extraction of lipids from supernatant fractions showed abundant amounts of unesterified fatty acids in all fractions (Fig. 31). When the supernatant fraction from ER incubated in the absence of ATP was subjected to density gradient fractionation, polypeptides and fatty acids were distributed similarly to those released in the presence of ATP, but FAS was lacking. The free fatty acid likely was not directly associated with FAS. The possible presence of fatty acid binding proteins and/or phospholipases warrants investigation.

C) ***Morphology and Immunocytochemistry:***

Electron micrographs of the LDFAS complex showed a homogeneous morphology of granular, symmetrical particles ranging in size from 40 nm to 170 nm in diameter (Fig. 32). These particles resemble low density lipoprotein (LDL) in morphology (Davis, 1991). Immunocytochemistry using antibody to rat liver FAS revealed distribution of FAS throughout the nonmembrane area of mammary epithelial cells as expected. Additionally, FAS was distributed at localized regions of the cytoplasmic surface of rough endoplasmic reticulum and on surfaces of intracellular lipid droplets (Fig. 33).

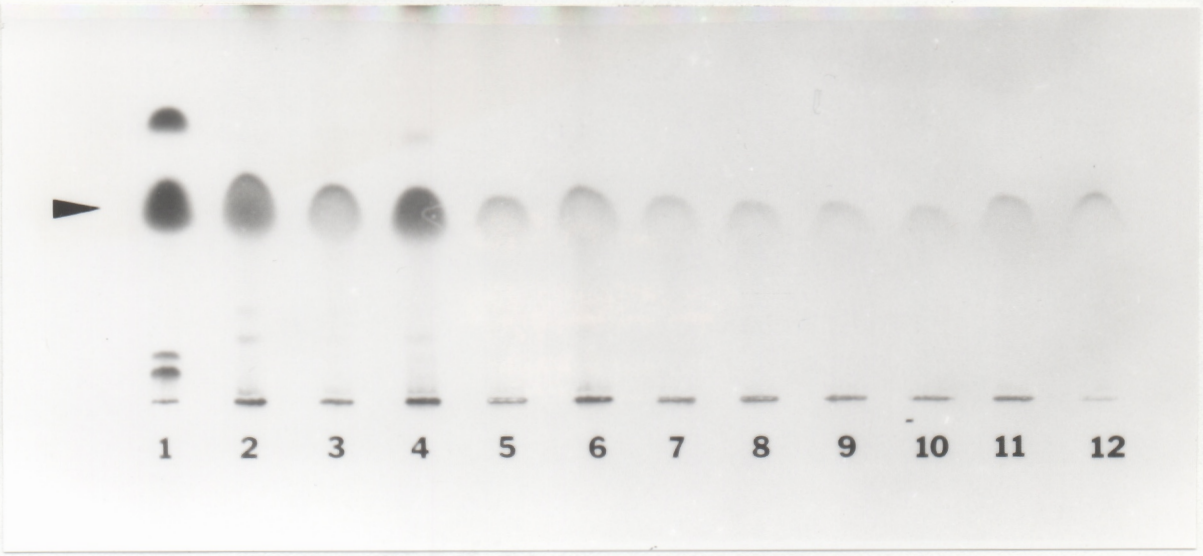

Figure 31: Mass distribution of nonpolar lipids in density gradient-separated supernatant fractions from incubation of ER with cell-free media containing ATP. Thin-layer plates were developed and charred. Lanes were numbered from the highest density fraction (lane 2) to lowest density fraction from KBr density gradient. Arrowhead denotes position of fatty acid constituents extracted from fractions. Lane 1 was standards, triacylglycerols, unesterified fatty acids, cholesterol, and the origin, respectively, from top to bottom. Amounts applied were the same as for polar lipid analysis (Fig. 6).

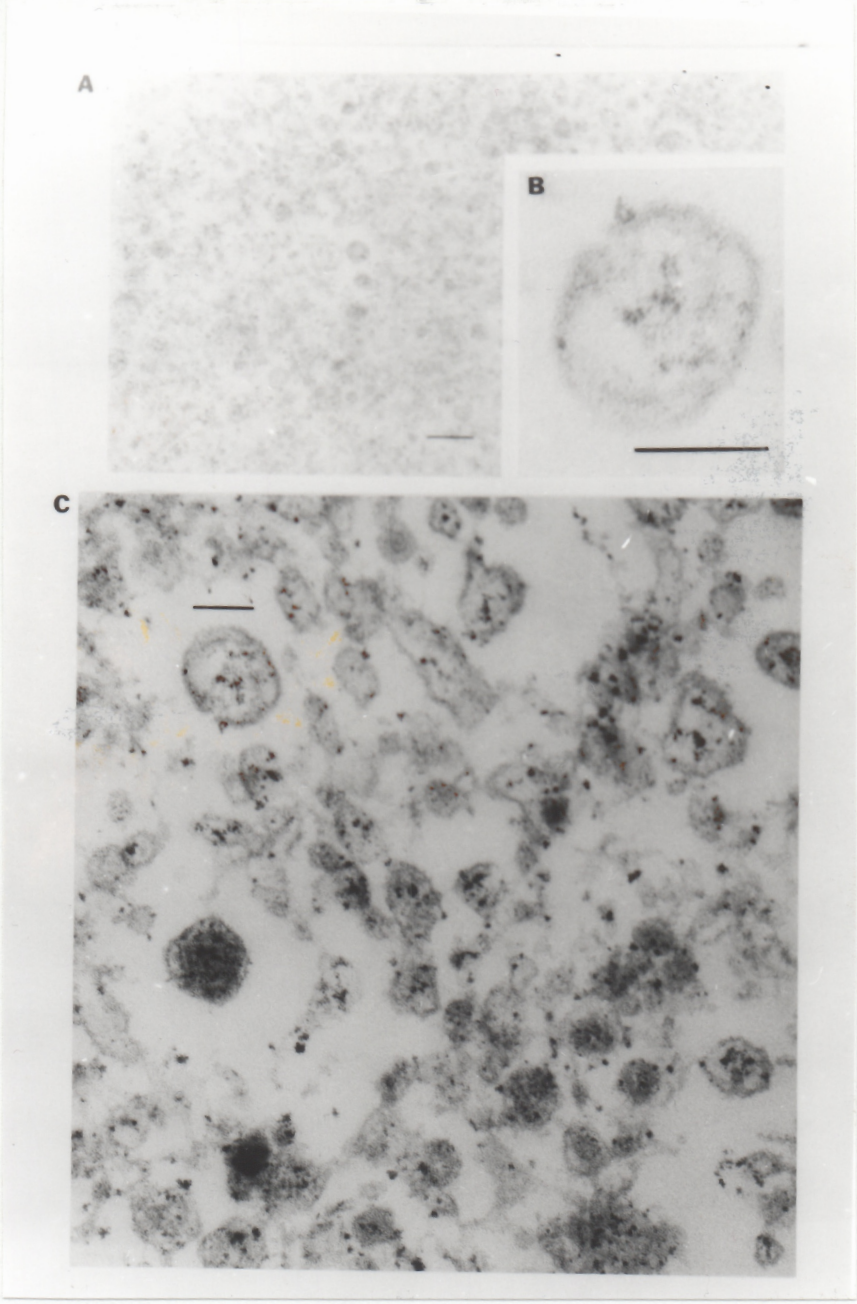

Figure 32: Electron micrographs of LDFAS. Samples were fixed sequentially with 2.5% glutaraldehyde on ice for 90 min and then with 1% osmium tetroxide overnight at 4°C and prepared for electron microscopic examination as described (Dylewski et al., 1984). Fig. A: bar = 200 nm, magnification was 50,000 x ; Fig. B: bar = 100 nm, magnification was 295,000 x ; Fig. C: bar = 100 nm, magnification was 134,000 x

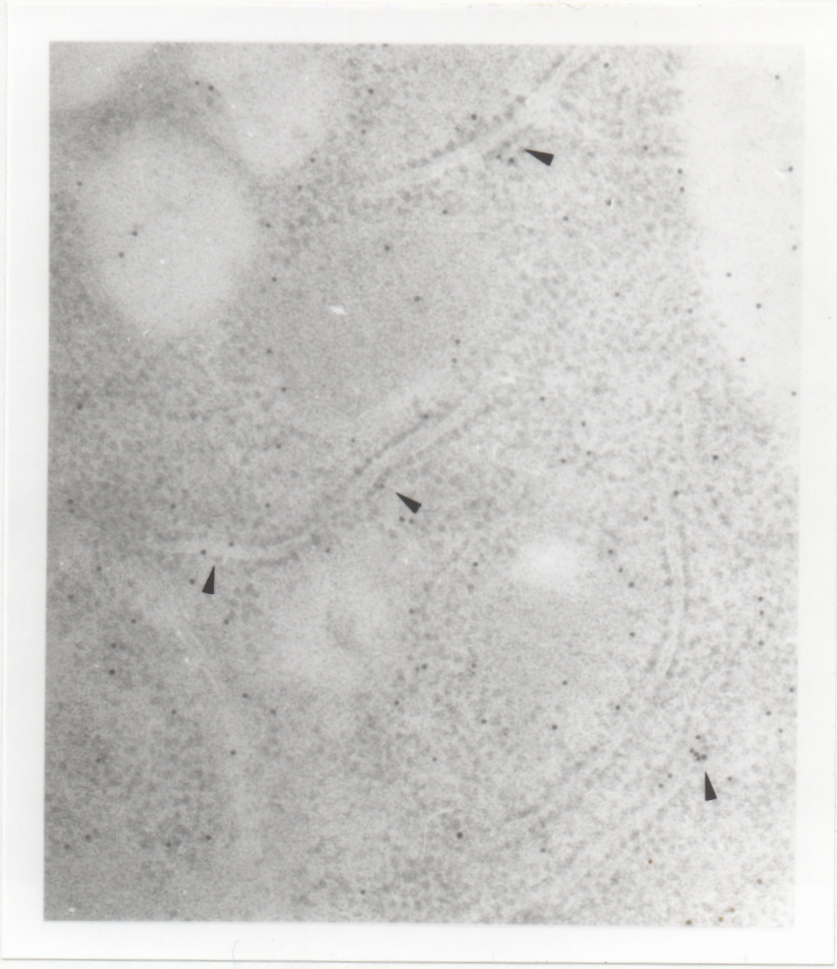

Figure 33: Immunogold localization of antigen recognized by antibody to rat liver FAS in rat mammary epithelial cells in situ. Arrowheads indicate distended regions of ER cisternae where antibody bound. Magnification was 83,000 x.

V

DISCUSSION

The initial goal of this research was to identify cytosolic constituent(s) that promoted the formation and release of precursor lipid droplets from ER. Electron micrographs consistently have shown intracellular lipid droplet precursors to be concentrated in the vicinity of rough ER, and the apparent budding of lipid droplets from ER has been observed in electron micrographs. Distended regions of ER have been interpreted as selected sites of triacylglycerol accumulation during formation of lipid droplets. Whether accumulation of triacylglycerol and encasement by protein and lipid occurs within the lumen of the ER, between the leaflets of the membrane bilayer, or by other mechanisms, has yet to be determined. Available data supports a mechanism for lipid accumulation between the bilayer. Such a mechanism makes sense with respect to the energetics of lipid interaction in an aqueous environment; for lipid to be released to the lumenal or cytoplasmic side of the ER, it must be stabilized by an amphiphilic surface. Phospholipids and amphiphilic proteins within the membrane would provide a protected environment for accumulation and, through their encasement of the lipid core upon release from the membrane, form a particle that is stable in the aqueous environment of the cell. No definitive evidence exists, however, to prove or

disprove such a mechanism, and the full range of factors involved in droplet formation remain unidentified. The finding that cell-free generation of lipid droplets from the ER was promoted by a cytosolic fraction from mammary gland homogenates represented the first evidence for a contribution by a soluble cytoplasmic factor in lipid droplet formation (Keenan et al., 1992).

While the initial goal of this study was to identify cytosolic factors necessary for promoting lipid droplet formation and/or release in a cell-free system, some degree of promotional activity was found to be present in several gel exclusion fractions of cytosol. The interesting observation, however, was that the fraction with the highest amount of activity also contained the bulk of membrane-free lipids present in mammary gland homogenates, and that these lipids were typical milk lipids. Additionally, this complex contained high molecular weight polypeptide constituents that had similar electrophoretic mobilities to proteins that were lactation-enriched. The focus of the experimentation thus turned towards the characterization of the high molecular weight lipoprotein fraction and towards attempts at understanding its involvement in the mechanisms of milk lipid secretion. This focus was undertaken with the awareness that this complex did not necessarily contain all the factors that collectively account for the ability of mammary gland cytosol to promote cell-free formation of nascent lipid droplets. Refinement of the cell-free system to alleviate its occasionally tenuous response would

facilitate definitive identification of other factors active in promoting lipid droplet formation *in vitro*.

In my experiments, lipid droplet promoting activity was shown to be present in a high molecular weight fraction of cytosol that contained the bulk of lipids recovered in membrane-free cytosol isolated from mammary gland homogenates. Both the polar and neutral lipids associated with this complex were typical of lipids found in milk, and these polar lipids could be transferred from cytosol to ER, to lipid droplets generated in the cell-free system, and to intracellular lipid droplets isolated from mammary gland homogenates. The major polypeptide constituent of this lipid-protein complex was the monomer unit of fatty acid synthase (FAS). Fatty acid synthase is a key enzyme in lipid metabolism and my experiments showed FAS to be abundant in mammary tissue from lactating animals relative to tissue from non-lactating animals. This finding was consistent with data reported in the literature and in accordance with the ability of the mammary gland to synthesize and secrete large amounts of lipid during lactation. Historically, FAS has been thought to occur in cells as a dimeric soluble enzyme. No lipid-associated or membrane-bound forms have been reported. The lipid associated form of FAS reported herein behaved differently from uncomplexed FAS when separated by size exclusion chromatography, density gradient centrifugation, and native PAGE. The complexed form of FAS behaved as a very large, low density, protein-lipid

aggregate. Morphological data of the low density FAS (LDFAS) complex was consistent with this interpretation. The complex appeared as symmetrical, granular particles, similar in appearance to plasma lipoproteins (Davis, 1991) but distinct in appearance from intracellular MLD's and CLD's. The LDFAS particles were more granular in appearance and had a less defined structure than lipid droplets. The particles did, however, appear to be limited by a peripheral region that stained more densely than the core.

In the LDFAS complex isolated from cow mammary gland cytosol, the two major milk lipid globule membrane proteins, xanthine oxidase and butyrophillin, as well as low molecular weight GTP-binding proteins, also were identified. Lack of suitable antibodies precluded detection of the corresponding milk lipid globule membrane proteins in rat LDFAS. However, a similar set of GTP-binding proteins were detected in the rat complex. Small GTP-binding proteins recently have been found in association with intracellular lipid droplets and with the MLGM (Ghosal et al., 1993). One of these proteins, an ADP-ribosylation factor (ARF), has been purified from lactating cow mammary gland cytosol and has been detected in rat LDFAS using Western blot analysis with anti-ARF antiserum (D.P. Ankrapp, personal communication). This family of GTP-binding proteins is known to play a role in vesicle-membrane targeting and recognition (Hall, 1990; Rothman and Orci, 1992).

Comparison of electrophoretic polypeptide profiles indicated that the LDFAS complex differed in polypeptide composition from intracellular lipid droplets. Although FAS was found in both MLD and CLD protein fractions, it was not a major component of either. The abundance of the FAS monomer as the major peptide constituent in LDFAS remained the distinguishing characteristic. The finding of FAS in association with ER and lipid droplets that originated from ER begged the question of a possible role for LDFAS as an intermediate in the formation of the precursor lipid droplets at the ER.

The source of the lipid found in the LDFAS complex remains to be determined. In liver, triacylglycerols are secreted in lipoprotein complexes by the normal secretory pathway for proteins (Davis, 1991). Apolipoprotein B (Apo B) is required for lipoprotein assembly and secretion. Apo B is translated on membrane-bound ribosomes and translocated into the ER lumen. In liver, formation of triacylglycerol cores is believed to occur between bilayer leaflets of the ER membrane. Apo B interaction with membrane phospholipid is thought to create a pocket in which nascent triacylglycerols can become concentrated prior to release into the ER lumen. The monolayer that surrounds lipoprotein triacylglycerol would subsequently be contributed by the extracellular leaflet. From the ER lumen, the lipoprotein is secreted by the typical protein secretory route, ER to Golgi apparatus to plasma membrane. Some evidence exists also for a direct route of lipoprotein secretion in liver,

from ER to plasma membrane (Morré, 1981; Twaddle et al., 1981). In mammary epithelial cells, the accumulation of triacylglycerol in the ER appears to occur by a mechanism similar to that for lipoproteins. The route of secretion for mammary gland lipid droplets is from ER to plasma membrane. No evidence has been obtained to suggest intermediate processing through the Golgi apparatus, and lipid droplets are not contained within secretory vesicles nor are they observed within the apical secretory cone defined by the contiguous Golgi apparatus dictyosomes (Dylewski et al., 1984b).

Although direct evidence does not exist, the absence of MLD in the ER lumen and the concentration of precursor MLD (some containing associated ribosomes) at the cytoplasmic surface of the ER favors the possibility that triacylglycerols accumulate between bilayer leaflets. In the liver, the lipid core does not appear to grow in size once released into the lumen, while the MLD core does, in fact, grow in volume after release from the ER. The greatest distinguishing factor between lipoprotein formation in liver versus mammary gland is that, in mammary gland, the lipid core is released to the cytoplasmic side of the ER. This distinction implies the need for a specific signalling process to direct triacylglycerol release to the lumenal or cytoplasmic side of the ER. Membrane differentiation through heterogeneous distribution of membrane polypeptides likely provides such signals. Differential localization of ribosomes translating specific protein mRNAs at these sites along the ER may

be involved in the signaling process as well.

Fatty acid synthase was found to be peripherally associated with endoplasmic reticulum isolated from homogenates of rat liver and of mammary gland from rat and cow. If membrane association of FAS is involved in expediating lipid synthesis at the ER, the presence of an ER-associated form of FAS in liver is not surprising, since liver also is active in lipogenesis. Treatment of ER with alkaline sodium carbonate to remove luminal and peripheral polypeptides resulted in the release of a portion of FAS into the soluble supernatant; however, as detected in coomassie stained gels, some of the protein remained associated with the ER membrane fraction after this treatment. Immunocytochemistry showed FAS to be distributed throughout the membrane-free portion of milk secreting cells, as expected. Additionally, polyclonal antibodies raised against rat liver FAS were bound at the cytoplasmic surface of rough ER as well as distended regions of the ER cisternae. Antibodies also were associated with surfaces of intracellular lipid droplets.

Fatty acid synthase is synthesized in the cytosol (Amy et al., 1989). Therefore, its association with lipid would require either targeting of FAS to the ER or other membranes or the targeted transfer of lipid from membranes to the FAS complex. The finding that FAS was associated with ER membrane favors the idea that FAS somehow is targeted to the ER. FAS may be targeted to the

ER during active lipogenesis to expediate the availability of fatty acids for incorporation into complex lipids. The sites at the ER where FAS associates may be sites selected for lipid droplet formation. If this were the case one also would expect to find other lipid biosynthetic enzymes in the same region. The coat material of lipid droplets appears to contain selected components of the ER membrane (Deeney et al., 1985). The lipogenic enzymes concentrated at the cytoplasmic side of the ER may become part of the surface monolayer of nascent lipid droplets. FAS has been localized to MLD, CLD, and MLGM. Acetyl CoA carboxylase has been detected by Western blot analysis in MLGM (Shriver et al., 1989). Neither enzyme was found to be active in MLGM; enzyme inactivation may occur upon removal of the secreted product from the cellular environment. Terminal acyltransferase activity, involved in triacylglycerol synthesis, has not been found in intracellular lipid droplets (Vallivulah et al., 1986). This may be due to possible localization of these enzymes to the lumenal leaflet of the ER. If the lipid droplets are in fact released in association with the cytoplasmic leaflet, their absence from lipid droplets would be expected.

Recently, investigators have identified membrane receptors (SNAREs: SNAP receptors) that recognize and bind the N-ethylmaleimide sensitive fusion factor (NSF) in its complexed form with soluble, NSF accessory proteins (SNAPs; Söllner et al., 1993). These researchers developed a system to purify

the SNAREs based on their observations that NSF/SNAP forms a complex with SNAREs only in the absence of Mg-ATP. Mg-ATP caused disassembly of the complex. In my experiments I noticed an ATP-driven disassociation of ER-bound constituents and that ATP decreased affinity of constituents of the soluble LDFAS complex for ER membrane and intracellular lipid droplets. The addition of ATP to suspensions of ER caused an increase in the release of FAS from ER. FAS also was released from ER membranes when incubated with the nonhydrolyzable ATP analog (AMP-PNP), but release was lower relative to ATP. Thus, ATP hydrolysis appeared to increase FAS dissociation from ER. Affinity reconstitution studies were consistent with this ATP response. Association of ^{125}I labeled LDFAS polypeptides to ER, MLD, and CLD occurred to some extent in the presence of ATP, but, in the absence of ATP, association of labeled LDFAS polypeptides was increased markedly. There were no apparent qualitative differences in the patterns of polypeptides that were bound in the presence versus the absence of ATP. This lack of selectivity suggests that LDFAS associates as a unit. The dual effect of ATP to cause specific release of ER polypeptides and to decrease the affinity of the LDFAS complex for ER and lipid droplets, suggested a possible role for an ATP-binding protein or ATPase in dissociation of LDFAS from the membrane.

Polypeptide constituents of the LDFAS complex and polypeptides released from ER have similar, but not identical, electrophoretic patterns. For both the LDFAS and the ER constituents, a polypeptide in the molecular weight of 65-75 kDa, partitioned into detergent. Most other major polypeptides in the LDFAS fraction also partitioned into detergent while similar sized polypeptides in the ER fraction did not. Separation of the ER constituents by KBr density gradient centrifugation resulted in distribution of the polypeptides throughout the gradient. Extraction of lipids from the KBr fractions showed large amounts of free fatty acids in all fractions and little to no detectable triacylglycerols or phospholipids. In comparison, the LDFAS complex contained only small amounts of unesterified fatty acids, while most of the lipid was triacylglycerols and phospholipids. These findings suggest that the constituents released from ER may be precursor LDFAS, i.e., the membrane-associated form of FAS may be active in fatty acid synthesis prior to the initiation of triacylglycerol synthesis and formation of lipid aggregates.

The approximately 60 kDa constituent released from ER has been isolated from SDS acrylamide gels and sequenced. Two N-terminal sequences were obtained. The data revealed homology in this region to protein disulfide isomerase (PDI) and to precursor PDI from rat liver. PDI was isolated, cloned, and sequenced previously, and several enzymatic activities have been assigned to it. The 57 kDa PDI polypeptide serves as the non-enzymatic

subunit of the triacylglycerol transfer protein (Wetterau et al., 1988). The 60 kDa protein was released from the ER by incubation in cell-free media with or without ATP, although more was released when incubated with ATP. Protein disulfide isomerase is known to be located in the lumen of the ER, but it has not been reported that PDI is released from ER in response to ATP. Whether the 60 kDa ER polypeptide is PDI or a protein containing a PDI-like domain remains to be determined. The possibility of an ATP-specific release of PDI would challenge the concept that PDI exists strictly as a lumenal protein. A polypeptide with an approximate molecular weight of 48 kDa, which cross-reacted strongly with polyclonal antibody to PDI, was released from ER only when incubated with ATP-containing media. Whether this polypeptide is an isoform of PDI or contains a PDI-like domain also remains to be determined. A polypeptide with similar electrophoretic mobility to the 48 kDa protein, which cross-reacted with PDI antibody, was phosphorylated in the ER and was released also upon incubation in media containing ATP. The soluble form of the polypeptide also was phosphorylated. Whether the phosphorylated polypeptide and the peptide that cross-reacted with PDI antibody are the same remains to be determined as well.

The data presented here do not provide direct evidence for a role of FAS in the lipid droplet secretory pathway. A further understanding of the origins of the LDFAS complex, its assembly, and its interaction with

intracellular components of lipid droplet secretion is necessary to define its role in lactating mammary gland. From the available data, I propose the following series of events, the careful testing of which will lead to a better understanding of lipid droplet secretion in the mammary gland. During active lipogenesis FAS is targeted to ER membrane by association with a signal or targeting peptide(s) in the cytosol. The signal peptide then binds to selected regions of ER where signal receptors reside. Binding of FAS may initiate synthesis and accumulation of triacylglycerol between ER membrane bilayers. Upon the achievement of a "critical mass", the lipid core may be released into the cytoplasm in an ATP-dependent fashion, surrounded by the membrane components that provided the hydrophobic pocket for lipid accumulation. Butyrophilin and the 65-70 kDa detergent-extractable constituent released from the ER and present in LDFAS are possible sources of such a function. Polypeptides from the cytosolic leaflet of the ER, and proteins peripherally associated with the leaflet then would comprise the polypeptide constituents of the lipid particle.

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PROTOPLASMA

Transferrin is a major cytosolic protein during early stages of involution of rat mammary gland

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Dedicated to Hilton H. Mollenhauer on the occasion of his reurement

Summary. Cytosol prepared from rat mammary gland contained a glycosylated protein with an estimated molecular weight of 80,000 which comigrated with transferrin upon electrophoretic separation. This protein was recognized specifically by polyclonal antibody to rat transferrin, and had an amino-terminal 17 residue sequence identical to that of rat transferrin. There was a pronounced developmental regulation of cytosolic transferrin levels in mammary gland. During early and mid stages of pregnancy, transferrin accounted for about 1% of cytosolic protein. During late pregnancy, and throughout lactation, transferrin accounted for about 2% of cytosolic protein. Within 2 days after initiation of mammary gland involution, transferrin accounted for more than 15% of cytosolic protein, and then declined during later stages of involution to below 1.5% of cytosolic protein. Pronounced increases in transferrin early in mammary gland involution may serve as a marker for commencement of gland regression.

Keywords: Transferrin; Iron transport; Cytosol; Mammary gland development; Involution.

Introduction

The only mammalian tissue not fully differentiated at birth is the mammary gland. Differentiation of tissue into functional epithelial cells begins at puberty and progresses toward terminal differentiation during the first pregnancy (Topper and Freeman 1980). Epithelial cells become fully differentiated and begin to secrete milk at or near parturition. At the natural end of the lactation period, or with initiation of involution by cessation of milk withdrawal, epithelial cell regression begins. During subsequent pregnancies, differentiation to functional epithelial cells is reinitiated. This cellular differentiation occurs in response to a complex interaction of extracellular matrix, growth factors, and ovarian plus pituitary hormones; a complete understanding of which still is lacking (Topper and Freeman 1980, Howlett and Bissell 1990, Aggeler et al. 1991). Transferrin, an iron-binding/iron transporting glycoprotein primarily synthesized in the liver and secreted into serum (Morgan 1981), also is synthesized by mammary epithelial cells (Bradshaw and White 1989). Transferrin is present in milk of some species together with lactoferrin, an iron-binding protein immunologically and structurally distinct from transferrin (Aisen and Leibman 1972). In milk, transferrin serves as a bacteriostatic agent by virtue of its ability to sequester metal ions necessary for bacterial survival. Transferrin has been implicated as an agent promoting tumor growth and cellular differentiation (Morgan 1981). The functional role of transferrin locally synthesized in mammary gland is unknown. That transferrin may be involved in mammary epithelial cell differentiation was suggested by Lee et al. (1987). Studies with mouse mammary epithelial cells in culture provided evidence that synthesis of transferrin is low in cells from virgin animals, but is elevated in cells from pregnant and lactating animals (Lee et al. 1987). Transferrin synthesis was not stimulated by lactogenic hormones with cells from pregnant or lactating mice or rabbits (Lee et al. 1987, Suard et al. 1989). This is in contrast to the major milk proteins, caseins and α-lactalbumin, the synthesis and secretion of which requires lactogenic hormones.

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Transferrin synthesis in mammary gland is high during pregnancy and decreases during lactation (Lee et al. 1987). Much of the transferrin in milk and mammary gland appears to be the result of local synthesis rather than import of transferrin secreted from liver (Bradshaw and White 1984).

There have been no published studies of transferrin levels in mammary gland throughout pregnancy, lactation, and involution. We have identified transferrin as a constituent of cytosol from rat mammary gland. This protein was present in cytosol from pregnant and lactating animals, and was enriched in cytosol from animals in initial stages of mammary gland involution. We suggest that transferrin may have a functional role in involution of mammary epithelial cells.

Materials and methods

Timed-pregnant, primiparous Sprague-Dawley rats were from Harlan Laboratories (Indianapolis, IN), were housed in temperature- and humidity-controlled quarters with 12 h light 12 h dark cycles, and were provided standard chow and water ad libitum. Animals were sacrificed in sets of three at seven day intervals throughout pregnancy, lactation, or involution. Sets of two rats were sacrificed on days 2 and 5 of involution as well. Pregnant animals carried between 8 and 14 fetuses, and lactating animals were nursing 8 to 12 pups. Involution was induced by removal of pups on the 22nd day of lactation. Nitrocellulose sheets were from S&S Scientific, Keene, NH. Protein molecular weight standards for electrophoresis were supplied by BioRad Laboratories (Richmond, CA). Crude IgG fraction of rabbit antiserum to rat fatty acid synthase was a gift of Dr. Stuart Smith, Children's Hospital, Oakland, CA. Affinity purified IgG fractions of sheep antisera to rat transferrin was from The Binding Site, Birmingham, England. Sigma (St. Louis, MO) provided affinity purified, alkaline phosphatase-linked, antibodies to sheep and rabbit IgG, solid-phase alkaline phosphatase substrate, rabbit-anti-human transferrin, and all other biochemicals.

Animals were sacrificed by cervical dislocation, mammary tissue was removed, placed on ice, and minced finely with scissors. Minced tissue was washed repeatedly in 0.25 M sucrose in cytosol buffer (10 mM Tris, 10 mM KCl, pH 7.0) until the wash solution remained clear. Cytosol was prepared as described (Keenan et al. 1992) after homogenization of tissue in a Polytron PT-20 for 45 s at 60% maximal speed, filtration through a single and then a double layer of cheesecloth, and centrifugation of the filtrate at 120,000 g for 90 min at 4 °C. Cytosol samples were frozen in liquid nitrogen and stored at − 70 °C until analysis.

Protein was measured with bicinconinic acid reagent (Pierce Chemical Company, Rockford, IL), using bovine serum albumin as standard. Electrophoresis (SDS-PAGE) was according to Laemmli (1970) in 10% acrylamide gels. Samples were loaded in equivalent protein amounts, stained with coomassie blue, and stained gels were scanned with a Shimadzu CS 9000 flying spot densitometer operated in the reflectance mode at 580 nm. For identification of glycoproteins, gels were stained by the periodic acid-Schiff procedure (Fairbanks et al. 1971). Proteins separated by electrophoresis were transblotted onto nitrocellulose and probed with antibodies (Towbin et al. 1979), or onto PVDF membrane for N-terminal sequence analysis. Amino-terminal sequencing was performed at the Protein Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO. Nitrocellulose sheets containing transblotted proteins were blocked by incubation in 3% bovine serum albumin in Tris-buffered saline (100 mM Tris, pH 7.6, 0.15 M NaCl) containing 0.02% Tween 20 (TBST) and incubated with appropriate dilutions of antiserum to rat liver transferrin or to rat liver fatty acid synthase. After washing with TBST to remove unbound antibody, detection was by incubation in species specific alkaline phosphatase conjugated anti-IgG, followed by reaction with solid-phase substrate. For quantification, enzyme-linked immunoadsorption assays using anti-rat liver transferrin were according to Clark and Engvall (1980).

Results

Separation of cytosolic proteins by SDS-PAGE showed a polypeptide of apparent M_r 80 kDa to be enriched

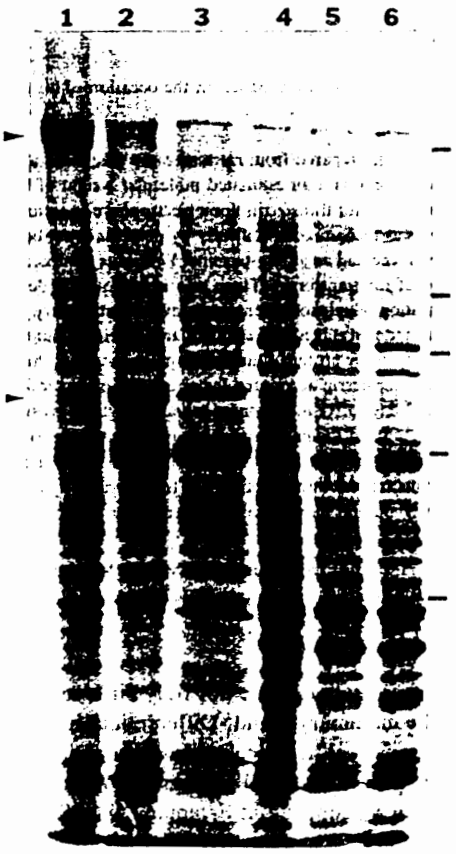

Fig. 1. Polypeptide composition of mammary gland (*1–3*) and liver (*4–6*) cytosol from lactating (*1* and *4*), 2 day involuted (*2* and *5*), and 5 day involuted (*3* and *6*) rats. Separation was by SDS-PAGE, and the 10% acrylamide gel was stained with coomassie blue. Bars along *6*, positions of 200, 116, 97, 66, and 45 kDa standards from top to bottom, respectively. Arrowheads along *1*, 220 kDa and 80 kDa constituents identified as fatty acid synthase and transferrin, respectively

markedly in cytosol from rats at day 2 of involution, relative to rats which were lactating or at day 5 of involution (Fig. 1). Cytosols from livers of the same animals were similar in polypeptide composition regardless of the physiological state of the mammary gland of the animal (Fig. 1). Cytosol prepared from mammary gland had amounts of polypeptide constituents comigrating with major caseins and α-lactalbumin which were not detected by coomassie blue staining. Caseins were detected only by autoradiography of gels used to separate proteins of cytosol from animals injected with mCi amounts [^{32}P]orthophosphatase several hours before sacrifice (Keenan et al. 1992). Concentrated mammary gland cytosol could not replace α-lactalbumin in a lactose synthetase assay (Keenan et al. 1970). Results from this assay suggested that α-lactalbumin, if present, accounted for under 0.1% of the cytosolic protein.

Heating of cytosol at 80 °C for 3 min proved to be an efficient and rapid way to enrich the 80 kDa protein for amino-terminal analysis. Most polypeptides other than the 80 kDa constituent were precipitated by this heat treatment. A 17 residue amino-terminal sequence was obtained by solid phase Edman degradation (Table 1), and this sequence was 100% identical to rat serum transferrin and 86% identical to human serum transferrin. The 80 kDa protein reacted positively when gels were stained for glycoprotein with the periodic acid Schiff reagent. The 80 kDa protein was specifically recognized by both anti-human and anti-rat transferrin in Western blots and by immunoadsorption assays.

Densitometric scanning of electrophoretic gels stained with coomassie blue was used to quantify transferrin (Fig. 2). Transferrin levels in cytosol were low at days 7 and 14 of pregnancy, increased slightly at day 21 of pregnancy and decreased at parturition. Transferrin levels remained low during lactation, but were elevated markedly at day 2 of involution. At day 7 of involution, transferrin had already declined to below pregnancy levels and was undetectable at day 21 of involution. The sharp increase in transferrin at day 2 of involution was paralleled by a marked decline in fatty acid synthase levels, which had risen at parturition and remained high throughout lactation. Western blot analysis of cytosols from these stages confirmed changes in transferrin and fatty acid synthase concentrations estimated by scanning gels (Fig. 3).

Fluctuations in concentration of cytosolic transferrin also were quantified by immunoadsorption assays using anti-rat transferrin (Fig. 4). These results agreed well with those reported above. At day 7 and 14 of pregnancy, transferrin accounted for about 1.1% of total cytosolic protein. Immediately prior to parturition, on day 21 of pregnancy, transferrin levels increased by 81% over early pregnancy levels. The

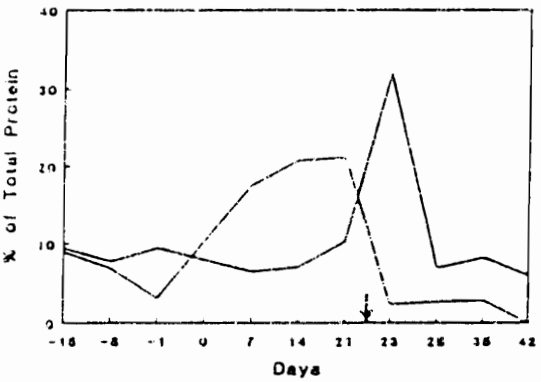

Fig. 2. Quantification of cytosolic transferrin and fatty acid synthase by densitometric scanning of electrophoretic gels (Fig. 1 is representative of gels which were scanned). Results are presented as percent of total coomassie blue stained protein scanned in each lane. Dashed lines represent fatty acid synthase bands and solid lines represent transferrin bands. Day 0 corresponds to the date of delivery of litters, and the arrow indicates the day on which pups were removed to initiate involution

Table 1. Amino-terminal sequence comparison of the 80 kDa protein from rat mammary cytosol with rat and human transferrins

Protein	Sequence
80 kDa protein	• P D K T V K • • A V S E H E N T
Rat transferrin	V P D K T V K W C A V S E H E N T
Human transferrin	V P D K T V R W C A V S E H E A T

Amino acid sequence (single letter abbreviations) was by solid-phase Edman degradation
• Unidentified or uncertainty in identification of residues in these positions. Rat and human transferrin sequences were from the protein sequence data bank

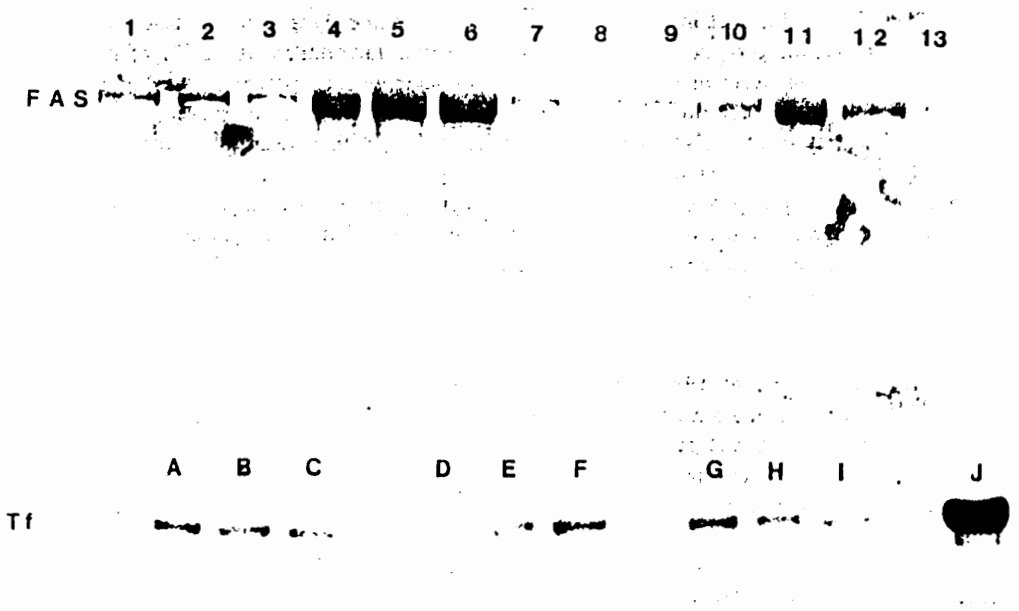

Fig. 3. Western blot analysis of cytosols from various stages of pregnancy, lactation, and involution. For detection of fatty acid synthase, blots were incubated with rabbit anti-rat liver fatty acid synthase, and for detection of transferrin, blots were incubated with sheep anti-rat liver transferrin. Detection of both antibodies was by incubation in alkaline phosphatase conjugated, species specific, anti-IgG, followed by reaction with solid-phase substrate. Samples were mammary gland cytosol from 7 day pregnant (*1* and *A*), 14 day pregnant (*2* and *B*), 21 day pregnant (*3* and *C*), 7 day lactating (*4*, *11*, and *D*), 14 day lactating (*5* and *E*), 21 day lactating (*6* and *F*), 7 day involuted (*7* and *G*), 14 day involuted (*8* and *H*), 21 day involuted (*9* and *I*), and 2 day involuted (*13* and *J*) rats, and liver cytosol, from the 7 day lactating (*10*) and 2 day involuted (*12*) rats

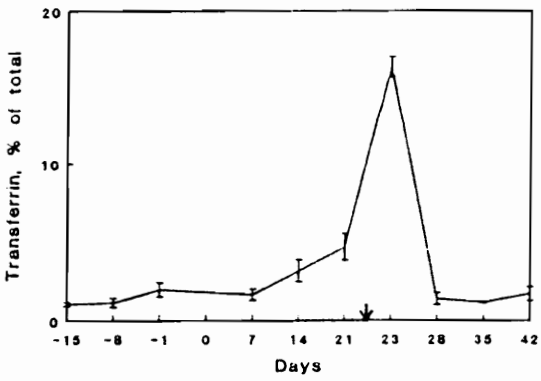

Fig. 4. Quantification of cytosolic transferrin by enzyme-linked immunoadsorption assay. Results are presented as percent of total cytosolic protein accounted for by transferrin, and are the means ± SEM of duplicate dilutions from three separate experiments. Day 0 corresponds to the date of delivery of litters, and the arrow indicates the day on which pups were removed to induce involution

amount of cytosolic transferrin remained constant throughout the first two weeks of lactation and then increased to about double the early pregnancy amount just prior to involution. On the 2nd day of induced involution, transferrin levels had increased markedly, and accounted for about 15.7% of total cytosolic protein. By day 7 of involution this value had diminished again to 1.4% of total cytosolic protein.

Discussion

Culture systems have been used extensively to study the effects of hormones, growth factors, and the extracellular matrix on ability of mammary epithelial cells to undergo differentiation. Synthesis and secretion of major milk proteins have served as markers for the physiological state of the cell. Caseins and α-lactalbumin are responsive to lactogenic hormones and their synthesis and secretion are reliable markers for differentiated mammary epithelial cells (Topper and Freeman 1980). Synthesis of transferrin by mammary epithelial cells appears to be unresponsive to lactogenic hormones (Lee et al. 1987). Liver appears to be the main source of transferrin, which is secreted into the serum to provide a means for iron transport into cells

(Morgan 1981, Octave et al. 1983). At one time it was thought that milk transferrin originated from blood, but subsequent findings showed that mammary epithelial cells can synthesize transferrin. In rabbits, less than 1% of milk transferrin originates from hepatocytes (Jordan and Morgan 1970). We found that, while transferrin levels in cytosol from mammary gland changed in response to the animal's physiological state, there was little or no change in transferrin levels in liver cytosol from these animals. Therefore, the dramatic rise in cytosolic transferrin observed at the onset of involution likely is a result of increased transferrin synthesis by the mammary gland. That involution had begun was marked by diminution in levels of fatty acid synthase, a cytosolic enzyme complex which is low in abundance during pregnancy, increases progressively through the first 15 days of lactation to reach levels 20 or more times those found during pregnancy, and then declines precipitously in late stages of lactation (Baldwin and Milligan 1966, Smith and Ryan 1979).

Results we obtained demonstrate that, in situ, transferrin is found in cytosol from cells of the mammary gland of rats, and that it is a major cytosolic protein in the early stages of involution. That this protein is transferrin and not lactoferrin, a protein similar to transferrin in size and iron binding activity, was suggested from immunoreactivity and N-terminal sequencing. Serum transferrin and lactoferrin reportedly do not show immunological cross-reactivity (Aisen and Leibman 1972). Human transferrin and lactoferrin have zero homology through the first 17 residues. The sequence we obtained for rat mammary transferrin had no homology with the first 17 residues of human lactoferrin, and one homology with mouse lactoferrin.

Whether the increase in transferrin early in involution is due to increased synthesis, or to stability to proteolytic degradation, remains to be established. Bradshaw and White (1984) studied transferrin synthesis in explants of mammary gland from pregnant and lactating rabbits. Their results demonstrated an increase in cytosolic transferrin in the later stages of pregnancy, and a further increase during lactation, especially during late stages of lactation. Bradshaw and White (1984) did not examine explants from animals in involution. As a secretory protein, transferrin should be compartmentalized in components of the endomembrane system for intracellular transit (Morgan 1981), and transferrin accumulated into cells should be compartmentalized in components of the endocytic pathway and not be released into the cytoplasm (Octave et al. 1983, Dautry-Varset et al. 1983). Why transferrin is present in cytosol is an interesting question which remains to be addressed. Leakage from compartments of the exocytic pathway apparently does not account for cytosolic transferrin, as cytosol prepared as described contains little or no detectable amounts of polypeptides migrating with major milk proteins by SDS-PAGE.

Our observation of a peak in mammary cytosolic transferrin at early involution leads us to believe that transferrin synthesized in mammary gland may be involved in cellular regression, and that elevated transferrin levels could conceivably serve as a marker for early stages of involution.

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