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PAS IV, an integral membrane protein of bovine milk-fat-globule membrane : an improved purification protocol and partial topological characterization

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membrane: An improved purification protocol and partial
topological characterization**

Jiwani, Nilofer Gulamhussein, M.S.

San Jose State University, 1991

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Ann Arbor, MI 48106

**PAS IV, An Integral Membrane Protein of Bovine Milk-Fat-Globule
Membrane : An Improved Purification Protocol and
Partial Topological Characterization**

A Thesis

Presented to

**The Faculty of the Department of Chemistry
San Jose State University**

In Partial Fulfillment

**of the Requirement for the Degree
Master of Science**

By

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August, 1991

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A B S T R A C T

PAS IV, AN INTEGRAL MEMBRANE PROTEIN OF BOVINE MILK-FAT-GLOBULE MEMBRANE : AN IMPROVED PURIFICATION PROTOCOL AND PARTIAL TOPOLOGICAL CHARACTERIZATION

by Nilofer G. Jiwani

In the present study, we present an attempt to characterize the topology of PAS IV which has been found to be localized in mammary epithelial and endothelial cells as well as capillary endothelial cells of liver, heart and other tissues. It is found to be highly resistant to proteolysis when attached to the phospholipid bilayer and amino acid analysis has revealed its pronounced hydrophobic nature. These data are the basis of our hypothesis that PAS IV is an integral membrane protein with multiple membrane-spanning regions. A new purification protocol and topographical data supporting this hypothesis have been presented here. The new purification protocol eliminates prior proteolytic treatment of the membrane and exploits some of the interesting properties of the non-ionic detergent, Triton X-114. Several topographical data obtained indicates the probable orientation of PAS IV based on which a partial model structure for PAS IV is presented here.

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I. INTRODUCTION

General Characteristics of Integral Membrane Proteins

Lipids and carbohydrates together with proteins constitute biological membranes. Lipid bilayers, 50 - 70 Å thick, are spontaneously formed under suitable conditions such that the polar headgroups are exposed to the aqueous phase and the hydrocarbon tails are buried. Proteins that can bind electrostatically to the surface of the membranes are named peripheral or extrinsic proteins. Proteins that are integrated into the lipid hydrocarbon continuum are called intrinsic or integral membrane proteins.

Integral membrane proteins play a pivotal role in such tasks as the transport of ions and protons, interaction between cells, etc. They must interact simultaneously with the hydrophilic headgroups and water as well as with the hydrophobic lipid. These special requirements make the architecture of integral membrane proteins not only interesting, but also rather specific and unique. Alpha - helical bundles that contain predominantly long stretches of hydrophobic amino acid residues or beta barrels are likely candidates to interact with the lipid medium. Integral membrane proteins can be divided into classes based on the structure and function of their transmembrane segments (1). Proteins such as the immunoglobulin M heavy chain (2), influenza virus hemagglutinins (3-7), and histocompatibility antigens (8-12), contain a single clearly hydrophobic transmembrane segment that serves primarily

to anchor the protein within the bilayer (1). Proteins responsible for transport, such as the acetylcholine receptor (13), the Ca^{+2} , Mg^{+2} - ATPase of sarcoplasmic reticulum (14) and the lac permease of Escherichia coli (15), are generally believed to traverse the bilayer several times, forming an assembly of conformationally ordered segments that can create a specific transport channel (16).

Current knowledge of PAS IV

The milk triglycerols, during lactation, are secreted into alveolar lumina of the mammary gland in the form of discrete droplets coated with a surface layer of membrane known as the milk-fat-globule membrane [MFGM] (17, 18). In a budding process, the intracellular fat droplets are progressively surrounded with plasma membrane derived from the apical pole of secretory alveolar cells (19, 20, 21). The lipid and enzyme composition of the membrane preparations isolated from milk evidences the cell surface origin of MFGM (22, 23, 24, 25). Thus MFGM provides a convenient supply of apically derived membrane proteins from terminally differentiated epithelial cells.

The MFGM proteins are usually identified and classified according to their mobilities during separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the proteins of MFGM from several species by SDS-PAGE leads to the resolution of eight to ten major components (17,18, 21, 22, 26-31). Two of these components, xanthine oxidase and

butyrophillin, account for nearly 50 % of the total proteins detectable by staining with Coomassie blue (21, 31-34). A glycoprotein with an apparent molecular weight (M_r) of approximately 80,000, known as PAS IV, comprises approximately 5 % of the total Coomassie blue - positive protein of bovine MFGM (31).

Using an immunofluorescence technique and affinity-purified polyclonal antibodies, bovine PAS IV has been found to be localized in mammary epithelial as well as endothelial cells and in capillary endothelial cells of liver, smooth muscle, heart and other tissues (35). The expression of PAS IV in capillary endothelial cells appears to vary between tissues. Capillaries of mammary gland, heart, liver, salivary gland, spleen, pancreas, and intestinal smooth muscle exhibit variable levels of antigen, while the capillary endothelial cells of brain and lung tissues, apparently do not express PAS IV. The reason for such tissue-specific distribution is unknown. However, these observations comply with the general hypothesis that different levels and complements of proteins are expressed by endothelial cells from different tissues and that these differences reflect the specific origins of capillary cells in embryonic tissues during development (36). In most tissues, PAS IV is restricted to capillary endothelial cells and seems to be tightly bound to cellular membranes, an evidence of the protein's pronounced hydrophobic properties. Thus this protein can serve as a convenient membrane marker for capillary endothelial cells in certain tissues.

PAS IV from bovine milk, is a relatively basic protein with an apparent molecular weight of approximately 80,000 as recognized previously by SDS-PAGE

and isoelectric focusing (28, 30, 31, 37). However, in tissues it is of slightly higher molecular weight [85,000] (35). This observation implies that the native form of PAS IV may have undergone limited proteolysis, either during or after secretion, leading to formation of the protein of M_r 80,000 in MFGM. By immunoblotting techniques, a protein of exactly double the M_r of PAS IV is obtained which is assumed to be dimeric form of PAS IV (35).

It has been hypothesized that PAS IV is an integral membrane protein based on several criteria :

- (1) Membrane bound PAS IV is resistant to proteolysis under conditions that completely degrade other MFGM proteins (30, 35, 38).
- (2) PAS IV is recovered in almost 100% yield from the detergent phase when total membrane extracts are partitioned in Triton X-114, a property of integral membrane proteins with amphiphilic properties (39).
- (3) Amino acid analysis of the purified protein reveals a high percentage of non-polar amino acid residues especially the branched chain amino acids. Almost 40 % of the amino acid content is accounted for by four highly hydrophobic residues : leucine, isoleucine, valine and phenylalanine.

(4) The treatment of 0.1 M Na₂CO₃ solutions at pH 11.5, conditions previously shown to effectively remove a large proportion of loosely adsorbed and peripheral membrane proteins (40, 41), was unable to dissociate membrane bound PAS IV from MFGM (35). PAS IV can therefore be classified as an intrinsic protein, suggesting that a part of the molecule is deeply embedded in the membrane matrix.

(5) The hydrophobicity index (42, 43) of PAS IV is estimated to be 1,174 which compares favorably with other well-characterized integral membrane glycoproteins [eg. 1,185 and 1,120 for bovine bacteriorhodopsin and rhodopsin respectively] (44, 42).

However, whether or not PAS IV spans the lipid bilayer cannot be determined from these data. Thus, topographical studies and complete sequence information of PAS IV will be essential to answer this question.

Specific Aims of Research

PAS IV appears to be a major constituent of the endothelial plasmalemma in cardiac and smooth muscles. Its function is not yet known. The objective of the research is to elucidate the structure of PAS IV and thereby help in defining its function. A large amount of data suggests that there is a correlation between a protein's structure and its function. For example, all membrane proteins with multiple spanning regions seem to be involved in direct transport (16). In contrast,

many surface receptors and enzymes commonly have only one membrane spanning region. Also, the topology of the amino and carboxyl termini may also be suggestive of the functional relationship as in the case of the erythrocyte glucose and anion transporters (45).

Importance of Research

Specific milk-fat-globule membrane proteins are potentially useful as markers of mammary tissue, mammary epithelial cells, and the apical surface of these cells. In addition, some proteins are specific for lactating mammary tissue and may therefore be useful as differentiation markers. Other MFGM proteins are expressed in cells other than mammary epithelial cells, most prominently endothelial cells of capillaries in many tissues, e.g. xanthine oxidase (21, 31-34).

As per immunofluorescence studies (35), PAS IV appears to be specific for endothelial cells in certain tissues and can therefore be used as a marker for studies on the origin and the development of organ microvasculature during embryogenesis, for studies on the relationship between parenchymal cells and the capillary bed in different tissues, and for studies of angiogenesis in solid tumors. The presence of an immunologically similar protein to PAS IV in human breast milk (35) suggests possible usefulness of PAS IV as a marker of capillary cells in human tissues.

The vascular endothelium, which is a monolayer of flattened cells, lines the

inner surface of blood vessels. Most of the membrane proteins of the surface of the endothelium are not characterized. It expresses the major histocompatibility complex cell surface antigens (46, 47) via an inducible immune accessory function. Previous studies suggest that endothelial cells from different sites, i.e. different tissues, differ both in their morphology as well as function. Examples of site-specific expression of endothelial cell antigens include the enzyme xanthine oxidase (32), angiotensin-converting enzyme (48), choline acetyl transferase (49) and histamine receptors (50). Recently, numerous monoclonal and monospecific polyclonal antibodies have been produced against endothelial cell-surface antigens, many of which are particularly specific for certain types of endothelia (51-56). The presence of site-specific endothelial cell-surface antigens has also been evidenced by lectin binding studies (57, 58, 59).

One of the best examples of endothelial cell specialization is the existence of unique high walled endothelial cells (HEV) which line post-capillary venules of certain lymphoid organs (60). It has been evidenced recently that these HEV contain unique receptors specific for different populations of lymphocytes (61, 62).

The normal complement of cell-surface antigens is also affected by the endothelial cell differentiation state. Thus even though very little biochemical data on the differentiation of capillary endothelial cells is available, several cell-surface antigens have been shown to be developmentally regulated both in vivo (55) and in vitro (63).

Thus PAS IV, which is specifically present on endothelial cells in certain

tissues, can be used as a model system to study the regulation of tissue-specific endothelial cell membrane protein expression. Structural information which will help in defining the function will then assist to achieve the eventual goals, which are to correlate the well-defined environmental stimuli with the expression of specific endothelial cell gene products and to understand the mechanisms of control involved. Such knowledge will advance our understanding of the functional bases of endothelial cell heterogeneity.

II. EXPERIMENTAL PROCEDURES

MATERIALS

Fresh bovine cream was obtained from a local dairy farm. Glycine, sodium dodecyl sulfate (SDS), Triton X-114, Triton X-100, Tris-base, Tris-HCl, phenylmethanesulphonylfluoride (PMSF), aprotinin, 6-amino-n-caproic acid, sodium thiocyanate, ammonium persulfate, bovine serum albumin (fraction V), Nitro Blue Tetrazolium, DEAE-cellulose, Concanavalin A lectin, and molecular weight protein standards were purchased from Sigma Chemical Co. (St. Louis, MO). N, N-dimethyl formamide was purchased from E M Science (Cherry Hill, NJ), Acrylamide was obtained from Spectrum Chemical Co. (Gardena, CA). Coomassie Brilliant Blue R 250, bisacrylamide, nitrocellulose paper, and affinity-purified goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (EIA grade) were purchased from Bio - Rad Laboratories (Richmond, CA). Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Ponceau S from Helena Labs (Beaumont, Texas). Bicinchoninic acid was purchased from Pierce Chemicals (Rockford, Illinois) and Endoglycosidase F from New England Nuclear Laboratories. 2-mercaptoethanol was obtained from Aldrich Chemical Company (Milwaukee, Wis.) and 5-Bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt (BCIP) from United States Biochemical Co. (Cleveland, Ohio). Centricon 30 microconcentrators were

purchased from Amicon Division, W. R. Grace & Co. (Danvers, MA).

METHODS

PAS IV - Purification

The purification of PAS IV essentially comprised two steps :

- I. Preparation of milk-fat-globule membrane (MFGM).
- II. Isolation of PAS IV using chromatographic and electrophoretic techniques.

Step I. Preparation of milk-fat-globule membrane :

Fresh cream was diluted 1:1 with 0.1 M citrate buffer, pH 7.0. After the temperature of the mixture had reached 10 - 11°C., it was homogenized in a Waring homogenizer at a rheostat setting of 30 until butter was formed (approximate time 20 - 30 mins.). The homogenate i.e. the buttermilk was filtered through four layers of cheesecloth. An orange-brown colored membrane pellet (MFGM) was obtained by centrifuging the filtrate for an hour at 26,500 rpm using a SW 27 rotor at 4°C. and resuspended in 10 mM Tris-HCl, pH 7.6. A Beckman model L8M microprocessor - controlled preparative ultracentrifuge was used for centrifugation.

Step II. Isolation of PAS IV :

(a) Solubilization and Phase-partitioning of MFGM proteins :

MFGM was solubilized in Triton X-114 at 4°C. by first pre-equilibrating 5 mg of membrane protein / ml in 10 mM Tris, pH 7.6, containing the protease inhibitors, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 1 % aprotinin (650 iu / ml), 1 mM 6-amino-n-caproic acid and 5 mM EDTA and 0.15 M NaCl for 30 mins. prior to addition of Triton X-114 to 1 % v/v final concentration. After gently stirring the mixture for half an hour at 4°C., the mixture was centrifuged at 26,500 rpm using a SW 27 rotor at 4°C. for an hour. Traces of white lipid material were aspirated from the top and the supernatant was then subjected to detergent phase partitioning as described by Bordier (39). In brief, the solubilized membrane was first warmed to 30°C. in a waterbath maintained at 37°C. and then the detergent-rich phase was recovered after a low speed centrifugation (1000 rpm using an IEC 870 rotor for 5 mins. at room temperature). The aqueous and detergent phases collected separately were washed either with Triton X-114 (1 % v/v, final concentration) or with a ten-fold excess of cold Tris-buffered saline (TBS), respectively. The two phases were then made up to equal volumes with TBS. A Bicinchoninic acid (BCA) assay was performed on the detergent phase to quantify the amount of protein present.

(b) DEAE - cellulose column chromatography :

The detergent phase of the extract was diluted 1: 5 with 10 mM Tris, pH 7.4, and dialyzed at 4°C. overnight against 10 mM Tris buffer, pH 7.6, containing 0.5 mM PMSF and 0.2 % Triton-X 114. The DEAE- cellulose resin was deaerated prior to packing into a 1.4 x 11.4 cm column and pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.6, containing 0.5 % (vol/vol) Triton X-100. The solubilized proteins were then chromatographed at 4°C. and after washing the column with multiple volumes of equilibration buffer, PAS IV was eluted using a 0 - 200 mM linear gradient of NaCl in 10 mM Tris-HCl buffer, pH 7.6, containing 0.5 % Triton X-100. A flow rate of 24 mls / hour was maintained throughout the experiment. Fractions were analyzed by SDS-PAGE and PAS IV enriched fractions were combined and dialyzed against 10 mM Tris buffer, pH 7.6, containing 0.15 M NaCl and 0.5 % Triton X-100.

(c) Concanavalin A lectin affinity column :

The dialyzed sample was adsorbed onto a 1.4 x 10 cm long Concanavalin A (Con A) column that had been previously equilibrated with a buffer containing 10 mM Tris, pH 7.6, 0.15 M NaCl, 10 mM CaCl₂, 10 mM MgCl₂ and 0.2 % (vol/vol) Triton X-100. Four column volumes of 10 mM Tris buffer, pH 7.6,

containing 0.15 M NaCl and 0.2 % (vol / vol) Triton X-100 were used to wash the column to remove unbound protein. PAS IV bound to the column was then eluted with 0.4 M alpha-methyl-mannoside in 50 mM Tris buffer, pH 7.6. PAS IV enriched fractions were combined, dialyzed against 10 mM Tris-buffered saline, pH 7.6, containing 0.2 % (vol / vol) Triton X-100 and the protein concentration was determined. The dialyzed sample was then concentrated by centrifugation in Centricon 30 microconcentrators at 6000 rpm using a SS 34 rotor, at 4°C., and stored at - 20°C.

(d) Electroelution :

PAS IV obtained after lectin-affinity chromatography was electrophoresed on a 7.5 % polyacrylamide denaturing gel of 1.5 mm thickness under reducing conditions. The band of PAS IV visualized by staining a slice of the gel with Coomassie blue, was excised and electroeluted overnight from gel in Tris-glycine buffer (25 mM Tris, pH 8.3, 192 mM glycine) at 200 mV, at 4°C., using an Elutrap device (Schleicher and Schuell, Inc. Keene, NH, USA). The electroeluted protein was dialyzed against TBS containing 0.2 % Triton X-100 and its content was determined by the BCA protein assay (64) .

Electrophoresis

Samples were separated by SDS-polyacrylamide-gel electrophoresis in 7.5 % (w/v) - acrylamide slab gels under reducing conditions (final concentration of mercaptoethanol : 2.5 %) as described by Laemmli (65). A constant current of 20 mA was applied until the dye front advanced to within 2 mm distance of the bottom of the gel. Proteins were stained with Coomassie blue (31). Destaining was performed first in a solution containing 50 % methanol / 10 % glacial acetic acid in water for 30 mins., followed by a solution containing 5 % methanol / 7 % glacial acetic acid overnight. Determination of the molecular weights of proteins by SDS-PAGE was as described (66), using the following proteins as standards : myosin (205,000), beta - galactosidase (116,000), phosphorylase B (97,400), bovine albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

Protein determination

Protein concentrations were determined by the method of Lowry et al. (67) or with the bicinchoninic acid (BCA) assay (64) using bovine serum albumin (BSA) as standard. The Lowry protein assay was modified by inclusion of SDS (final concentration of 1.0 %) whenever the protein samples to be assayed contained Triton X-114.

Preparation of polyclonal antiserum to PAS IV

Polyclonal rabbit antibody to PAS IV was prepared by the Berkeley Antibody Company Inc., Richmond, CA, using the monomeric form of purified PAS IV obtained after electroelution.

Preparation of a PAS IV-Affinity Column

PAS IV was covalently linked to cyanogen-bromide activated Sepharose 4B as described below :

2.4 gram of cyanogen-bromide activated Sepharose 4B was swollen in 10^{-3} M hydrochloric acid (HCl) for 15 mins. The HCl was removed while transferring the resin into a column, by filtering under vacuum and washing the resin with 100 ml of distilled water. The washed resin slurry was then equilibrated with coupling buffer containing 0.1 M NaHCO_3 / 0.5 M NaCl, pH 8.7, prior to coupling with 12 mg of PAS IV (dialyzed overnight against coupling buffer) for 2 hours at room temperature. In order to achieve maximum coupling of the protein to the resin, the mixture was shaken on an orbital shaker during the coupling period. The unbound protein solution was then drained off from the column and resin was washed with coupling buffer. The remaining reactive sites of the resin were blocked by 6.0 mls of ethanolamine, pH 9.0, for 2 hours at 4°C. The ethanolamine was then removed and the resin was washed 3 times with the following buffers, alternately :

(i) 25 ml of 0.1 M acetate, 1.0 M NaCl, pH 4.0.

(ii) 25 ml of 0.1 M borate, 1.0 M NaCl, pH 8.0.

The final washing was 200 mls of phosphate buffer saline (PBS) prior to storage in 0.1 % sodium azide in PBS at 4°C. until further use. The efficiency of coupling procedure was checked by analyzing the effluent by SDS-PAGE as well as protein assay.

Purification of PAS IV-specific antibodies

Rabbit antisera (30 ml) was dialyzed against Tris-buffered saline containing 0.2 % Triton X-100. The dialyzed serum was then centrifuged at 4°C. at 26,500 rpm for 30 mins. using a SW27 rotor and fractionated at 4°C. on the above mentioned PAS IV-affinity column which was preequilibrated with dialysis buffer. After removing nonspecifically bound antibodies by washing the column for three hours with dialysis buffer, PAS IV specific antibodies were eluted with 30 ml of 3 M sodium thiocyanate (NaSCN) in TBS, pH 7.6, containing 0.2 % Triton X-100 at the rate of 12 mls / hour and dialyzed overnight against 4 liters of TBS, pH 7.6, at 4°C. The column was then washed with TBS, pH 7.6, containing 0.2 % Triton X-100 and stored in 0.02 % sodium azide solution. The fractions were dialyzed overnight against TBS, pH 7.6, containing 0.2 % Triton X-100. UV measurements at 280 nm were made on all fractions to determine the peak of elution, followed by protein determination by BCA assay. SDS-PAGE was used to

analyze the purity of PAS IV-specific antibodies.

The specificity of affinity-purified antibodies was checked by Immunoblotting technique as described below using membrane.

Immunoblotting technique

Protein samples separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper, as described by Towbin et al. (68). The transferred proteins were visualized by staining with Ponceau S stain and marked with pencil. The nitrocellulose paper was then blocked overnight with 2 % w/v BSA / TBS and incubated with affinity - purified polyclonal antibodies to PAS IV, diluted 1:1000 in 2 % (w/v) BSA / TBS (0.65 μ g protein / ml) for an hour. The unbound antibodies were removed by washing the paper sequentially with 0.5 % (v/v) Triton X-100 / TBS (for 30 mins.); 0.5 M NaCl / TBS (for 30 mins.); TBS (for 30 mins.) and 0.1 % (w/v) BSA / TBS (for 30 mins.). The bound PAS IV specific antibodies were then located by incubating the paper for an hour with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase. The unbound antibodies were removed by sequential treatment with 0.1% w/v BSA / TBS (for 30 mins.); 0.1 % (v/v) Triton X-100 / TBS (for 30 mins.); 0.5 % (v/v) Triton X-100 / TBS (for 30 mins.); 0.5 M NaCl / TBS (for 30 mins.) and two changes of TBS (for 30 mins. each). PAS IV was then visualized by addition of 5-Bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt (BCIP) together with Nitro Blue Tetrazolium

(NBT) [0.1 ml BCIP at a concentration of 5 mg / ml in dimethyl formamide, 1.0 ml NBT at a concentration of 1 mg/ml in barbital buffer, pH 7.0, 20 µl of 2 M magnesium chloride and 9.0 ml of barbital buffer, pH 7.0] as a distinct blue colored band. No colored band was observed in control immunoblots (Control serum, 1: 500 dilution).

Densitometry

The relative amounts of individual bands of protein in representative SDS-polyacrylamide gels were determined by densitometric tracings after drying the gels between layers of Gel Bond and Gel Gard films. The dried gels, fixed to the Gel Bond were then scanned at 600 nm on a Beckman AppraiseTM densitometer.

Endoglycosidase F experiment

The number of N-linked oligosaccharides present in PAS IV was estimated by their removal by Endoglycosidase F (Endo F). The deglycosylation of PAS IV was carried out under two different experimental conditions :

(a) Varying the enzyme concentration while keeping the period of incubation constant .

0.06 µg (3 µl) of purified PAS IV was incubated serially with 100, 200, and 500 milliunits of Endo F at 37°C. for 20 hours in a reaction mixture containing 1 µl

of 1 mM PMSF, 1 μ l of 5 mM iodoacetamide, 1 μ l of mercaptoethanol and 29 μ l of 50 mM Tris-HCl buffer, pH 8.6 at 37°C. (and pH 8.9 at 25°C.) containing 50 mM EDTA, 0.5 % Triton X-100 and 0.1 % SDS.

(b) Varying the period of incubation while keeping the concentration of enzyme constant .

0.06 μ g of purified PAS IV was incubated with 200 milliunits of Endo F at 37°C. for 12, 24, 36, and 48 hours in a reaction mixture containing 1 μ l of 1 mM PMSF, 1 μ l of 5 mM iodoacetamide, 1 μ l of mercaptoethanol and 29 μ l of 50 mM Tris-HCl buffer, pH 8.6 at 37°C. (and pH 8.9 at 25°C.) containing 50 mM EDTA, 0.5 % Triton X-100 and 0.1 % SDS.

The reaction in both experimental conditions was terminated by boiling for one minute after addition of an equal volume (35 μ l) of SDS-PAGE sample buffer containing 5% mercaptoethanol. The samples were then separated by SDS-PAGE, transferred to nitrocellulose paper and immunoblotted as described before.

Sequence Analysis

The purified sample was submitted to Dr. Ken Watt, Cetus Corporation, Emeryville, CA for sequence determination. N-terminal sequence and several internal sequences from Endoproteinase Lys-C (lysine-specific protease)-derived peptides were obtained. The sequence analysis was performed using an Applied

Systems 470A gas-phase sequencer with an on-line model 120A PTH amino acid analyzer (69).

Hydrophobic moment calculations

The hydrophobic moment (μ_H) of the putative alpha-helix of N residues was calculated by formula :

$$\mu_H = \{ [\sum_{n=1}^N H_n \sin(\vartheta_n)]^2 + [\sum_{n=1}^N H_n \cos(\vartheta_n)]^2 \}^{1/2}.$$

where H_n is the hydrophobicity of the nth residue and ϑ , the angle at which successive side chains emerge from the backbone, is 100° (70). The value of μ_H gives the amphiphilicity of alpha-helix. The hydrophobicity values used were as given in (71).

III. RESULTS

Preparation of pure PAS IV

The purification procedure steps are briefly summarized in Fig.1, evidenced in Fig. 2 and quantitated in Table 1. The SDS-PAGE pattern of milk-fat-globule membrane prepared from fresh bovine cream is shown in Fig. 2. With Coomassie blue-staining, at least 15 clearly distinguishable bands were observed. The total membrane protein concentration determined as per Lowry (67) was 984 mg / 37 ml MFGM or 984 mg / liter of cream (Table 1).

In two determinations with membrane samples prepared at different times, the amount of protein solubilized in 1 % Triton X-114 in 10 mM Tris buffer, pH 7.6, containing protease inhibitors, was 22 % of the total membrane protein incubated with detergent, 50 % of which (i.e. 11 % of the total MFGM protein) was partitioned into the detergent phase and 50 % in the aqueous phase (Table 1). The schematic diagram of membrane solubilization and phase-partitioning is shown in Fig. 3. Comparison of the PAS IV band in detergent and aqueous phases indicates that approximately 95 % of PAS IV was partitioned into the detergent phase (Fig. 2). An approximately five-fold enrichment of PAS IV was obtained by the partitioning procedure (Fig. 4a, 4b). The membrane proteins phase-partitioned in the detergent phase were then fractionated on an ion-exchange column and eluted by a linear NaCl gradient. Total of 56 fractions of 3.0 mls each were collected and

FIGURE 1 : Schematic diagram of PAS IV Purification protocol

FIGURE 2 : Analysis of PAS IV Purification Procedure steps by SDS-PAGE in reducing conditions : Samples were resolved on a 7.5% w/v polyacrylamide slab gel (0.75 mm thickness) as follows : lane 1, untreated MFGM; lane 3, detergent phase; lane 4, PAS IV after purification by DEAE-cellulose column and lectin-affinity chromatography; lane 5, molecular weight standards. Proteins were stained with Coomassie blue R 250. The molecular weight standards from top to bottom of the gel were as follows : myosin (205 kdaltons), beta-galactosidase (116 kdaltons), phosphorylase B (97.4 kdaltons), bovine albumin (66 kdaltons), ovalbumin (45 kdaltons), and carbomic anhydrase (29 kdaltons).

TABLE 1 : Quantitation of PAS IV Purification Procedure@

Steps	Total Protein (mg)	PAS IV (mg)	% recovery
MFGM	984.20	49.21*	100
Solubilized membrane	216.50	-	-
Detergent Phase	108.26	29.34	59.62
DEAE-column Chromatography	37.20	-	-
Con A-column Chromatography	17.63	15.27	31.03
Electroelution	12.00	12.00	24.39

@ Numbers presented here represent experimental values and not true values.

* Based on the observation that 5% of total membrane protein is PAS IV.

PHASE PARTITIONING OF PAS IV

FIGURE 3 : Schematic representation of phase partitioning of PAS IV.

FIGURE 4a : Densitometric tracing of untreated MFGM (lane I of Fig. 2) : The gel was scanned at 600 nm with a Beckman Appraise™ densitometer. The slit width used was 4 mm and the scan length was 45 cm. The shaded area indicates the position of PAS IV. PAS IV constitutes 5.7% of the total peak area.

FIGURE 4b : Densitometric tracing of detergent phase (lane 2 of Fig. 2) : The gel was scanned at 600 nm with a Beckman Appraise™ densitometer. The slit width used was 4 mm and the scan length was 45 cm. The shaded area indicates the position of PAS IV. PAS IV constitutes 27.1% of the total peak area.

separation of each of the fractions on an analytical scale by electrophoresis in polyacrylamide gels containing SDS was obtained. Elution of PAS IV was seen from fraction 16 onwards. PAS IV appeared to elute at a NaCl concentration of 50 - 80 mM of salt. PAS IV-enriched fractions 17 through 27 were combined (Fig. 5). These fractions contained butyrophillin, a doublet at approximate M_r 60,000 daltons, and a lower M_r (~ 15,000 daltons) band as contaminants. The PAS IV-enriched fractions containing 37.2 mg of protein / 28 mls were then adsorbed on a lectin-affinity column as PAS IV is known to bind Concanavalin A. On elution with 0.4 M alpha-methylmannoside, the 15,000 dalton-contaminant was removed by Con A, but a slight contamination of butyrophillin and the doublet still persisted. The pooled fractions from Con A shown in Fig. 6 were concentrated at 4°C., electrophoresed (Fig. 7) and electroeluted to obtain pure PAS IV. Analysis of purified PAS IV by SDS-PAGE showed a single major band of protein. Approximately 25 % of theoretical yield of PAS IV was obtained.

Covalent linkage of purified PAS IV and Isolation of PAS IV-specific antibodies

10.8 mg of purified PAS IV was coupled to CNBr-activated Sepharose resin as described earlier in the Experimental section. The efficiency of coupling procedure was checked by analyzing the effluent by SDS-PAGE [data not shown] as well as

FIGURE 5 : SDS-PAGE in reducing conditions in 7.5 % acrylamide gel (0.75 mm thickness) of DEAE-cellulose column chromatography fractions : The fractions were collected by elution of the proteins of the detergent phase with a NaCl gradient. Fractions 17 through 27 represent the pooled fractions used for the following step i.e. adsorption on Concanavalin A lectin-affinity column. The gel was stained with Coomassie Blue R 250. Mr standards used were as described in Fig. 2.

FIGURE 6 : SDS-PAGE pattern of fractions eluted from the Concanavalin A-lectin affinity column : The fractions 2 through 9 were combined and concentrated for electroelution. Coomassie Blue R 250 was used for staining the gel. Mr standards used were as described in Fig. 2.

FIGURE 7 : Densitometric tracing of purified PAS IV (lane 3 of Fig. 2) : The gel was scanned at 600 nm with a Beckman Appraise™ densitometer. The slit width used was 4 mm and the scan length was 45 cm. The shaded area indicates the position of PAS IV. PAS IV constitutes 86.6% of the total peak area.

Lowry protein assay (percentage coupling of the purified PAS IV was determined to be 90 %). This PAS IV-affinity column was used for obtaining anti-affinity purified-PAS IV antibodies from rabbit antisera. The fractions containing PAS IV-specific antibodies were determined from absorbance measurement at 280 nm. The elution profile of purification of antibodies is shown in Fig. 8. Fractions 2, 3 and 4 were combined which upon analysis with SDS-PAGE displayed two bands [heavy chain and light chain] (Fig. 9). From 30 ml serum, 17 mg of affinity-purified antibodies were obtained which were then aliquoted for further use in Immunoblotting procedures.

Deglycosylation of PAS IV :

To confirm the presence and to estimate the number of N-linked oligosaccharides, microgram amounts of purified PAS IV were treated with Endo F in the presence of EDTA in various experimental conditions as described in detail in the Experimental section. A substantial and gradual decrease in apparent molecular weight of PAS IV from 85,000 to 75,000 was observed (Figs. 10a, 10b, 11a, 11b). In fact, a flattening of the curve was seen with the increase in period of incubation indicating complete removal of N-linked carbohydrates of PAS IV (Fig. 11c). Assuming that 1) the purified PAS IV was as glycosylated as in vivo, 2) the ultimate product of the experiment is totally deglycosylated, 3) the N-linked oligosaccharides are of approximately equal size (2,500 daltons) (72), and 4) the

Anti-PAS IV Elution Profile

FIGURE 8 : Elution profile of affinity-purified antibodies specific to PAS IV : UV measurements at 280 nm were made of 5 ml fractions collected during elution of PAS IV-specific polyclonal antibodies with 3M NaSCN from the PAS IV-affinity column which was loaded with rabbit antisera. Fraction 1 represents 3-hour wash and fractions 2 through 4 represent PAS IV-specific polyclonal antibodies eluted by 3M NaSCN after 3-hour wash.

FIGURE 9 : Purification of PAS IV-specific antibodies using the PAS IV-affinity column : The purified antibodies were analyzed by SDS-PAGE using 7.5 % w/v polyacrylamide slab gel (0.75 mm thickness) as follows : lane 1, Mr standards; lane 2, gamma-globulin fraction; lane 3, purified antibodies; lane 4, blank; lane 5, 3-hour wash. The proteins bands were visualized by staining with Coomassie Blue R 250. The molecular weight standards used were as described in Fig. 2.

FIGURE 10a : Immunoblot of deglycosylation of PAS IV at various concentrations of Endoglycosidase F : Digestion of PAS IV for 20 hours with different concentrations of Endoglycosidase F was carried out at 37° C. The samples shown are in the following order : lane 1, M_r standards; lane 2, untreated pure PAS IV, as a control; lane 3, PAS IV band on digestion with 100 milliunits of Endo F; lane 4, PAS IV band on digestion with 200 milliunits of Endo F; lane 5, PAS IV band on digestion with 500 milliunits of Endo F. The molecular weight standards used were as described in Fig.2.

FIGURE 10b : Immunoblot of deglycosylation of PAS IV for different incubation periods : Samples of pure PAS IV when digested at 37°C., with 200 millunits of Endo F for different periods of incubation : lane 1, untreated pure PAS IV, as a control; lane 2, after 12-hours digestion; lane 3, after 24-hours digestion; lane 4, after 36-hours digestion; lane 5, after 48-hours digestion. The Mr standards used were as described in Fig. 2.

FIGURE 11a : Endoglycosidase F digestion plot (constant incubation period) :
 A plot showing a substantial decrease in apparent molecular weight of PAS IV during its digestion with Endo F for 20 hours at 37°C. at different endoglycosidase concentrations. * denotes untreated PAS IV (Mr 85,000); + represents after digestion with 100 milliunits of Endo F (Mr 80,000); • represents after digestion with 200 milliunits of Endo F (Mr 75,000); Δ represents after digestion with 500 milliunits of Endo F (Mr 71,500). The Mr standards used were as described in Fig.2. The ordinate gives the logarithm of molecular weight and the abscissa gives the Rf values calculated from the ratio of the distance travelled by protein-band to that travelled by the solvent-front. The apparent molecular weights of the samples were estimated by comparing their Rf values with those of Mr standards.

Endo F digestion - constant [enzyme] (200mUnits)

FIGURE 11b : Endoglycosidase F digestion plot (constant enzyme concentration) : A plot showing the gradual decrease in apparent molecular weight of PAS IV during its digestion with Endo F (200 mUnits) for different periods of incubation at 37°C. * denotes untreated PAS IV (Mr 85,000); • denotes PAS IV after 12-hour digestion (Mr 78,000); + denotes PAS IV after 24-hour digestion (Mr 75,000); Δ denotes PAS IV after 36-hour digestion (Mr 75,000); ◇ denotes PAS IV after 48-hour digestion (Mr ,000). The Mr standards used were as described in Fig. 2.

Molecular weight vs Incubation period

FIGURE 11c : Molecular weight vs Incubation period : A plot displaying gradual decrease in an apparent molecular weight of PAS IV with respect to time on digestion with Endo F (200 mUnits).

lack of aberrant migration in SDS-PAGE, these results suggest that PAS IV contains at least four N-linked oligosaccharides which are either high mannose or complex type or both.

Sequence Information

Various sequences (i.e. the N-terminus and some of the internal sequences obtained by lysine-specific proteolytic digestion) of purified PAS IV are shown in Fig.12 and Table 2. The average hydrophobicity (H) of each of the internal sequences was calculated and tabulated (Table 3). The probable transmembrane helix from the N-terminal sequence was identified as per the algorithm of Eisenberg et al. (71). In short, a candidate transmembrane segment was first located by running a 21-residue "window" along the sequence and selecting as a "candidate" the one with the highest mean hydrophobicity ($\langle H \rangle$) value, followed by tentative characterization of the chosen membrane-associated segment by running a window of 11 residues through the sequence to obtain a set of successive windows with values of $\langle H \rangle$ and mean hydrophobic moment $\langle \mu_H \rangle$ (Table 4). The window with the highest $\langle \mu_H \rangle$ was then selected. This window's $\langle H \rangle$ and $\langle \mu_H \rangle$ were plotted on the hydrophobic moment plot (Fig.13). The boundary lines are arbitrary, selected only because they separate proteins of different functional classes

N - TERMINAL SEQUENCE OF PURIFIED BOVINE PAS IV

GLY - VAL - ASN* - ARG* - ASN* - SER* - GLY - LEU - ILE -
ALA - GLY - ALA - VAL - ILE - GLY - ALA - VAL - LEU -
ALA - VAL - PHE - GLY - GLY - ILE - LEU - MET - PRO -
LYS - ? - ILE - ? - SER*.

FIGURE 12 : N-terminal sequence of PAS IV : The probable alpha- helical membrane-spanning region is underlined. The residues marked * are the hydrophilic residues. The membrane-spanning region is thought to be anchored by positively charged residues. The residues identical in N-terminal sequences of both GP IV and PAS IV are highlighted in 'bold' letters.

TABLE 2 : List of the various sequences of PAS IV obtained by lysine-specific proteolytic digestion.

Number	Internal sequences
# 1076	V-F-V-L-E-E-D-I-S-A-F-K-K
#1107	S-I-(E)-L-M-F-Q-T-R-T-L-K
# 1097 (major)	R-L-Q-V-N-M-Y-K-G-R-V-L-D-I-G-K
# 1097 (minor)	V-A-I-K-E-T-L-V-Y-P-K
# 1096	T-G-G-D-D-S-Y-?-Q-F-(S)-K
# 1089 (major)	R-L-Q-D-V-Y-R-Q-F-W-I-F-D-V-Q-N-P-D-E-V-T-V-V-S-S-K
# 1089	T-G-T-V-N-M-L-V-K-P-A-K
# 1082 (major)	R-L-Q-D-V-Y-R-lost-F-W-I-F-D-V-Q-N-P-D-E-V-T-V-V-D-S-K
# 1082 (minor)	H-G-Y-I-E-S?-Y-lost-Q-L-(L)-E-(T)-(G)-W-I-I-?-?-K

TABLE 3 : Average hydrophobicities of obtained sequences

Internal sequences	< H >
# 1076	0.480
# 1089 (minor)	- 0.129
# 1089 (major)	- 0.072
# 1096	- 0.300
# 1097 (minor)	0.487
# 1097 (major)	- 0.561
# 1107	- 0.860

TABLE 4 : Selection of N-terminal segment with the highest mean hydrophobic moment

Sequence positions	Hydrophobic moment ($\langle \mu H \rangle$)	Mean hydrophobicity ($\langle H \rangle$)
Asn 5 - Gly 15	0.113	0.602
Ser 6 - Ala 16	0.050	0.729
Gly 7 - Val 17	0.120	0.844
Leu 8 - Leu 18	0.082	0.896
Ile 9 - Ala 19	0.127	0.856
Ala 10 - Val 20	0.154	0.829
Gly 11 - Phe 21	0.194	0.881
Ala 12 - Gly 22	0.180	0.881
Val 13 - Gly 23	0.200	0.868
Ile 14 - Ile 24	0.247 *	0.869
Gly 15 - Leu 25	0.199	0.866
Ala 16 - Met 26	0.178	0.881
Val 17 - Pro 27	0.149	0.835

* Segment with the highest $\langle \mu H \rangle$ and was found to be located in the region representative of transmembrane proteins as shown in Fig. 13.

FIGURE 13 : Hydrophobic moment plot : The point labeled x represents a 11-residue probable alpha-helix segment with highest $\langle \mu_H \rangle$ selected from N-terminus sequence of purified PAS IV. The ordinate gives the mean hydrophobic moment per residue $\langle \mu_H \rangle$ and the abscissa shows the mean hydrophobicity per residue $\langle H \rangle$. The curve defines the outer limit of possible points as per Ref. 71.

as helices of different functional types tend to cluster in different regions of such a plot. The hydrophobicity and hydrophobic moment values of the N-terminal sequence were calculated to be 0.869 and 0.247, respectively (Ile14 - Ile24), and imply an amphiphilic nature for the N-terminal sequence which is characteristic of membrane-spanning region of integral membrane proteins which contain multiple membrane-spanning helices (64). Residues 7 - 27 of N-terminal sequence constitute a hydrophobic region ($\langle H \rangle = 0.828$) which may represent a membrane-spanning helix. A "Helical wheel" diagram of the putative membrane-spanning sequence of PAS IV was constructed as in (73) (Fig. 14). In a "helical wheel" diagram, an alpha-helical segment of the protein is viewed from an "end-on" position, as if the reader were looking directly down the central axis from the N-terminal end of the helix. Since there are 3.6 residues per turn of helix, adjoining residues appear 100° apart on the wheel. The most distinctive feature of the helix is the clustering of glycine residues (at positions 11, 15, 22, and 23) on one side of the membrane-spanning helix.

The putative alpha helical orientation of amino-terminal sequence of PAS IV was also identified by another commonly used secondary structure analysis where membrane-buried helix parameter was plotted as a function of residue sequence number . A curve of the buried-helix parameter versus sequence number was produced first and then smoothed using a sliding average over a fixed sequence length (i.e.three, five, and seven-residue segments) along the N-terminal sequence.

PAS IV : Helical Wheel

FIGURE 14 : Helical wheel of the N-terminal sequence of PAS IV : Residues 7 - 26 are drawn in form of a "helical wheel". Polar residues are highlighted.

The profiles obtained by plotting the position of a reference amino acid (i.e. the middle amino acid in the region being summed) instead of sequence number are shown in Fig.15. The 7-residue window-profile displays a pronounced peak implying a probable alpha helix in the N-terminal region of PAS IV.

Smoothing curves of N-terminal sequence

FIGURE 15 : Smoothing curves obtained by sliding average technique : The plot was computed for N-terminal sequence of PAS IV using variable window widths (i.e. 3, 5, 7 and 11 residues). The values given for membrane-buried helix parameter profiles represent the average for the corresponding window, the value is printed at the middle residue of the window.

IV. DISCUSSION

The milk-fat-globule membrane (MFGM) contains glycoproteins as membrane constituents. Even though the function of these glycoproteins in secreted milk is obscure, as components of epithelial or endothelial cell membranes they may have important roles in cell recognition (74), adhesion (75), transport (79, 124, 125), immunological recognition (77), and as receptors for viruses (78) and hormones (79). Studies on glycoproteins of the MFGM, therefore, are expected to offer valuable information about structural and / or biochemical properties of the mammary epithelial cell and its membrane as well as endothelial cells of various tissues. The endothelial cells line arteries, veins, capillaries, and lymphatic vessels and form a continuous lining between circulating blood and surrounding tissues. The location renders endothelium an important role in several biological functions and disease states and has made endothelium the target for extensive studies (57, 80). The role of the endothelium of blood and lymph vessels is substantial in many biologic processes, such as the regulation of hemostasis and selective permeability of blood vessels. Blood group antigens are expressed in the endothelial cells and blood group-specific antisera have been used as light microscopic and ultrastructural markers for endothelia in kidneys (81) and other tissues (82). Abnormalities of endothelial cells are prominent factors involved in the pathogenesis of diseases such as atherosclerosis and thrombosis (83, 84). Also, an interaction of endothelial cell surfaces with malignant cells may be a critical step in

the process of metastatic invasion (85). However, since the identification of cell type based on morphology alone is quite difficult in culture, the increasing use of endothelial cell cultures from various sources has accentuated the need for specific markers. These would allow monitoring of the state of differentiation as well as an unambiguous identification of endothelial cells in primary cultures. Identification of endothelial cells will also be required in the study of other cell types, since primary cultures from various organs may be contaminated with them. Antisera raised against Factor VIII-related antigen (86, 87, 88, 89), angiotensin-converting enzyme (90, 91, 92) and, as has been shown by Holthofer et al. (57) and Miettinen et al. (93), a ligand recognized by the *Ulex europaeus* I lectin (UEA I) have been used for identifying cultured endothelial cells of various sources. Thus PAS IV which is present on endothelial cells of certain organs can be of use as a marker.

In the present study, an attempt to elucidate the structure (to characterize the topology) of Bovine PAS IV, an integral membrane glycoprotein of milk-fat-globule membrane, previously shown to be specifically localized in capillary endothelial cells in myocardial and smooth muscle tissues (35) has been made. The project deals first, with an improvement in the purification protocol and second, with collection and interpretation of data relative to the topological orientation of PAS IV in the membrane. The milk-fat-globule membrane preparation, instead of microsomal membranes prepared from the tissue, was used as a source of PAS IV, as 1) it is easier to obtain, 2) can be isolated in a large quantities, and 3) is a homogenous membrane. Due to the cellular heterogeneity of

tissues, microsomal membranes contain impurities like endoplasmic reticulum, mitochondria, etc. which makes the isolation of specific molecules difficult.

Improvement of the purification protocol

An advantage of the presence of PAS IV as a constituent of milk-fat- globule membrane was taken in the purification protocol. The preparation of MFGM involves the removal of fat globules from milk and the release of membrane material from the surface of the droplets. As shown by Basch et al. (94), the removal of casein micelles present in fat globules can be facilitated by the inclusion of salt in the washing buffer. However, as evidenced earlier, the membrane may be lost from the surface of the globules during this procedure, presumably as a result of mechanical erosion during the resuspension and centrifugation steps (95, 96). This was resolved by directly homogenizing the cream with 0.1 M citrate buffer as casein micelles are destabilized by citrate so that after centrifugation of the membrane, they remained in the supernatant and thus were easily removed (97). The efficiency of the citrate buffer was determined by the absence of casein in MFGM using SDS-PAGE (data not shown).

PAS IV has been previously purified (35). However, the percentage yield of pure PAS IV obtained by the purification procedure described here is almost double that of previous method (Table 1). In the previous protocol, the MFGM preparation was first digested with the protease papain [as PAS IV has been found

to be resistant to papain proteolysis (35)]. The proteolytic step was followed by solubilization of the washed membrane with the detergent Triton X-100. The solubilized proteins were not phase-partitioned as the cloud point (the temperature at which distribution of proteins between detergent and aqueous phases occurs) of Triton X-100 is very high (64°C.) and generally proteins, with few exceptions, are unstable at such a high temperature.

In the new procedure, without prior proteolytic treatment, the milk-fat-globule membrane was directly solubilized and phase - partitioned with the non-ionic detergent Triton X-114, instead of Triton X-100. In this process, advantage was taken of some of the interesting properties of Triton X-114. Below its cloud point (20°C.), Triton X-114 forms micellar solutions and is an efficient membrane proteins solubilizing agent (98). Also, because of its low solubility above the cloud point, separation of proteins into two phases can be easily achieved.

During the solubilization procedure, most of the lipid molecules that were in contact with the hydrophobic domain of the integral membrane protein were replaced by the nonionic detergent Triton X-114 which led to the formation of a soluble protein - detergent mixed micelle (99, 100) at 4°C. At 30°C., i.e. above the cloud point of Triton X-114, the solution turned turbid because of an increase in micellar molecular weight due to aggregation of small micelles. This resulted in two clear phases, 1) detergent-enriched containing hydrophobic proteins and 2) detergent-depleted, i.e. the aqueous phase containing hydrophilic proteins. Approximately 95 % of PAS IV was phase-partitioned in the detergent phase

(Fig. 2, compare lane 2 with lane 5). A five-fold enrichment of PAS IV was observed on phase-partitioning (compare Fig. 4a with 4b). PAS IV was found not to be degraded during this procedure as was evidenced by the absence of a detectable difference between the molecular weight of PAS IV band in MFGM and that in the detergent phase, as Triton is a "mild" detergent (99, 100, 101) and also as an extra precaution, protease inhibitors were used during solubilization.

The advantages of this modified protocol are as listed below :

- (1) Simplicity in operation.
- (2) Elimination of use of protease which otherwise may increase the chances of degradation of protein of interest.
- (3) Phase-partitioning of the hydrophobic and hydrophilic proteins of solubilized MFGM into detergent and aqueous phase, respectively (because Triton X-114 exhibits a cloud point at about 20°C.), results in complete removal of hydrophilic protein contaminants. For example, lactoferrin, a soluble glycoprotein, very similar to PAS IV (same molecular weight, isoelectric point, and Con A-binding efficiency) is partitioned into the aqueous phase.
- (4) Enhancement in electrophoretic resolution. The contaminating soluble proteins frequently dominate electrophoretograms of membrane preparations, and since many integral membrane glycoproteins stain poorly with Coomassie blue, a real (true) picture of the relative amounts of integral membrane proteins present may not be obtained. Thus, as solubilization and phase-partitioning with non-ionic detergents freed the integral membrane proteins from contamination by adsorbed

soluble proteins, the resolving power of electrophoretic techniques was greatly increased.

(5) An approximately five-fold enrichment of PAS IV was obtained by the partitioning procedure.

Topographical data

Based on several criteria, PAS IV has been hypothesized to be an integral membrane glycoprotein. The membrane-bound form of PAS IV was found to be resistant to proteolysis, under conditions that completely remove the cytoplasmic peripheral proteins (35). Also, the treatment of membrane preparations with 0.1 M Na_2CO_3 solutions at pH 11.5, conditions previously shown to remove a large proportion of peripheral and loosely adsorbed membrane proteins, was unable to dissociate PAS IV from MFGM (40, 41). These data imply that PAS IV is neither a peripheral protein of the cytoplasmic coat nor a protein loosely adsorbed to the external membrane but is closely associated with the membrane phospholipid bilayer. Two pieces of evidence supporting the hypothesis that PAS IV is an integral membrane protein as well as implying that it has multiple membrane-spanning regions were obtained : (1) Sequence information, and (2) Endoglycosidase F digestion of PAS IV.

Sequence information. Since the amino acid sequence contains the complete

structure information of a protein (102), it should theoretically be possible to derive the spatial structure from the sequence alone without X-ray analysis. Normally, the first and the foremost step in this direction is to try to bridge the gap between the two lowest levels of structural organization in proteins, i.e. between sequence and the secondary structure. To a rough approximation, the secondary structure of an alpha helix is a function of the constituent amino acids alone (103). As we suspect PAS IV is an integral membrane protein with many alpha-helices, the identification of a putative alpha-helix was undertaken. Thus an attempt has been made to propose the partial spatial arrangement of PAS IV based in part on prediction of the secondary structure of the N-terminal sequence obtained from the purified protein.

It has been observed that hydrophobic segments, often 18 to 24 residues in length, of many membrane-associated proteins are mostly membrane-penetrating alpha helices (16, 104-107). Such a length seems to be adequate for spanning a membrane as 21 residues coiled into an alpha-helix approximate the thickness of the apolar portion of a lipid bilayer (50 - 70 Å). Also, a nonpolar environment is more likely to be preferred by a fully hydrogen-bonded backbone of the alpha-helix than the non-hydrogen-bonded conformation. A region in a membrane protein has a high probability of being a membrane-spanning region if it is 18 - 26 residues long (16, 104, 106, 107) with an average hydrophobicity of > 0.68 (71). However, it cannot be ruled out that other regions of the protein with a lower hydrophobicity index value also span the membrane as they can be stabilized in membranes via co-operative association with other chains in the membrane; for example,

chain-forming segments are stable in the membrane because they enjoy some association with each other (2, 71, 109-115).

An algorithm developed by Eisenberg et al. (71), which distinguishes alpha helices involved in the interactions of membrane proteins with lipid bilayers from helices in soluble proteins as well as helices of membrane proteins which contain one versus multiple membrane-spanning helices, was utilized for the interpretation of the sequence data to predict the probable topological orientation of PAS IV. The algorithm states that an "initiator" is necessary for transmembrane association. This initiator can be either a very hydrophobic single helix (mean hydrophobicity $\langle H \rangle \geq 0.68$) or a pair of helices of moderate hydrophobicities (whose $\langle H \rangle$ values sum to 1.10). Other helices within the same polypeptide even though less hydrophobic, if they are above a threshold hydrophobicity ($\langle H \rangle \geq 0.42$) can also probably be transmembrane. The mean hydrophobicity ($\langle H \rangle$) of the N-terminal sequence of PAS IV was calculated to be 0.828, which satisfies the condition of the candidate to be considered as transmembrane segment. Thus a probable potential initiator for transmembrane association was identified.

Since various classes of membrane-association (e.g. one- or more membrane-penetrations, surface-association, etc.) can be detected from amino acid sequences (71, 116), the N-terminal sequence, after being selected as potential transmembrane helix, was then classified with the aid of the hydrophobic moment plot, on which the hydrophobic moment of each helix was plotted as a function of its hydrophobicity. The magnitude of hydrophobicity is a measure of the affinity

of the helix for the membrane interior, while that of hydrophobic moment gives an amphiphilicity of the helix (and hence its tendency to seek a surface between hydrophobic and hydrophilic phases). Helices of the different protein classes exhibit a tendency to cluster in different regions of the plot, as shown in Fig. 11. For example, alpha helices from globular, surface and transmembrane proteins occupy characteristic regions, although the regions do not have clearly defined boundaries. In fact, multimeric and monomeric transmembrane proteins are found in separate distinct regions. The point in the plot representing PAS IV (Fig. 11) represents a single, 11-residue alpha-helix which is the fragment of the 21-residue N-terminal putative helix, identified as described in the Results section, as having the largest hydrophobic moment. The rationale for plotting 11-residue helices is that as their length of about three turns represents the approximate distance over which neighboring helices generally interact, the tendency, if any, to shield hydrophilic residues from surrounding membrane lipid might be more obvious in 11-residue rather than in 21-residue helices. The location of the 11-residue segment, and thus the putative alpha-helix of N-terminal sequence in the multimeric transmembrane region, supports the hypothesis that PAS IV is an intrinsic membrane protein with multiple-spanning regions. The pronounced amphiphilicity characteristic of the adjacent helices of "channel-forming" proteins was observed in the N-terminal sequence of PAS IV. Therefore, we propose a multimeric transmembrane structure of PAS IV with a potential transport function(s).

The smoothed curve of the 'Chou-Fasman' membrane-buried helix preference

parameter as a function of residue sequence number (117) also supports the prediction of an alpha-helix structure for the N-terminal sequence. This parameter was chosen because of its accuracy and efficiency. It can discriminate and identify all the helices in bacteriorhodopsin, a well-characterized integral membrane protein, while hydropathy was able to sharply delineate only five of the seven transmembrane regions, with the separation of the two C-terminal helices being unclear (106). The buried-helix parameter for a particular amino acid is defined as the ratio of its composition in the predicted helices to its composition in all sequence regions of the integral membrane protein. As the profile due to the shortest span (3 residues) was found to be noisier, only the 7-residue span-profile was utilized for interpretation of the data (Fig. 14). The presence of the pronounced peak suggests a probable alpha helix in the N-terminal region of PAS IV.

It has been emphasized by several recent studies that membrane-associated proteins may contain helical regions in which there is a relatively sharp segregation of polar and nonpolar amino acid side chains on the surface of the helix (73, 118, 119). There is evidence that such an arrangement confers structural stability (118) as well as functional specificity (119). It is a general observation that multiple-spanning membrane proteins are transport proteins. Their membrane-buried helices accommodate the charged, hydrophilic amino acids by orienting themselves in such a way that they are not in contact with the lipid medium, but are situated in the center of the molecule, possibly forming a hydrophilic channel for transport or face the adjacent helix or helices, whereas the

hydrophobic, uncharged residues are directed outward towards the lipid environment [for example, bacteriorhodopsin] (73, 118, 119). Thus, in short, the protein has an "inside-out" arrangement when compared with the organization of soluble proteins. A helical wheel diagram of the N-terminal sequence of PAS IV (Fig. 12) demonstrates a distinct demarcation into polar, hydrophilic and nonpolar, hydrophobic faces. Both ends of the probable helix are flanked by positively charged anchoring residues. Charged or polar amino acid residues near the membrane-spanning segments could be used for the translocation of ions, as has been suggested for bacteriorhodopsin (118, 120), a protein that pumps protons across a hydrophobic membrane environment. All of the glycine residues and all except one alanine residue are found to be located on the same side of the helix, implying the high probability of this relatively hydrophilic side facing the adjacent helix or helices or may be in contact with an aqueous environment defined by other helices of PAS IV. This suggests that PAS IV could have an inside-out arrangement, forming a channel with the polar and hydrophilic amino acids in the adjacent alpha helical regions grouped toward the center of the protein molecule or facing the adjacent helix or helices and the nonpolar residues in the alpha-helices on the protein surface in contact with membrane lipids. The helical wheel diagrams of each of the internal sequences obtained by lysine-specific proteolytic treatment of purified PAS IV display a random distribution of polar amino acids implying their possible location in exterior and / or cytoplasmic domain(s), or their possible role in channel formation (for example, lining the internal wall(s) of the channel,

involvement in helix-helix interactions) [data not shown].

Our inability to detect transmembrane segments from internal sequences obtained could be due to either: (1) the sequences represent portions of the membrane-spanning regions; or (2) the sequences represent an external or cytoplasmic domain(s); or (3) their membrane-embedded regions form beta-structures, such as a beta-barrels, which are not detectable by this algorithm.

Another parameter that has been commonly used to predict secondary structures in integral membrane proteins is the propensity to form particular secondary structures namely P_{α} [propensity to form alpha helix] and P_{β} [propensity to form beta sheet] (121). As per this propensity parameter, residues 3 through 28 form beta sheets (data not shown) as oppose to helical orientation predicted as per algorithm of Eisenberg et al. (71) and smoothed curve of the Chou-Fasman's membrane-buried helix preference parameter. This inconsistency displays insufficient present-day knowledge essential to predict an accurate protein structure with certainty.

The N-terminal sequence analysis reveals an unblocked glycine residue followed by a sequence of six hydrophilic amino acids, which in turn is followed by an amphiphilic 20-residue putative membrane-spanning region. The first 28 residues of the N-terminal domain of PAS IV are shown in Table 2 where 20-amino acids extended sequence of hydrophobic amino acids is underlined.

The N-terminal sequence showed no appreciable sequence homology with any of the known protein sequences (as evidenced by a search through Brookhaven

Protein Data Bank as well as Protein Identification Resource). However, the recently published N-terminal sequence of human platelet glycoprotein, GP IV (88,000 daltons) (122), displays a great similarity to the N-terminal sequence of bovine MFGM PAS IV. Both sequences possess an unblocked glycine residue as an N-terminal residue. Both exhibit an identical juxtaposed stretch of 22-residues from Gly-7 to Val-28. Residues identical in both sequences are highlighted in 'bold' letters (Fig. 12). Glycoprotein IV (GP IV) is one of the major glycoproteins of the blood platelet cell surface (123). Some of the very recent studies demonstrate its role as receptor for erythrocytes infected with the mature *Plasmodium falciparum* parasite (124), collagen fibrils (125) and thrombospondin, a large adhesive protein required for irreversible platelet aggregation (126, 127). GP IV seems to have a rather wide range of distribution. Besides platelets, GP IV has been found in endothelial cells, monocytes and a variety of tumor cell lines including the C32 melanoma cell line (54, 122, 126). It also displays immunological cross-reactivity to the leukocyte differentiation antigen designated as CD 36 (128). The cDNA-derived amino acid sequence of CD 36 (129) displays homologies to N-terminal and some of the obtained internal sequences of PAS IV (Eugene software program of Baylor College of Medicine, Houston, TX was used for sequence homology determination). CD 36, like GP IV, has recently been shown to bind to a cytoadherence ligand present on the surface of *Plasmodium falciparum* - infected erythrocytes (124, 129). Although the reactivity of PAS IV to extracellular matrix components (for example, thrombospondin and collagen) is not

yet been analyzed and its exact function is not yet known, there is possibility of both PAS IV and GP IV having similar function(s), i.e. as receptors of extracellular matrix components. Based on inability of GP IV to bind fibronectin and its binding to thrombospondin via sequence other than Arg-Gly-Asp sequence, GP IV seems to be unrelated to the integrin family of adhesive proteins (126). Thus, this indicates the possibility of PAS IV and GP IV belonging to an absolutely new group of cell-adhesion proteins.

The comparison of several characteristics revealed many similarities between bovine MFGM PAS IV and human platelet GP IV such as (1) an almost identical amino-terminal sequence; (2) presence of an unblocked glycine residue as an N-terminal residue; (3) upon analysis the N-terminal sequence of both exhibited an identical 21-residue putative membrane-spanning regions with pronounced amphiphilicity adjacent to short hydrophilic hexapeptide; (4) lack of N-linked glycosylation site (i.e. Asn-X-Thr/Ser) on N-terminal sequence of both proteins; (5) high resistance to proteolysis (35, 125); (6) pronounced hydrophobic nature and high efficiency to partition into the detergent phase of the detergent Triton X-114. [These characteristics are the fundamental bases of their purification protocols (this study, 122, 127)]; (7) both are integral membrane proteins based on their overall hydrophobicity (revealed from amino acid analysis) and resistance to proteolytic digestion. These homologies are indicative of a probable relationship between PAS IV and GP IV.

However, inspite of these similarities PAS IV differs from GP IV in several

respects. Their isoelectric points are different [Bovine PAS IV - 7.8 to 8.5 and GP IV - 4.4 to 6.3 and 4.5 to 5.5] (35, 126, 130). They vary in carbohydrate content. Bovine PAS IV contains approximately 5 -10% total carbohydrate while human platelet GP IV contains 26% total carbohydrate. The absence of N-acetylgalactosamine in bovine PAS IV indicates absence of O-linked oligosaccharides whereas in human platelet GP IV approximately one-third of carbohydrate is in O-glycosidic linkages (35, 127, 136). Also, the M_r of PAS IV and GP IV differ by approximately 3,000. However, these dissimilarities between PAS IV and GP IV could likely be due to cell-specific glycosylation variability.

Endoglycosidase F digestion. Various specific glycosidases are found to be useful in elucidation of the structures, structure-function relationships, and biosynthetic pathways of biologically important glycoproteins (132-136). Two of the most useful are the endoglycosidases endo H (132) and endo D (133), which cleave high-mannose and complex glycans, respectively, from glycoproteins and glycopeptides. However, unlike endo H and endo D, endo F (endo-N-acetyl-glucosaminidase F) appears to have broader specificity in as much as it can deglycosylate both types of N-linked sugars, i.e. complex and high-mannose glycans (Fig. 16) from asparagine-linked glycoproteins (137). Also, cleavage by endo H and endo D occurs between the adjacent N-acetylglucosamine residues linking the glycan moiety to the asparagine of the peptide or protein

Various types of sugar chains of glycoproteins.

FIGURE 16 : Structure of high mannose and complex oligosaccharides.

backbone (132,133), while hydrolysis by endo F occurs at the glycosylamine junction. By hydrolyzing the glycosidic bond of the carbohydrate core structure N-linked to asparagine, it removes the bulk of the glycan moiety *en bloc*, i.e. both core N-acetylglucosamine residues remain attached with the released oligosaccharides, while the peptide or protein asparagine gets converted to an aspartic acid residue at the point of cleavage.

PAS IV is found to contain 5 -10 % total carbohydrate [wt / wt] (35), but the exact nature and the location of the glycosylation sites and the number of carbohydrate moiety (ies) have not yet been determined. The endo F digestion showed a decrease in apparent molecular weight of PAS IV by approximately 10,000 daltons, indicating the presence of several (approximately four) N-linked sugars. However, it should be noted, this result does not comply with recent data (Greenwalt, unpublished observation) where a decrease of 20,000 daltons is observed. This discrepancy could be due to susceptibility to evaporation of the experimental conditions utilized in this study leading to premature termination of endoglycosidase reaction. Asparagine-linked glycosylation sites can be recognized by the sequence Asn-X-Ser/Thr. Even though all asparagine-linked glycosylation sites have been shown to have this sequence, it is not necessary that all such sequences be glycosylated (reviewed in 138). None of the deduced amino acid sequences revealed any potential glycosylation sites upon analysis (refer Table 3). However, as glycosylated residues of membrane proteins are restricted to the luminal side of the membrane (139, 140), the absence of the potential glycosylation

site(s) in the N-terminal sequence (assuming the amino-terminal is in the external domain) implies the presence of at least two membrane-spanning regions (including the putative N-terminal membrane-associated region). A return to the exterior surface of the membrane via additional membrane-spanning regions is essential because of the requirement of glycosylation in the exterior domain at site(s) distal to the N-terminal membrane-spanning region. However, to confirm this interpretation, the determination of the exact location of N-terminus is required. The basis of the assumption of the N-terminus being in the exterior domain is the observation of slightly higher molecular weight PAS IV band in tissues (35) implying a possibility of limited proteolysis of native PAS IV, either during or after secretion, leading to formation of the protein of lower molecular weight in MFGM.

V. CONCLUSION

We report here an attempt to improve the purification protocol and to elucidate the structure of bovine PAS IV, an integral membrane glycoprotein of milk-fat-globule membrane. Eliminating the use of proteolysis, PAS IV can be purified in good yield by making use of its pronounced hydrophobic properties. The sequence analysis reveals an amino terminal sequence comprised of a short, hydrophilic, six-residue terminus followed by a 20 residue putative membrane-spanning region. The secondary structure prediction studies suggest a putative alpha-helix structure spanning the phospholipid bilayer for the amino terminus of PAS IV. The pronounced amphiphilicity characteristic of the adjacent helices of "channel-forming" proteins was observed in the N-terminal sequence of PAS IV which suggests a probable multimeric transmembrane structure of PAS IV with potential transport function(s). The distinctive feature of the putative helix is the presence of all of the seven hydrophilic residues positioned on one side of the helix implying its possible interaction with an adjacent PAS IV membrane-spanning helix or with an aqueous environment defined by the other helices of PAS IV. An algorithm designed to distinguish between membrane proteins with one versus multiple membrane-spanning helices also supports this prediction. The endo F digestion of PAS IV reveals presence of several N-linked oligosaccharides and presence of glycosylation site(s) distal from N-terminus. The absence of a glycosylation site in N-terminus sequence itself is suggestive of presence of more

than one membrane-spanning regions as the proteins are glycosylated only on their external surface. Thus, this is one more bit of evidence for PAS IV as an integral membrane protein with multiple membrane-spanning regions.

In order to construct a model of the protein, one needs to know the orientation of the protein in the lipid bilayer. Based on two assumptions -- (1) the NH₂ terminus of the PAS IV is located on the extracellular surface of the membrane and (2) the assignment of the membrane-spanning region based on secondary structure prediction studies is correct -- and from determination of an approximate number of N-linked carbohydrate moieties, it is possible to propose a partial model with the partitioning of the PAS IV polypeptide chain into the extracellular, internal, and cytoplasmic domains of the lipid bilayer (refer Fig. 17). This structural information will be helpful in extending the molecular architecture of PAS IV. For the model presented, further experimentation is needed in order to test the specific predictions of the model. The predictions can be tested by obtaining the complete sequence of PAS IV from overlapping sequences obtained by several proteases, localizing an amino and carboxy termini, determining the positions of the glycosylation sites, and establishing the membrane-spanning regions of the protein. One should also consider the aspect that PAS IV could be an oligomeric complex and the possible interactions among the subunits have not been considered in our interpretation. Once the sequence data become available and the validity of structural predictions based on amino acid sequence data is established, a detailed model of PAS IV can be made. Such a model should prove useful in determining relationships among

Model Structure Of Bovine PAS IV

FIGURE 17 : Model structure of bovine PAS IV : Model depicting possible partial partitioning of the polypeptide chain of PAS IV in the lipid bilayer. The potential asparagine-linked oligosaccharides are marked as CHO.

structure, function, and regulation of PAS IV. It surely will be of great assistance in studying the correlation between protein conformation and biological activity(ies) as well as an aid to crystallographers in interpreting X-ray data. Integral membrane proteins, as a rule, are insoluble in water and are difficult to crystallize.

Nevertheless, good single crystals of porin (141) and of a photosynthetic reaction center (142), suitable for three-dimensional X-ray crystallographic study, have been obtained by the use of special detergents (like octylglucosides) and amphiphiles (143, 144).

Information from the studies of the structure, topography and function of PAS IV will be useful in understanding the mechanisms involved in (1) its integration in the membrane bilayer, (2) its processing to its final conformation and orientation, and (3) its transfer to the final location on the surface of the cell. Specific structural and topographical information will also be useful for an understanding of the interactions of various domains of PAS IV with different compartments of the cell; for example, interactions of the hydrophobic domain with the lipid bilayer, interactions of the carbohydrate moieties with the external region, etc.

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