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Purification and characterization of human GP-80 from milk fat globule membrane

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San Jose State University, 1989

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PURIFICATION AND CHARACTERIZATION OF HUMAN GP-80
FROM MILK FAT GLOBULE MEMBRANE

A Thesis
Presented to
The Faculty of the Department of Chemistry
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science

By
On-Yee So
August, 1989

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF HUMAN GP-80 FROM MILK FAT GLOBULE MEMBRANE

by On-Yee So

A human membrane protein with an apparent molecular weight of 80 kD was purified from human milk fat globule membrane and partially characterized. The amino acid sequence of the first 29 residues in the N-terminus contains a peptide segment of 21 hydrophobic and neutral residues which may define a transmembrane segment. The hydrophobic nature of human GP-80 was revealed by its complete partitioning in the detergent phase of Triton X-114 solution, and by its large calculated hydrophobicity value. The interactions between the transmembrane sequence and the membrane interior was analyzed with the aid of the helical wheel and the hydrophobic moment plot. Functional type analysis of human GP-80 suggests that the protein is a multimeric transmembrane protein. Similarity between human GP-80 and the multifunctional human platelet membrane protein human GP IV is suggested by the highly homologous N-terminal amino acid sequences. The apparent homology of GP-80 and GP IV may be important in breast cancer cell metastasis.

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I) Introduction

The study of the chemistry of membrane proteins and their biological functions in living organisms has developed into an important aspect of protein chemistry. Most intercellular communications and many essential biochemical reactions involve membrane proteins. Proteins in living organisms, generally speaking, can be divided into three categories: structural proteins such as collagen, soluble proteins including soluble enzymes and hormones, and membrane proteins. The membrane proteins are as important as the other two kinds of proteins in participating in biological reactions of cells, and much effort has been made in studying them.

In spite of the effort, much less is known about membrane proteins than about the other two kinds of proteins because X-ray crystallography and other biophysical tools have not been successfully applied to membrane proteins. Structural models and functional studies of membrane proteins in certain species, however, have been developed (Eisenberg 1984). These models include the glucose transporter of the plasma membrane of erythrocytes (Mueckler et al. 1985), $\text{Na}^+\text{-K}^+\text{-ATPase}$ in mammalian kidney and the electric organ of eels (Cantley 1981), and chlorophyll a/b protein of the chloroplast thylakoid membrane involved in the transformation of light

into chemical forms in plants (Bennett 1979; Li 1985).

The best known classical model of a membrane protein is bacteriorhodopsin, a globular membrane protein located in the bacterium Halobacterium halobium living in the salt beds of San Francisco bay (Brestcher 1985). The structure of bacteriorhodopsin was determined in 1975 by Negel Unwin and Richard Henderson (Henderson 1975). Their model showed that the polypeptide chain of bacteriorhodopsin spanned the phospholipid bilayer seven times, and each embedded segment was an alpha helix. In the membrane, the helices of bacteriorhodopsin were packed together to form a globular structure (Brestcher 1985). It is believed that bacteriorhodopsin of Halobacterium holobium serves as a proton channel, which captures photons from sun light and exploits the photoenergy to pump protons across the membrane against a concentration gradient. It is the potential energy stored in the proton gradient which drives the synthesis of ATP in the bacteria cell. The break down of ATP then provides energy for the biosynthetic activities of the bacteria.

The MFGM system

In mammals, an important model for membrane proteins is the milk-fat-globule membrane (MFGM) system. MFGM is simply a piece of epithelial membrane of the mammary gland, enveloping a droplet of triglyceride, the milk fat. During the milk secretion process, milk-fat droplets are secreted

by budding from the apical surface of the mammary secretory gland, bringing along an area of the epithelial cell membrane, and finally, the milk fat droplets are completely enveloped by the membrane to become individual milk-fat-globules secreted along with the milk (Anderson & Cawston 1975). With peripheral proteins loosely bound to the extracellular surface and integral membrane proteins present within the phospholipid bilayer, the milk-fat-globule membrane system is considered as a suitable model for the study of membrane proteins.

By using the MFGM system as a model, investigations of membrane proteins have been conducted and have mostly concentrated on the purification and characterization of selected proteins. Xanthine oxidase, a membrane protein with a molecular weight about 155 kD, and the membrane glycoprotein butyrophilin with an apparant molecular weight about 66 kD have been purified from the milk fat globule membrane of bovine milk (Mather et al. 1980; Johnson & Mather 1985). Some other studies including the surface differentiation antigens of human mammary epithelial cells (Roberto et al. 1977), and the expression of selected MFGM proteins in different tissues have also been carried out (Greenwalt & Mather 1985).

The GP-78 membrane protein

Recently, a new membrane protein named bovine GP-78 was purified from bovine milk-fat-globule membrane. Bovine

GP-78 is a glycoprotein with an apparent molecular weight of 78 kD (Greenwalt & Mather 1985). Some characteristics of this protein have been revealed in previous work. Those characteristics include the unusual highly hydrophobic amino acid composition, the resistance to being washed off from the membrane by 0.1 M Na_2CO_3 , the resistance to protease digestion before being extracted from the membrane, and the susceptibility to detergent extraction.

Another important observation about bovine GP-78 is its uneven distribution in tissues. Immunolocalization results from previous studies showed that bovine GP-78 was detected in capillary endothelial cells of muscle, heart, and liver, in both epithelium and capillary endothelium of mammary gland as well as in the epithelium of bronchioles. This protein, however, was not found in capillary endothelial cells of lung, kidney and brain (Greenwalt et al. 1985). Given its unique nature, bovine GP-78 is considered as a tissue-specific marker protein of the endothelium system.

Endothelial cells and associated proteins

The endothelium is a single layer of flattened cells always found lining the inner surface of capillaries of organs. It plays important roles in regulating the transport and the exchange of molecules between cells and tissues. It is reported, for instance, that the transfer of iron from transferrin to hepatocytes is regulated by the

capillary endothelium of liver (Tavassoli et al. 1988). In the field of the cardiovascular study, attentions are now drawn to the exploration of the biochemistry of membrane proteins and their effects on the physiological functions of tissues. In addition to regulating the exchange of fluid and molecules between the blood and extravascular tissues, the vascular endothelium provides a laminal surface to which blood cells usually do not adhere. Most of the membrane proteins of the vascular endothelium remained uncharacterized. The study of one of the constituent proteins, bovine GP-78, of the vascular endothelium may be used as a model for the understanding of membrane proteins and their functions. Knowledge of endothelial membrane proteins may also lead to further understanding of the physiology of the cardiovascular system.

The GP-80 protein

Interestingly, in certain human tissues, a protein with approximately the same molecular weight as that of the bovine protein was located. This protein was named human GP-80 (Greenwalt & Mather 1985). Characteristics of human GP-80 very similar to that of bovine GP-78 were observed in previous preliminary work (Greenwalt & Mather 1985). Human GP-80 had not been purified from human MFGM and very little was known about it in terms of its amino acid composition, its structure, and its biological function in human cells.

As suggested by previous studies, there are possible similarities between human GP-80 and bovine GP-78. The comparison of these two proteins may contribute to the further understanding of the biochemistry of membrane proteins and their roles in endothelial biochemistry.

II) Materials and Methods

Materials

Tris base, tris-HCl, 4,4'-dicarboxy-2,2'-biquinoline (Bicinchoninic acid), Triton X-114, Triton X-100, epsilon-amino-N-caproic acid, phenylmethylsulphonyl fluoride (PMSF), nitroblue tetrazolium, butylated hydroxytoluene, barbital buffer, sodium dodecyl sulphate (SDS), Aprotinin, and glycine (free base anhydrous) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bis(N,N'-methylene-bis-acrylamide), affinity purified goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate, Coomassie brilliant blue R-250, and nitrocellulose membrane were purchased from Bio-Rad (Richmond, CA, U.S.A.). DEAE-Sephacrose CL-6B (45-165 μ m), and CM-Sephacrose CL-6B (45-165 μ m) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Methanol, sodium chloride, and N,N-dimethyl formamide were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Acrylamide was purchased from Spectrum Chemical MFG. Corp. (Gardena, CA, U.S.A.). Magnesium chloride was purchased from Spectrum Chemical MFG corp. (Redondo Beach, CA, U.S.A.). 5-bromo-4-

chloro-3-indolyl-phosphate p-toluidine salt was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.). Ponceau S was purchased from Helena Laboratories (Beaumont, TX, U.S.A.). EDTA was obtained from Curtis Matheson Scientific, Inc. (Houston, TX, U.S.A.). Centricon 30 micro-concentrators were purchased from Amicon Division, W.R. Grace & Co., (Danvers, MA, U.S.A.). Standard cellulose dialysis tubing with a molecular cut off at 12,000 D to 14,000 D was purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA, U.S.A.).

Methods

Source of milk and preparation of cream.

Fresh pooled human milk was collected from the Valley Medical Center milk bank in San Jose, California. The cream was separated from the milk by 2500 xg centrifugation at 4 °C for 10 minutes. The skim milk was discarded. The cream was then washed by resuspending it in five volumes of TBS (Tris buffer saline) at 30 °C, recentrifuged and stored in liquid nitrogen.

Preparation of milk fat globule membrane.

The washed cream was diluted with equal volume of 10 mM Tris buffer (pH 7.4 at 4 °C). The diluted cream was then warmed to 30 °C in a water bath and then cooled down to 4 °C. A Waring blender connected to a speed controller "Powerstat" made by The Superior Electric Company (Bristol,

CT, U.S.A.), with a setting of 25 was used to stir the cream until butter was formed. The bright yellow butter was resuspended in 30 °C Tris buffer to release the membrane fragments trapped in the butter. The butter milk and the butter wash were combined and centrifuged at 26,500 rpm (98,000 X g) at 4 °C for one hour with a SW 27 swinging bucket rotor. A brown membrane pellet with an overlaying thin layer of soft white material, the "white membrane," was obtained. The membrane pellet was homogenized in a glass homogenizer immersed in ice, diluted with a 10 mM citrate buffer, pH 7.0 containing 1 mM epsilon-amino-N-caproic acid, and recentrifuged for one hour. The washed membrane was then homogenized in TBS, pH 7.4, and a BCA protein assay was performed to determine the total protein content of the membrane (Smith et al. 1985).

Preparation of the 10.8% Triton X-114 solution

The detergent used in the MFGM extraction was an aqueous solution of 10.8% Triton X-114. Twenty ml of the 100% Triton X-114 was dissolved in 980 ml of TBS containing 16 mg of butylated hydroxytoluene, an anti-oxidant, at 4 °C by stirring. The solution was then warmed to 30 °C and maintained at that temperature for 5 minutes. The cloudy Triton X-114 solution separated into the aqueous phase and the detergent phase in a separatory funnel left in the dark overnight (Bordier 1981). The detergent phase was collected and washed by using the same procedure. The

percentage of the Triton X-114 solution was determined by comparing the U.V. absorption of the original Triton X-114 at 280 nm to the Triton X-solution obtained.

Extraction of human GP-80 from the membrane.

The homogenate of human brown and white membrane was diluted with TBS, pH 7.4 and adjusted to a final concentration of 1 mg protein per ml. The protein solution was stirred in an extraction solution containing 1 mM EDTA, 5 mM epsilon-amino-N-caproic acid, 0.5 mM PMSF, 1% aprotinin, and 0.15 M NaCl for 30 minutes at 4 °C. Triton X-114 solution was added to the protein solution to a final concentration of 1.0 % and the solution was stirred for one hour. The unsolubilized membrane was pelleted by centrifugation again at 98,000 xg at 4 °C for one hour. The volume of the supernatant was recorded and a BCA protein assay was performed on a small aliquot of the supernatant to determine the amount of protein solubilized from the membrane.

Phase partitioning.

The membrane extract was warmed to 30 °C in a 37 °C water bath. The solution was kept at 30 °C for 15 minutes with occasional stirring. The cloudy extract was centrifuged at 5000 xg in a fixed angle rotor pre-warmed to 30 °C. The aqueous phase was removed by aspiration and discarded. The volume of the detergent phase was recorded and the total protein content of the detergent phase was

calculated after a BCA protein assay.

DEAE ion exchange chromatography

The detergent phase was diluted five fold in 10 mM Tris buffer, pH 7.4, and dialyzed against 10 mM Tris buffer, pH 7.4 containing 0.1% Triton X-100 at 4 °C overnight. The dialyzed detergent phase was mixed with 10 ml of deaerated and equilibrated DEAE-Sepharose resin, and incubated in siliconized glass tubes at 4 °C on a shaker for half an hour. The mixture was then transferred to a siliconized column, and the unbound proteins were eluted from the column. After washing the column with three volumes of Tris buffer (10 mM, pH 7.4), human GP-80 was selectively eluted by the same Tris buffer containing 75 mM NaCl. Two to three milliliter fractions were collected. SDS reducing mini-gel PAGE was run on each fraction to determine which fractions contained human GP-80. BCA protein essays were performed on each fraction containing significant amounts of human GP-80.

Electroelution

Most of the time, one or more contaminants of lower molecular weight were eluted along with human GP-80. To obtain pure human GP-80, electroelution was necessary. The combined and concentrated GP-80-rich DEAE fractions were loaded onto a 1.5 mm thick, 10% acrylamide SDS reducing mini-gel with one well, and electrophoresis was performed. A thin strip was cut in the middle of the gel and stained

in Coomassie blue for a short time. The strip was then destained until the protein bands were clearly shown on the gel. The strip was washed with TBS to remove the methanol and acetic acid from the destain solution, then placed back into the gel. The human GP-80 band on the stained gel strip was used as a guide to cut the GP-80 band from the unstained original mini-gel. The unstained GP-80 gel slices were further cut into 1 cm pieces and stacked in the elution chamber of the Elutrap electroelution device made by Bio-Rad (Richmond, CA, U.S.A.). After eluting the protein at 200 volt at 4 °C for 8 hours, pure human GP-80 solution was trapped in a small chamber with a total volume of about 400 to 800 microliters (Jacobs & Clad 1986). A reducing SDS PAGE and a BCA protein assay were performed on a small aliquot of the recovered protein sample to determine the purity and the recovery of human GP-80, respectively.

Desitometry

PAGE gels containing molecular weight standards, crude membrane homogenate, and fractions collected after each purification step were stained by Coomassie Blue and dried on a piece of gel supporting film for the purpose of determining the recovery of human GP-80 after each purification step. The bands on each lane were scanned individually with a Beckman "Appraise" desitometer.

Production of polyclonal antibody

Polyclonal antibody was made to human GP-80 by Berkeley Antibody Company, Inc. (Berkeley, CA). A rabbit was immunized with pure human GP-80 protein (mixed with RIBI's adjuvant) in a dosage of 150 ug. The animal was boosted twice with 75 ug of human GP-80, and the immune serum was collected after each boost by bleeding the rabbit. Control serum was collected from the rabbit before it was immunized with pure human GP-80.

Western blotting

The activity of the polyclonal antibody was examined by Western blotting (Renart, J., & Sandoval, I. V., Methods in Enzymology (1984) 104 Part C 455-460). Two identical sets of protein samples were run electrophoretically in a 10% polyacrylamide SDS reducing mini-gel with pure human GP-80 next to the molecular weight standard. After electrophoresis, the polyacrylamide gel was soaked in deionized water for 15 minutes and then in transfer buffer (75 mM Tris buffer, 0.19 M glycine, 20% methanol, pH 8.3) for 15 minutes. Proteins on the gel were transferred to a piece of nitrocellulose paper pre-wetted in the transfer buffer at 0.1 Amp overnight. After the transfer, the gel was discarded and the nitrocellulose paper was stained by Ponceau S. Protein bands were marked on the nitrocellulose paper after rinsing off the background color. The nitrocellulose paper was cut in half with one set of proteins on each piece. Both pieces were incubated in a 2%

BSA/TBS solution to block all the nonspecific binding sites on the nitrocellulose paper. To the experimental set, the immune serum was added at a one to five hundred dilution in TBS containing 0.2% BSA and incubated for one hour. The control set was incubated in the rabbit control serum at the same dilution ratio for the same period of time. These two sets were then washed by 0.5% Triton X-100/TBS solution, 0.5 M NaCl/TBS and TBS for a period of 15 minutes for each wash. The secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate diluted at a one to one thousand ratio with TBS containing 0.2% of BSA, was applied to both the experimental and the control sets. The wash steps were repeated after the one hour incubation period. The substrate, which contained 0.1 ml 5-Bromo-4-chloro-3-indolyl phosphate in a concentration of 5 mg/ml in dimethyl formamide, 1 ml Nitro-Blue tetrazolium in a concentration of 1 mg/ml in barbital buffer, 20 μ l of 2 M Magnesium chloride, and 9 ml of barbital buffer at pH 7.0, was applied to both sets.

Amino acid sequencing of human GP-80

Purified human GP-80 (100 micrograms) was sent to Cetus Corp. in Emeryville, California for sequencing. The amino acid sequence of the first twenty-nine amino acids on the N-terminus was obtained through a sequencer based on the technique of Edman degradation (Edman & Begg 1967; Naill 1973)

Homology search of human GP-80

A homology search was performed on the amino acid sequence of the N-terminus of human GP-80 by using a Sun 4/110 computer and the software "Eugene[™]" (Baylor College of Medicine, Houston, TX). The available amino acid sequence of human GP-80 was compared to the known sequences of 9,138 proteins from the data bank. The score of homology was calculated based on the Dayhoff conservative subtraction system (Needleman & Wunsch 1970).

III) Results

A) Purification of human GP-80

1) Membrane preparation

The amount of 97.2 grams of cream collected from fresh human milk were used for membrane preparation (procedure was described in materials and methods). A brown membrane pellet with a layer of soft white material, the "white membrane," on top of it was obtained. After homogenizing the brown and the white membrane in 0.1 M citrate buffer (pH 7.4 at room temperature) to solubilize caseins, the major soluble milk proteins, a BCA assay was performed on the brown and the white membrane homogenate, separately. There were 189.42 mg of total protein detected in the brown membrane homogenate, and 42.63 mg of total protein detected in the white membrane homogenate (Table 1).

2) Triton X-114 extraction

The brown and the white membranes were homogenized separately and diluted in TBS. Triton X-114 extractions were performed on each as described in materials and methods. The total protein solubilized in the brown membrane Triton X-114 extract and the white membrane Triton X-114 extract were determined by BCA protein assay. In the protein assay, the same amount of Triton X-114 as in the

Table 1. Proteins recovered from each purification step

Protein recovery purification step	Total protein recovery (mg)	% total protein	Human mg	GP-80 %	% GP-80 recovery
MFGM homogenate	Brn 189.42	100.00	-	-	-
	Wht 42.63	100.00	-	-	-
Triton X-114 extraction	Brn 170.10	89.80	2.29	1.35	100.0
	Wht 34.33	80.50	0.72	2.1	100.0
Phase Partitioning	Brn.D 15.54	8.20	2.27	14.6	99.1
	Wht.D 4.89	11.47	0.69	14.1	95.8
DEAE ion exchange	7.07	3.04	1.61	22.8	53.4
Electroelution	0.78	0.81	0.78	100.	25.9

samples was added to the protein standards. There were 171.10 mg and 34.33 mg of solubilized protein from the brown and the white membrane, respectively. The respective yields of solubilized protein was 89.80% for brown membrane, and 80.50% for white membrane (Table 1).

3) Phase partitioning

The supernatants of the brown membrane Triton X-114 extract and the white membrane Triton X-114 extract phase partitioned in a 37 °C water bath, separately. The initial temperature of the protein solution was 4 °C. As the temperature rose to 23 °C, the protein solution suddenly turned turbid, indicating the formation of large micelles in the protein-detergent solution. To determine the efficiency of the phase partitioning, a BCA protein assay and a SDS-PAGE were performed on the detergent and aqueous phases obtained from the brown and the white membrane Triton X-114 extract. There were 15.54 mg and 4.98 mg of total protein partitioned in the detergent phase of the brown membrane and the white membrane Triton X-114 extract, respectively. The yield of protein recovered in the detergent phase was 8.20% for the brown membrane, and 11.47% for the white membrane (Table 1). Figure 1 shows a SDS reducing PAGE gel, in which human GP-80 as well as other hydrophobic proteins which partitioned into the detergent phase are shown on lane 3 and lane 5 for the brown and the white membrane, respectively. Hydrophilic

proteins which partitioned into the aqueous phase, including xanthine oxidase, lactoferrin and a significant amount of butyrophilin are shown on lane 4 for the brown membrane, and lane 6 for the white membrane (Figure 1).

Densitometry of the dried gels demonstrated that the percentage of human GP-80 in the detergent phase was 14.6% for the brown membrane (Figure 2a), and 14.1% for the white membrane (Figure 2b).

4) DEAE ion exchange chromatography

The detergent phases of the brown and the white membranes were combined and diluted five-fold with 10 mM Tris-HCl, pH 7.4. After the diluted detergent phase was dialyzed overnight against 10 mM Tris buffer containing 0.1% Triton X-100, it was mixed with 6 ml of DEAE-Sepharose and incubated for 30 minutes on a shaker at 4 °C. The bound human GP-80 was eluted from the DEAE resin with 10 mM Tris-HCl, pH 7.4, containing 75 mM NaCl (Fig 3). The total protein obtained from DEAE-sepharose anion exchange was 7.07 mg, as determined by BCA protein assay. Protein recovered though this step constituted 3.04 % of the total protein in the human MFGM crude homogenate, and human GP-80 was 22.8% of the total protein collected in the DEAE-Sepharose fractions according to densitometry measurements (Figure 4).

5) Electroelution

The combined fractions from the DEAE-Sepharose ion

Figure 1. Distribution of human milk fat globule membrane proteins after phase partitioning in Triton X-114.

- lane 1, molecular weight standards (kD);
- lane 2, human brown membrane Triton X-114 extract;
- lane 3, detergent phase of brown membrane Triton X-114 extract;
- lane 4, aqueous phase of brown membrane Triton X-114 extract;
- lane 5, detergent phase of white membrane Triton X-114 extract;
- lane 6, aqueous phase of white membrane Triton X-114 extract.

Figure 2a. Distribution of human GP-80 in the detergent phases of the brown membrane.

The darkened area represents GP-80 present in the brown membrane detergent phase. The GP-80 peak area is 14.6% of the total peak area.

Figure 2b. Distribution of human GP-80 in the detergent phase of the white membrane.

The darkened area represents the presence of GP-80 in the white membrane detergent phase. The GP-80 peak area is 14.1% of the total peak area.

Figure 3. Elution profile of human MFGM detergent phase proteins from DEAE-Sepharose ion exchange resin.

Lane 1, molecular weight standards (as in Figure 1);
lane 2, human MFGM homogenate;
lane 3, diluted detergent phase combination;
lane 4, first elution from the DEAE-Sepharose resin by 10 mM Tris containing 75 mM NaCl;
lane 5, second elution from the DEAE-Sepharose resin by 10 mM Tris containing 75 mM NaCl;
lane 6, the third elution from the DEAE-Sepharose resin by 75 mM NaCl.

The fourth elution did not contain significant amount of GP-80 and is not shown here.

Figure 4. Densitometry results of protein distribution in combined DEAE fractions.

The peak area of GP-80 is 22.8% of the total peak area.

exchange resin containing significant amounts of human GP-80 were concentrated to about 2 ml by using a "Centricon-30" micro-concentrator. Approximately 0.7 ml samples of the concentrated human GP-80 enriched fractions were run on SDS polyacrylamide gels, and human GP-80 was then electroeluted as described in materials and methods. The purity of the recovered protein sample was determined by electrophoresis on a reducing SDS polyacrylamide gel. Human GP-80 is the only protein band shown on the gel after Coomassie Blue staining (Figure 5). The total amount of protein obtained from the electroelution was 783 mg as determined by BCA protein assay.

B) Amino acid sequence of human GP-80

A sample containing 162 ug of pure human GP-80 was sent to Cetus Corporation (Emeryville, CA) for amino acid sequencing. The sequence of the first twenty-nine amino acids in the N-terminus was determined (Figure 6). The peptide segment from the eighth amino acid (Leu) to the twenty-eighth (Val) is rich in hydrophobic residues.

C) Homology search of the amino acid sequence of human GP-80

A homology search was carried out on the amino acid sequence of human GP-80 on a Sun 4/110TM micro computer by using the program "Eugene," developed in the Department of

Figure 5. The purity of human GP-80 after electroelution.

lane 1, molecular weight standards;

lane 2, human MFGM homogenate;

lane 3, detergent phase of human brown membrane;

lane 4, DEAE anion exchange eluent;

lane 5, human GP-80 after electroelution;

lane 6, bovine MFGM detergent phase.

Ser-X-Asp-X-Asn-Ser-Gly-Leu-Ile-Ala-Gly-Ala-Val-Ile-Gly-
Ala-Val-Leu-Ala-Val-Phe-Gly-Gly-Ile-Leu-Met-Pro-Val-Gly

Figure 6. The amino acid sequence of the first twenty-nine amino acid residues from the N-terminus of human GP-80.

The three letter codes are used as the abbreviations of the corresponding amino acids. X denotes an uncertain amino acid in the sequence.

Biology, Baylor college of medicine. In this program, a homology score was calculated using Dayhoff conservative subtraction system (Needleman & Wunsch 1970). The score has a range from zero to ten. The score of ten indicates the maximum similarity between the sample protein and a particular known protein in the data bank. A value of three or below suggests no significant similarities. The sequence of the first twenty-nine amino acids from the N-terminus of human GP-80 was compared with 9,138 known protein sequences in the data bank. The homology scores of all comparisons were below the value three, indicating that human GP-80 is a unique protein.

D) Hydrophobicity and hydrophobic moment analysis of the human GP-80 sequence

An algorithm developed by Eisenberg for quantitatively measuring the hydrophobicity and classifying a protein as either a surface protein or membrane protein was applied to the known sequence of human GP-80. Five peptide segments generated by running a window covering twenty-one residues along the sequence were examined (Eisenberg et al. 1984). The mean hydrophobicity, $\langle H \rangle$ was calculated for each segment in the series by taking the average of the hydrophobicity values of each type of amino acid in the segment. In this calculation, a normalized consensus amino acid hydrophobicity scale was used

(Eisenberg et al. 1982). Among the calculated $\langle H \rangle$ values of the series, the highest was 0.87, located in the segment containing amino acid number eight to amino acid number twenty eight. This segment was identified as a putative transmembrane segment (Table 2).

The transmembrane segment was further classified as a helix from either soluble, surface, or membrane protein by hydrophobic moment analysis. Hydrophobic moment $\langle U_h \rangle$ is a parameter to measure the amphiphilicity of the transmembrane helix. It is a numerical value obtained from calculations applying an equation developed by Eisenberg. In the calculation of the mean hydrophobic moment, a series of undecamers were generated by running a window covering eleven residues along the transmembrane sequence identified in the previous step. The equation used for the calculation of the U_H of each undecamer in the series was $U_H = \{ [\sum H_n \sin(\theta n)]^2 + [\sum H_n \cos(\theta n)]^2 \}^{1/2}$. U_H denotes the calculated hydrophobic moment of an undecamer, and $\langle U_H \rangle$ is the mean of U_H obtained by dividing U_H by eleven, the number of amino acids in the undecamer. Theta (θ) is the angle in radians at which successive amino acid residues emerge from the backbone when the sequence wound down along the axis of an alpha-helix. The value for theta was 100° according to Eisenberg (Eisenberg et al. 1984). In this calculation, it was assumed that the peptide segment was in an alpha-helix. H_n expresses the

Table 2. Calculations of Hydrophobicity and Hydrophobic Moment

No a.a	n Hs	Mean Hs W 21	sin θ	cos θ	Hn sin(en)	Hn cos(en)	Undecamer Series	Mean Hs W 11	Sum Hn sin(en)	Sum Hn cos(en)	U _h	<U _h >
1 ser	-0.18		0.9848	-0.1736	-0.1773	0.0313						
2 xxx	0.00				0.0000	0.0000						
3 asp	-0.90		-0.8660	0.5000	0.7794	-0.4500						
4 xxx	0.00				0.0000	0.0000						
5 asn	-0.78		0.6428	-0.7660	-0.5014	0.5975						
6 ser	-0.18		-0.8660	-0.5000	0.1559	0.0900						
7 gly	0.48		-0.3420	0.9397	-0.1642	0.4511						
8 leu	1.10		0.9848	0.1736	1.0833	0.1910						
9 ile	1.40		-0.0000	-1.0000	-0.0000	-1.4000						
10 ala	0.62		-0.9848	0.1736	-0.6106	0.1077						
11 gly	0.48		0.3420	0.9397	0.1642	0.4511						
12 ala	0.62		0.8660	-0.5000	0.5369	-0.3100						
13 val	1.10		-0.6428	-0.7660	-0.7071	-0.8426						
14 ile	1.40		-0.6428	0.7660	-0.8999	1.0725						
15 gly	0.48		0.8660	0.5000	0.4157	0.2400	5-15	0.6109	-0.5271	0.6481	0.8354	0.0759
16 ala	0.62		0.3420	-0.9397	0.2121	-0.5826	6-16	0.7382	0.1863	-0.5320	0.5637	0.0512
17 val	1.10		-0.9848	-0.1736	-1.0833	-0.1910	7-17	0.8545	-1.0529	-0.8130	1.3302	0.1209
18 leu	1.10		0.0000	1.0000	0.0000	1.1000	8-18	0.9109	-0.8887	-0.1641	0.9037	0.0822
19 ala	0.62		0.9848	-0.1736	0.6106	-0.1077	9-19	0.8673	-1.3614	-0.4628	1.4379	0.1307
20 val	1.10		-0.3420	-0.9397	-0.3762	-1.0337	10-20	0.8400	-1.7376	-0.0964	1.7403	0.1582
21 phe	1.20		-0.8660	0.5000	-1.0392	0.6000	11-21	0.8927	-2.1663	0.3959	2.2022	0.2002
22 gly	0.48		0.6428	0.7660	0.3085	0.3677	12-22	0.8927	-2.0219	0.3126	2.0459	0.1860
23 gly	0.48		0.6428	-0.7660	0.3085	-0.3677	13-23	0.8800	-2.2503	0.2549	2.2647	0.2059
24 ile	1.40		-0.8660	-0.5000	-1.2124	-0.7000	14-24	0.9073	-2.7557	0.3975	2.7842	0.2531
25 leu	1.10	0.76	-0.3420	0.9397	-0.3762	1.0337	15-25	0.8800	-2.2320	0.3587	2.2606	0.2055
26 met	0.64	0.83	0.9848	0.1736	0.6303	0.1111	16-26	0.8945	-2.0174	0.2299	2.0305	0.1846
27 pro	0.12	0.84	-0.0000	-1.0000	-0.0000	-0.1200	17-27	0.8491	-2.2295	0.6925	2.3345	0.2122
28 val	1.10	0.87	-0.9848	0.1736	-1.0833	0.1910	18-28	0.8491	-2.2295	1.0745	2.4749	0.2250
29 gly	0.48	0.84	0.3420	0.9397	0.1642	0.4511	19-29	0.7927	-2.0653	0.4255	2.1087	0.1917

assigned hydrophobicity value of the amino acids in the undecamer. The highest mean hydrophobic moment $\langle U_H \rangle$, calculated from the undecamer series was 0.253, corresponding to the segment covered from residue number 14 to residue number 24 (Table 2).

E) Immunochemical characterization

1) Production of anti-human GP-80 polyclonal antibody

Polyclonal antibodies were made to human GP-80 by BAbCO, a biotechnology company in Berkeley, California. Three hundred and seventy-five ug of pure human GP-80 was used in the production of antibodies. Three batches of rabbit-anti-serum were obtained by bleeding the rabbit after immunizing the animal with 150 micrograms GP-80 and three boosts of 75 ug of the protein. A Western blot was carried out to verify the presence of anti-human GP-80 antibody in the collected rabbit serum. One ug of pure human GP-80 was electrophoretically run on a reducing SDS PAGE mini-gel. Then the protein was transferred to a piece of nitro cellulose paper for the blotting. As shown in Figure 7, human GP-80 was visualized as a dark band on the nitrocellulose paper after a series of incubations with the applied human-GP-80 antibody, the enzyme-conjugated secondary antibody, and the substrate of the enzyme (Figure 7). Control serum was applied on the control set. There was no detectable band on the control blot. The positive

Figure 7. Examination of the activity of anti-human GP-80 antibody by Western blotting.

Two 1.0 ug aliquots of purified human GP-80 were used in the 8% SDS PAGE under reducing conditions. The protein was transferred onto a nitrocellulose paper and Western blotting was carried out.

- a) Rabbit immune serum was applied as the primary antibody at 1:500 dilution.
- b) Control: Rabbit control serum was applied at 1:500 dilution.

result on the experimental set and the negative result on the control set indicate the presence of anti-human-GP-80 antibodies in the rabbit serum.

2) The cross-reactivity of anti-human GP-80 antibody with bovine GP-78 was examined. One microgram of pure human GP-80 and one microgram of pure bovine GP-78 were loaded onto adjacent lanes of an SDS-PAGE gel. The proteins were transferred to a piece of nitrocellulose paper after electrophoresis. Anti-human-GP-80 polyclonal antibody was applied to the nitrocellulose paper on which both human GP-80 and the bovine GP-78 were transferred. Two black bands were observed on the nitrocellulose paper after applying the anti-human GP-80 antibody, the secondary antibody and the substrate as described previously. The band of human GP-80 was darker than the band of bovine GP-78, indicating that anti-human GP-80 antibody has a higher affinity for the human GP-80 than the bovine GP-78. Also as shown on Figure 8, the band of Human GP-80 is higher than the band of bovine GP-78, indicating that the apparent molecular weight of human GP-80 is higher than that of bovine GP-78 (Figure 9).

Figure 8. Cross reactivity of anti-human GP-80 antibody with bovine GP-78 protein.

a) lane 1, 0.5 ug of bovine GP-78 protein was run on a 8% SDS polyacrylamide gel under reducing condition. The protein was transferred onto a nitrocellulose paper, and the Western blotting was carried out by using anti-human GP-80;

lane 2, 2.0 ug protein of human brown membrane detergent phase was used in the Western blotting under the same conditions as described in lane one. It was estimated that about 1/4 of the total protein in the detergent phase was human GP-80. The amount of human GP-80 present in lane 2 was about 0.5 ug.

b) control

Figure 9. Determination of molecular weight of human GP-80.

IV) Discussion

A) Purification of human GP-80

Previous studies have shown that a particular protein of approximately the same molecular weight as bovine GP-78 was present in human MFGM. In addition, it has also been shown to react with an antibody to bovine GP-78 (D. E. Greenwalt & I. H. Mather 1985). Based on these observations, a purification protocol for human GP-80 was developed from that of bovine GP-78 with modifications. The purification of human GP-80 involved a five-step procedure including milk-fat-globule membrane (MFGM) preparation from human cream, solubilization of the membrane proteins with Triton X-114, a non-ionic amphiphile, phase partitioning, DEAE anion exchange chromatography, and electroelution. Since preliminary studies suggest that human GP-80 is an integral membrane protein which constitutes less than 2% of the total membrane protein, the extraction of human GP-80 from the membrane and its recovery after subsequent purification steps are crucial for a successful purification of the protein. Triton X-114 extraction and phase partitioning serve the purpose of solubilizing hydrophobic membrane proteins, separating them from hydrophilic proteins, as well as concentrating them in the detergent phase.

During the extraction of membrane proteins, the non-ionic detergent Triton X-114 molecules replace most of the lipid molecules which are in contact with the hydrophobic domains of the integral membrane proteins, and dissolve the proteins by forming soluble protein-detergent mixed micelles.

Proteins partition between the hydrophobic interior of micelles and the aqueous environment outside of the micelles according to their hydrophobicity. Hydrophobic proteins including human GP-80 preferentially partition within the micelles, while hydrophilic proteins remain in the aqueous environment. The protein-detergent micelles grow in size, and associate into large aggregates as the micelle solution reaches its "cloud point" during the incubation period. The cloud point is defined as the temperature at which a micelle solution becomes cloudy. The cloud point varies in different systems depending on the nature of the protein as well as the degree of hydrophobicity of the detergent (Bordier 1981).

The key points of phase partitioning are the formation of detergent-protein micelles, and the association of the micelles into large aggregates at the cloud point. The aggregation of micelles leads to the microscopic phase separation in the solution so that human GP-80 (and other hydrophobic proteins) separates from

hydrophilic proteins, and becomes enriched in the detergent phase.

To effectively extract and recover human GP-80 from the crude membrane homogenate, the choice of detergent is very important. Triton X-114 is a suitable reagent for the extraction and the phase condensation. The Triton X series is a group of non-ionic detergents consisting of both hydrophilic oxyethylene units and a hydrophobic octylphenyl residue. The number of the hydrophilic groups significantly affects the critical micelle formation temperature (cloud point) of the detergent-protein micelle solution. For example, the cloud point of Triton X-100, which has 10 hydrophilic oxyethylene units, is about 60 °C (Bordier 1981; Corti 1985), while the cloud point of Triton X-114, which has seven to eight oxyethylene units, is about 20 °C (Bordier 1981; Goldfarb & Sepulved 1969). The advantage of using Triton X-114 in the purification of human GP-80 is its relatively low cloud point, which allows the formation of large protein-detergent aggregates and the phase partitioning of proteins at a low temperature to minimize the possibilities of irreversible protein denaturation and enzymatic degradation.

One indication of an effective phase partitioning is the absence of human GP-80 from the aqueous phase (Figure 1), and the presence of human GP-80 as one of the major proteins in the Triton X-114 detergent phase. As shown in

Figure 1, human GP-80 is barely detectable in the SDS PAGE gel of the crude membrane homogenate. The band of human GP-80 is clearly seen on the Coomassie Blue stained gel of the detergent phases of both human brown and white membrane, and is about 14% of the total protein determined by densitometry (Figure 2).

Another indication of the effectiveness of phase partitioning is the absence of lactoferrin, a hydrophilic protein with an apparent molecular weight about 85 kD in human MFGM, from the detergent phase. As shown in Figure 1, the band of lactoferrin is invisible in the Coomassie Blue stained SDS PAGE gel of the detergent phases of both human brown and white membrane. Lactoferrin, however, shows as a strong band on the Coomassie SDS PAGE gel of the aqueous phase of human MFGM (Figure 1). It is important to separate lactoferrin from human GP-80 in the phase partitioning, because lactoferrin is difficult to remove in the subsequent purification steps due to the similarity of its size as well as its net charge with that of human GP-80.

A faint doublet is seen below the band of human GP-80 on both lane 2 and lane 4 of Figure 1 (the detergent phase of human brown and white membrane, respectively), indicating the presence of butyrophilin, a membrane protein with an apparent molecular weight about 68 kD (Johnson et

al. 1985). It is expected that about 75% of the butyrophilin would partition into the aqueous phase, and about 25% of it would remain in the detergent phase (Johnson et al. 1985). Because of the difference in the isoelectric point between human GP-80 and butyrophilin, butyrophilin can be removed easily by ion-exchange chromatography.

B) Hydrophobicity and hydrophobic moment analysis and functional type characterization of human GP-80

The hydrophobic nature of human HP-80 was confirmed by the experimental result that the protein partitioned exclusively in the detergent phase (Figure 1). The hypothesis that human GP-80 was a transmembrane protein was raised and examined by a hydrophobicity analysis of the known amino acid sequence (Eisenberg et al. 1984). The possible conformation of human GP-80 was predicted based on the hydrophobicity data, the hydrophobic moment analysis, and the hydrophobic moment plot.

In the analysis, it was assumed that the peptide segment of human GP-80 was in an alpha-helix structure. This assumption was based on the fact that the fully hydrogen-bonded backbone of the alpha-helix was more likely to seek the apolar part of the membrane than a non-hydrogen bonded conformation (Eisenberg et al. 1985). The most important steps in the analysis are locating the candidates

of transmembrane helices, establishing their status, and the characterization of membrane-associated segments.

In the first step, a moving window with the length of 21 residues is run along the entire sequence of the protein to generate a series of peptide segments. The rationale for the moving window covering 21 residues is that the length of an alpha-helix formed from coiling 21 residues is approximately the thickness of the apolar portion of the lipid bilayer.

The second step is to establish the status of the potential transmembrane segments. The mean hydrophobicity of each peptide is calculated by taking the summation of the numerical hydrophobicity values of the amino acids in the peptide. These hydrophobicity values are from the normalized consensus scale of Eisenberg et al. (Eisenberg et al. 1982b), and determined from comparing the Gibb's free energy required for transfer of the amino acid from an aqueous environment to a hydrophobic environment. The calculated hydrophobicity value is the reflection of the affinity of the peptide for the hydrophobic interior of the membrane, which helps to identify the transmembrane sequence of a protein. Any segment with calculated mean hydrophobicity higher than 0.68 or any two segments whose sum of the calculated hydrophobicity is equal to or greater than 1.10 are considered as potential transmembrane helices, because a relatively high hydrophobicity is an

important criteria for helices penetrating the membrane, and initiating the cooperative binding with neighboring helices, if there are any, in the membrane.

In the final step, the putative membrane spanning segment is further characterized as either the helix of a monomeric transmembrane protein, or a helix of a multimeric transmembrane protein. This is performed with the aid of a hydrophobic moment analysis and hydrophobic moment plot.

The hydrophobic moment is a numerical value calculated from a undecamer generated from running a moving window along the entire sequence of an identified transmembrane helix. The magnitude of the hydrophobic moment represents the amphiphilicity of the transmembrane segment. The larger the hydrophobic moment, the greater the amphiphilicity of the membrane spanning helix. The amphiphilicity of a membrane spanning helix results from an amino acid residue's arrangement such that the hydrophilic and hydrophobic residues are clustered on opposite sides of the undecamer helix. The hydrophilic side of the helix is considered disfavored in making contact with the hydrophobic membrane interior, but likely to have interactions with another local hydrophilic environment, probably the hydrophilic side of another transmembrane helix.

The rationale of using a undecamer in the hydrophobic moment analysis is that a three-turn helix coiled from

eleven amino acid residues is the approximate distance over which neighboring helices, if there are any, generally would interact (Eisenberg et al. 1984).

In the determination of the transmembrane segment(s) of human GP-80, the local maximum hydrophobicity, which is 0.87, is located in the sequence segment covering from residue number eight (Leu) to residue number twenty eight (Val). This segment is identified as the transmembrane sequence because of its largest hydrophobicity in the candidate series (Eisenberg et al. 1984) (Table 2).

In the hydrophobic moment analysis of human GP-80, the undecamer which covers from the fourteenth amino acid (Ile) to the twenty-fourth amino acid (Ile) shows the highest mean hydrophobic moment value $\langle U_H \rangle$, which is 0.253. As suggested by Eisenberg's previous studies on other proteins, the high hydrophobic moment indicates the amphiphilic nature of the helix, and possible interactions between this helix and its local hydrophilic environment, probably other neighboring amphiphilic helices via ionic or other forces.

The interactions between the transmembrane helix of human GP-80 and its environment is further analyzed by an algorithm developed by Argos et al. (Argos et al. 1982). A helical wheel is constructed with the known amino acid sequence of human GP-80 (Figure 10). Based on the arrangement of amino acids on the wheel and the physical

characteristics of the twenty amino acids, the possible interactions between the transmembrane helix and its environment is predicted. As shown in Figure 10, most of the hydrophobic amino acid residues of the known sequence of human GP-80 cluster on one side of the wheel, while most of the less hydrophobic residues including all five glycines and three of the four alanines are on the other side of the wheel. As glycine and alanine are the smallest amino acids, one side of the helix may appear as a hole, which is able to couple with bulky amino acids on the neighboring helix. The amino acid arrangement on the helical wheel shows that the transmembrane alpha-helix of human GP-80 may interact with the membrane interior on its hydrophobic side, and aligns with an adjacent membrane spanning helix, interacting via a "hole and knob" relationship.

As indicated by both hydrophobic moment and helical wheel analysis, the identified transmembrane helix of human GP-80 shows a strong possibility of having interactions with a local hydrophilic environment in the hydrophobic interior of the membrane. This possible hydrophilic site in human GP-80 is most likely due to the presence of other amphiphilic membrane spanning helices in human GP-80, such that the amphiphilic helices can bury their hydrophilicity by interacting with each other on their hydrophilic side. The possibility of human GP-80 having multiple

Figure 10. Helical wheel of human GP-80

transmembrane helices was further evaluated with the aid of a hydrophobic moment plot.

In the hydrophobic moment plot, the highest numerical value of the hydrophobic moment calculated from a undecamer in the series is plotted as a function of its corresponding calculated hydrophobicity. The coordinates of the undecamer in the hydrophobic moment plot determine whether the evaluated membrane spanning segment is a helix of a soluble protein, or the helix of a membrane protein with a monomeric transmembrane sequence, or a helix of a protein with multimeric membrane spanning regions. According to Eisenberg's previous study on the functional type characterization on other proteins, the helix of a monomeric transmembrane protein usually lies in the region of highest hydrophobicity and lowest hydrophobic moment in the plot. Mouse histocompatibility antigens class I H-2d No 2, for example, have a hydrophobicity as high as 0.94 and the hydrophobic moment as low as 0.13, suggesting the minimum sidedness of the transmembrane helix and the maximum hydrophobic interaction between the helix and the interior of the lipid bilayer in all directions (Kvist et al. 1981)

Helices presumed to be paired, such as the transmembrane M segment of surface immunoglobulins, or helices bundled together in the membrane, such as bacteriorhodopsin, are found to have higher hydrophobic

moment and smaller hydrophobicity values within the ranges of 0.59 to 0.62, and 0.21 to 0.25, respectively (Khorana et al. 1979). The relatively low mean hydrophobicity value indicates the presence of a considerable amount of hydrophilic amino acid residues in the transmembrane segment, while the high hydrophobic moment suggests the amphiphilicity of the alpha-helix, probably due to the arrangement of the polar and non-polar amino acid residues on opposite sides of the alpha-helix.

The coordinates of the undecamer selected from the identified transmembrane segment of human GP-80 fall into the region characteristic of a membrane spanning helix of a protein with several transmembrane helices (Figure 11), suggesting that there are more membrane spanning helices in human GP-80 interacting with each other. It has been reported that an ion channel of acetylcholine receptor consists of amphiphilic helices (Finer-Moore et al. 1984). Taking into consideration the amphiphilic nature of the human GP-80 transmembrane helix, and the multimeric transmembrane characteristic revealed by its coordinates on the hydrophobic moment plot, it is possible that the membrane spanning helices of human GP-80 are paired or bundled together to form a channel with a central hydrophilic pore (Agros et al. 1982), facilitating the transfer of ions and other charged molecules through the hydrophobic lipid bilayer of the membrane.

Figure 11. Functional type analysis of human GP-80 by hydrophobic moment plot

+ represents the coordinates of the undecamer covering from Ile¹⁴ to Ile²⁴

C) Comparison of human GP-80 and bovine GP-78

The high similarity between human GP-80 and bovine GP-78 is suggested by their highly homologous N-terminal amino acid sequences, and the cross-reactivity of their antibodies to each other (Figure 8a). Some evidence shows, however, that human GP-80 and bovine GP-78 may not be identical proteins. As indicated by their names, human GP-80 has an apparent molecular weight of 80 kD, while the apparent molecular weight of the bovine protein is about 78 kD. When these two proteins are run on adjacent lanes of a 12% reducing SDS polyacrylamide gel, the band of human GP-80 is above the band of bovine GP-78 (Figure 5). There are two possible explanations for this observation.

One of the possibilities is that the human GP-80 protein is actually larger than bovine GP-78 by 2 kD, which is equivalent to about 17 amino acid residues (the average molecular weight of an amino acid is about 120). Since the amino acid sequence of the first twenty-eight residues of human GP-80 and that of bovine GP-78 are highly homologous, and are even identical from residue number seven to residue number twenty-seven, the possibility of human GP-80 having extra amino acids on the N-terminus is ruled out. It is possible, however, that human GP-80 carries the extra seventeen amino acid residues on the C-terminus or somewhere else in the peptide chain. To examine these

possibilities, the most promising approach is to obtain and compare the entire sequences of these two proteins.

Another explanation for human GP-80 showing a larger apparent molecular weight than bovine GP-78 is their different glycosylation patterns. During glycosylation, a post-translation modification process of protein synthesis, certain kinds of carbohydrate units are attached to the protein at specific sites recognized by glycosylation enzymes. Mannose, glucose, galactose, acetylglucosamine, acetylgalactosamine and sialic acid are the most common carbohydrate units found on membrane proteins (Marshall, 1972).

The binding of the carbohydrate units to the protein is via covalent linkages which include the O-linked attachment of galactose to the hydroxyl groups of serine or threonine and the N-linked attachment of glucose and/or its derivative acetylglucosamine to the amide group on the side chain of asparagine in the specific sequence Asn-X-Ser or Asn-X-Thr (X denotes any amino acids except proline) (Kronquist & Lennarz 1978; Geetha-Habib et al. 1988; Welpy et al. 1983).

It is reported that carbohydrate units found on bovine GP-78 are mostly neutral monosaccharides and their derivatives, including mannose, galactose, glucose, and N-acetylglucosamine, as well as a significant amount (20% by mole) of negatively charged sialic acids (Greenwalt &

Mather 1985). Even though the carbohydrate content of human GP-80 has not been examined, it is possible that the glycosylation pattern of human GP-80 is different from that of bovine GP-78. It is reported that the oligosaccharide patterns of prothrombin, α_1 -acid glycoprotein and cold, insoluble globulin of cattle and human origin showed remarkable species differences in the carbohydrate moieties of these glycoproteins (Mizuochi et al. 1979; Mizuochi et al. 1981; Yoshima et al. 1981; Takasaki et al. 1979; Takasaki et al. 1980).

The type and the quantity of sugar moieties on the protein may affect the migration profile in SDS PAGE (Leach 1980; Tanaka 1981). In SDS PAGE of non-glycosylated proteins, SDS molecules bind to proteins proportionally to the surface area of the protein via hydrophobic interactions between the hydrophobic groups of proteins and the hydrocarbon chains of SDS, forming protein-SDS complexes. With the negatively charged sulfate groups in contact with the aqueous environment, the net charge of the SDS-protein complexes is negative, and the difference of charge density on the complexes is leveled off regardless of the charge difference of the original proteins. The migration of the SDS-protein complexes in SDS-PAGE is therefore strictly determined by their sizes.

With glycosylation, the polar hydroxyl groups of sugar units and the negatively charged sialic acids on the

protein surface disfavor the binding of SDS onto the protein. The more the sugar units, the fewer the SDS molecules bound to the protein. Because of the possible difference in the glycosylation pattern of proteins, the amount of SDS bound to the protein surface is no longer proportional to the surface area of the protein, and differences in surface charge density of the glycoproteins are not leveled off. The migration profile of the SDS-protein molecules is no longer strictly size dependent, but determined by both charge and size of the SDS-glycoprotein units. Therefore, if the glycosylation pattern of human GP-80 is different from that of bovine GP-78 (eg. human GP-80 is more glycosylated than bovine GP-78), human GP-80 could appear larger than bovine GP-78 even though their actual molecular weights are identical.

The possible difference in glycosylation pattern between human GP-80 and bovine GP-78 could be due to the number of available glycosylation sites resulting from species-specific differences and/or point mutations during evolution. Substitution of asparagine with another basic amino acid, such as lysine, in the sequence of Asn-X-Thr and/or Asn-X-Ser in the N-linked glycosylation site may affect the amount of sugar units linked to the proteins. It is reported that the extent of protein glycosylation is determined by several factors including the adequate accessibility of the glycosylation sites to the

glycosylation enzymes, the availability of the lipid-linked oligosaccharide donor, the rate of the peptide chain folding during the protein synthesis, as well as the activity of the glycosylation enzymes (Pless & Lennarz 1977; Carson et al. 1981). As discussed previously, the quantity of polar and/or charge groups on carbohydrate units of the glycoproteins could be responsible for proteins showing abnormal migration profiles in SDS-PAGE. To determine the actual size of human GP-80 and bovine GP-78, carbohydrate-free protein samples treated with enzymes which specifically cleave off all sugar units on glycoproteins, such as the enzyme endo-beta-N-acetylglucosaminidase, could be examined in SDS PAGE. Again, the comparison of the entire amino acid sequence of these two proteins, or DNA sequencing could be very helpful in revealing the true identity of these two proteins.

D) Comparison of protein GP-80 purified from human milk fat globule membrane and protein GP IV purified from human platelet membrane.

Purification, characterization and functional studies of a platelet membrane glycoprotein GP IV has been reported recently (McGregor et al. 1989; Tandon et al. 1989). Some evidence suggests that human platelet membrane protein GP IV and human MFGM protein GP-80 could be closely related

proteins. First of all, both human GP-80 and human platelet GP IV are resistant to proteolysis before being extracted from the membrane, indicating the transmembranous and hydrophobic nature of these two proteins (Greenwalt & Mather 1985; Tandon et al. 1989). Secondly, the N-terminal amino acid sequence of human MFGM GP-80 and human platelet GP IV is highly homologous in the first 28 amino acids, except for a couple of uncertain residues in the hydrophilic hexapeptide terminal sequence (Tandon et al. 1989). These two proteins even have identical amino acid sequences in the segment from amino acid number 7 to amino acid number 28. These residues consist exclusively of neutral and hydrophobic amino acids, and are considered as a transmembrane domain (Tandon et al. 1989; Figure 6).

Another possible similarity between human GP-80 and human platelet GP IV is their conformation in the membrane. The multi-transmembrane alpha-helix configuration of human GP-80 is predicted by hydrophobic moment plot and the helical wheel analysis (Argos et al 1982). Likewise, the possibility of human platelet GP IV having multiple transmembrane loops between the periplasmic and the cytoplasmic space is suggested by Jamieson et al (Tandon et al. 1989). They reported that human platelet GP IV can be iodinated in intact platelets, indicating the presence of tyrosine in the periplasmic domain of the protein. It is also known that the N-terminal domain of the protein lacks

tyrosine (Jamieson et al. 1989). Based on this data, Jamieson argued that the human platelet GP IV must be a protein with more than one transmembrane domain (Tandon et al. 1989). However, Jamieson's argument is valid only if the N-terminal domain of human platelet GP IV is in the periplasmic space. Since the location of the N-terminus has not been reported, it is possible that human platelet GP IV is a monomeric transmembrane protein with its N-terminus buried in the membrane, and the iodination occurs at a periplasmic domain of the protein. A better argument that human GP IV is a multimeric transmembrane protein can be made based on its large hydrophobicity. If the protein has only one membrane spanning region, a major portion of this protein should be hydrophilic because it has to be exposed to the periplasmic and cytoplasmic environment. As reported by McGregor et al., human platelet GP IV completely partitions in the detergent phase of Triton X-114 (McGregor et al. 1989), indicating that the protein is highly hydrophobic and a major portion of this protein should be in a hydrophobic environment, ie, embedded in the membrane. Since one membrane spanning region is only 21-23 amino acid residues, the protein is likely to have many membrane spanning regions.

On the other hand, there are differences between human GP-80 and human platelet GP IV. One of the observations is the different apparent molecular weight

between these two proteins. The molecular weight of human platelet GP IV determined by SDS-PAGE is about 88 kD, which is 8 kD larger than that of human MFGM GP-80. As discussed previously, the difference in apparent molecular weight between these two proteins could be again due to the substitution of amino acid residue(s), and/or the difference in carbohydrate structure resulting from tissue specific biosynthetic pathways (Furukawa et al. 1989). It has been reported that some glycoproteins show organ-specific difference in carbohydrate structure and content. gamma-glutamyltranspeptidase, an enzyme purified from neonatal mouse liver and adult mouse kidney have different glycosylated carbohydrate chains (Yamashita et al. 1983).

The biological function of human platelet GP IV has been studied. It is reported recently that human platelet GP IV is a multi-functional membrane protein which specifically binds thrombospondin, which is an adhesive protein that plays an essential role in platelet aggregation during the blood clotting process (Asch et al. 1987), adheres platelets to collagen (Tandon et al. 1985a), attaches and spreads human melanoma cells (Roberts et al. 1987).

The biological function of human GP-80 remains to be clarified. Considering that human GP-80 and human platelet GP IV are located in different tissues, their carbohydrate structure and content may differ from each other due to

tissue-specific biosynthetic pathways (Yamashita et al. 1983). It is believed that the carbohydrate structure plays important roles in the regulation of biological functions of glycoproteins (Furukawa et al. 1989). Because of the tissue-specific difference, human GP-80 purified from milk fat globule membrane may have a unique biological function such as an ion transport channel, and/or mediating the cell-to-cell, cell-to-matrix interactions in the tissue. Human GP-80 may or may not be related to platelet GP IV.

V) Conclusion

Human GP 80 is a membrane glycoprotein with an apparent molecular weight about 80 kD purified from human milk-fat-globule membrane. This protein exhibits an extremely hydrophobic nature by completely partitioning in the detergent phase of a non-ionic detergent, Triton X-114. The amino acid sequence of the first twenty nine amino acids in the N-terminus consists of a short hydrophilic hexapeptide sequence followed by a hydrophobic domain from gly⁷ to gly²⁹. The sequence from Leu⁸ to Val²⁸ is considered to be a transmembrane helix because of its high hydrophobicity and its relatively large calculated hydrophobic moment, meaning that the arrangement of amino acid residues on the helix is such that the hydrophilic and hydrophobic amino acid residues are on opposite sides. The amphiphilicity of the transmembrane helix implies possible interactions between this helix and a local hydrophilic site, which is hypothesized to be another amphiphilic membrane spanning sequence of human GP-80 in the membrane interior.

The amino acid arrangement and the type of possible interactions between this helix and its environment were further analyzed from the helical wheel developed by Argos et al. (Argos et al. 1982). The side view of the helix demonstrates a characteristic pattern of

the distribution of amino acids glycine and alanine, the residues with the smallest size, on the transmembrane helix. The clustering of the small sized amino acids on one side of the helix suggests that the helix may be aligned with a second adjacent membrane helix, and they interact via a "hole-and-knob" relationship.

The hypothesis of human GP-80 as a membrane protein with multiple membrane spanning sequences is supported by the functional type characterization from Eisenberg's hydrophobic moment plot. The high coordinates of the hydrophobic moment and the corresponding hydrophobicity of the examined undecamer peptide fall into the region characteristic of multimeric transmembrane protein.

Human GP-80 is a protein very similar to Bovine GP-78 and human platelet GP IV based on the highly homologous N-terminal amino acid sequences. The difference in apparent molecular weights between these three proteins could be due to the substitution of amino acid residue(s) at potential glycosylation sites, and/or the variation of carbohydrate structure of these proteins synthesized by different species and/or by different cell types (Furukawa et al. 1989).

The biological function of human GP-80 remains to be clarified. Even though human GP-80, bovine GP-78 and human platelet GP IV may share some common features and physical

properties, they are, however, expressed in different tissues and/or different species. Therefore, it is possible that besides their observed similarities, these proteins may have their unique biological functions in their host tissues.

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