Characterization of an Apically Derived Epithelial Membrane Glycoprotein from Bovine Milk, Which Is Expressed in Capillary Endothelia in Diverse Tissues

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ABSTRACT A glycoprotein (PAS IV) of apparent M_r 76,000 was purified from bovine milk-fat-globule membrane and partially characterized. PAS IV contained mannose, galactose, and sialic acid as principal sugars (~5.3% total carbohydrate [wt/wt]) and existed in milk in at least four isoelectric variants. The glycoprotein appeared to be an integral membrane protein by several criteria. PAS IV was recovered in the detergent phase of Triton X-114 extracts of milk-fat-globule membrane at room temperature. When bound to membrane, PAS IV was resistant to digestion by a number of proteinases, although after solubilization with non-ionic detergents, the protein was readily degraded. Amino acid analysis of the purified protein revealed a high percentage of amino acids with nonpolar residues. The location of PAS IV was determined in bovine tissues by using immunofluorescence techniques. In mammary tissue, PAS IV was located on both the apical surfaces of secretory epithelial cells and endothelial cells of capillaries. This glycoprotein was also detected in endothelial cells of heart, liver, spleen, pancreas, salivary gland, and small intestine. In addition to mammary epithelial cells, PAS IV was also located in certain other epithelial cells, most notably the bronchiolar epithelial cells of lung. The potential usefulness of this protein as a specific marker of capillary endothelial cells in certain tissues is discussed.

During lactation, milk triacylglycerols are secreted from mammary epithelial cells in the form of discrete droplets coated with a surface layer of membrane (2, 61). This membrane layer, commonly referred to as the milk-fat-globule membrane (MFGM),[1] is derived from the apical pole of secretory alveolar cells by a budding process in which intracellular lipid droplets are progressively enveloped with apical plasma membrane (6, 7, 21). Some membrane may also be acquired directly from secretory vesicle membrane within the cell (77, 78), although the magnitude of the contribution to MFGM from this source remains controversial (21, 53). As may be expected, the cell-surface origin of MFGM is reflected in the enzyme and lipid composition of membrane preparations isolated from milk. Typical plasma membrane components enriched in MFGM include sphingomyelin, 5′-nucleotidase, phosphodiesterase, and alkaline phosphatase (14, 43, 44, 53). MFGM thus provides a convenient source of membrane protein and lipid from a specific epithelial cell type in an heterogeneous population of cells.

Separation of the proteins of MFGM from several species by SDS PAGE leads to the resolution of eight to ten major components (2, 14, 21, 23, 31, 46, 50, 52, 55, 61). Two of these components, xanthine oxidase and butyrophilin, account for nearly 50% of the total protein detectable by staining gels with Coomassie Blue (21, 40, 54–56). Butyrophilin is firmly bound to MFGM and appears to be largely restricted to the apical plasma membrane of secretory epithelial cells (21, 31). Other glycoproteins that appear largely restricted to the apical surface include a highly sialylated protein with an M_r of over 400,000 in several species (references 4, 12, and 20 and data in press) and glycoproteins of M_r 100,000 and 55,000 in the cow and guinea pig, respectively (unpublished data).

In contrast, xanthine oxidase is present throughout the

[1] *Abbreviations used in this paper*: MFGM, milk-fat-globule membrane; PMSF, phenylmethylsulphonyl fluoride; TBS, Tris-buffered saline; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone.

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cytoplasm of secretory epithelial cells, although the enzyme appears in higher concentrations towards the apical pole of these cells (40). Secreted fat globules contain large amounts of xanthine oxidase in both a soluble and a membrane-bound form (11, 40, 54, 56). Xanthine oxidase is also present in a soluble form in capillary endothelial cells in mammary tissue and in other tissues appears largely restricted to this cell type (11, 40).

MFGM preparations from human, cow, and guinea pig also contain a glycoprotein with an M_r of ~75,000–80,000 (PAS IV of reference 55). Approximately 5% of the total Coomassie Blue–positive protein of bovine MFGM is composed of this component (55). We have purified PAS IV to homogeneity and determined the distribution of this glycoprotein in bovine tissues by immunofluorescence microscopy. The antigen is concentrated on the apical surfaces of secretory epithelial cells in mammary tissue but is also present, in apparently significant amounts, in mammary endothelial cells. In other tissues, with some exceptions, PAS IV is restricted to capillary endothelial cells and unlike xanthine oxidase appears to be firmly bound to cellular membranes, a reflection of the protein's pronounced hydrophobic properties. Thus, this protein serves as a convenient membrane marker for capillary cells in certain tissues. In this present communication, we describe the purification of PAS IV from bovine milk, the biochemical characterization of this protein, and the determination of its distribution in bovine tissues using immunological procedures.[2] Part of this work has been previously published in abstract form (27, 28).

MATERIALS AND METHODS

Materials: Aprotinin, 6-amino-*n*-caproic acid, phenylmethylsulphonyl fluoride (PMSF), *n*-propyl gallate, Histopaque, BSA, DEAE-cellulose, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), neuraminidase (type X), pronase (type XIV), papain (type IV), trypsin (type XI), and pepsin were obtained from Sigma Chemical Co. (St. Louis, MO). Chymotrypsin was purchased from ICN Pharmaceuticals, Inc. (Cleveland, OH) and amino acid standards were from the Pierce Chemical Co. (Rockford, IL). Triton X-100 and X-114, SDS, ampholines, acrylamide, nitrocellulose paper, and M_r protein standards were obtained from Bio-Rad Laboratories (Richmond, CA). Ultrodex resin was purchased from LKB Instruments, Inc. (Gaithersburg, MD). Concanavalin A/Agarose was purchased from E-Y Laboratories, Inc. (San Mateo, CA) and ^{125}I-labeled goat anti-rabbit IgG was from New England Nuclear (Boston, MA). Freund's complete adjuvant, V-8 proteinase, rabbit antimouse IgG, fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG and guinea pig antibodies to bovine prekeratin were obtained from Miles Laboratories, Inc. (Elkhart, IN). Fluorescein isothiocyanate-conjugated avidin and biotinylated goat anti-rabbit IgG and horse anti-mouse IgG were purchased from Vector Laboratories, Inc. (Burlingame, CA). Rabbit antibody to human factor VIII-associated protein was obtained from Calbiochem-Behring Corp. (La Jolla, CA) and cyanogen-bromide–activated Sepharose 4-B was from Pharmacia Fine Chemicals (Piscataway, NJ). O.C.T. compound for embedding frozen specimens was from Lab-Tek Div., Miles Laboratories, Inc. (Naperville, IL).

Preparation of Membrane and Milk Fractions: "Plasmalemma-enriched" membrane fractions from bovine mammary tissue obtained from Holstein cows at slaughter were prepared by modification of a procedure described by Huggins et al. (37). Mammary tissue was homogenized for 20 s in 4 vol (wt/vol) of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose) using a Sorvall Omni-mixer (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT) at a speed setting of 5. The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 750 *g*. The resulting membrane pellet was then resuspended and homogenized in 1 vol of homogenization buffer with a Dounce, type A, homogenizer, and centrifuged as above. After centrifugation, the supernatant was recentrifuged for 7.5 min at 32,000 *g*, and subsequently for 75 min at 100,000 *g*. The final membrane pellet was resuspended in homogenization buffer, adjusted to a final sucrose concentration of 40% (wt/vol), and centrifuged for 4 h at 100,000 *g* under a sucrose gradient, prepared by overlaying the sample with 9 ml each of 36, 32, and 0.9% (wt/vol) sucrose in 20 mM Tris-HCl buffer, pH 7.4. The membrane fraction that collected at the 32/0.9% interface was designated as the "plasmalemma-enriched" fraction. The alkaline phosphatase activity of this fraction was enriched 16- to 17-fold over the activity in the total homogenate. Similar preparations from lactating guinea pig mammary tissue have been extensively characterized. Activities of the following enzymes were significantly enriched over total homogenates in these fractions: 5′-nucleotidase (17-fold), alkaline phosphatase (14-fold), galactosyl transferase (19- to 20-fold), and uridine diphosphatase (10-fold). Preparations were devoid of the mitochondrial marker enzyme, succinate/iodonitrotetrazolium reductase.

Crude microsomal membrane fractions were also prepared from lactating mammary glands. Tissue was minced with scissors and homogenized in 3 vol (wt/vol) of Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 5 mM 6-amino-*n*-caproic acid, 0.5 mM PMSF, and aprotinin, 650 kallikrein inhibitor units [iu]/ml) in a Sorvall Omni-mixer at maximum speed for 30 s at 4°C. Homogenates were centrifuged at 1,000 *g* for 10 min and the resulting supernatants were further centrifuged for 10 min at 10,000 *g*. The 10,000-*g* supernatants were then centrifuged at 95,000 *g* for 1 h and the final membrane pellet designated "crude microsomal membrane fraction."

Bovine MFGM and soluble Triton X-100 extracts were prepared as previously described (55). The soluble fraction of milk was prepared by centrifuging skim milk at 90,000 *g*, for 90 min at 4°C. The clear aqueous supernatant was retained and designated "soluble whey fraction." Both the thin layer of residual fat and the white casein pellet were discarded.

To resolve adsorbed, peripheral proteins from integral membrane protein, we resuspended membrane fractions (≤0.5 mg protein/ml) at 0°C in 0.1 M Na_2CO_3 (24, 35) and subjected them to ultrasound a total of four times, every 10 min for 30-s periods. The suspensions were then centrifuged at 114,000 *g* for 1 h at 4°C and membrane pellets were resuspended in 10 mM Tris-HCl buffer, pH 7.4.

Purification of PAS IV: PAS IV was purified by a four-step procedure involving proteinase treatment of bovine MFGM, solubilization of the proteinase-resistant proteins in Triton X-100, DEAE-cellulose chromatography, and adsorption to and elution from concanavalin A/Agarose.

Bovine MFGM (2 mg of membrane protein/ml; 0.5–1 gram total protein) was incubated at pH 6.2 in 5 mM cysteine containing 2 mM EDTA and papain (0.75 IU/mg membrane protein) for 2 h at 37°C. Proteinase inhibitors (0.4 mM PMSF and 0.04 mM TLCK, final concentrations) were then added and the mixture was centrifuged at 96,000 *g* for 1 h at 4°C (for data on the inhibition of papain with these compounds, see reference 75). The membrane pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing 0.02 mM PMSF and 0.02 mM TLCK and dialyzed at 4°C overnight against 4 liters of the same mixture. The protein composition of the treated membrane at this stage is compared with untreated MFGM in lanes *1* and *2* of Fig. 1. Following dialysis, the papain-digested membrane suspension was extracted with 1% (vol/vol) Triton X-100 as described (55) but with the addition of 0.4 mM TLCK, and the solubilized proteins were collected by centrifugation at 100,000 *g* for 2 h. Triton X-100 extracts contained a fraction of all the major proteins in the proteinase-treated membrane (compare Fig. 1, lanes *2* and *3*) including a substantial enrichment in PAS IV over the untreated membrane. The solubilized proteins were then fractionated on DEAE-cellulose that had been previously equilibrated with a mixture of 10 mM Tris-HCl buffer, pH 7.5, and 0.5% (vol/vol) Triton X-100. After washing the column of ion-exchange resin (2 × 30 cm) with several volumes of equilibration buffer, PAS IV was eluted with a 0–100 mM linear gradient of NaCl in 10 mM Tris-HCl buffer, pH 7.5. Collected fractions were analyzed by SDS PAGE and samples enriched in PAS IV (eluted between 30 and 50 mM NaCl) were pooled (Fig. 1, lane *4*) and adsorbed onto a column of concanavalin A/Agarose (1.5 × 10 cm). At least 5 column volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.5% (vol/vol) Triton X-100 were passed through the column and PAS IV was eluted with a solution of 0.4 M α-methylmannoside in 50 mM Tris-HCl buffer, pH 7.5 (Fig. 1, lane *5*). This final step served to concentrate the protein fractions and remove minor contaminants with molecular weights just below that of PAS IV. Purified protein samples were dialyzed against TBS or other appropriate buffers and stored at −20°C. Yields of PAS IV were at least 10% of the theoretical yield assuming a 20-fold purification from untreated MFGM.

Preparation of Monoclonal and Affinity-purified Polyclonal Antibodies to PAS IV: We isolated two hybridoma cell lines, designated E-1 and E-3, by fusing spleen cells from mice immunized with purified PAS IV and SP-2/0 myeloma cells (71). The positive hybrids were cloned four times and grown as ascites tumors in BALB/c mice. Specificity of these monoclonal

[2] Throughout the text, for convenience, we refer to this antigen as one protein, regardless of tissue location. This assumes that PAS IV is essentially the product of one gene or closely related genes.

antibodies was determined by solid-phase immunoassay and immunoaffinity chromatography. Full details of these procedures will be published separately (D. E. Greenwalt, V. G. Johnson, and I. H. Mather, manuscript in preparation). All the work reported in this present paper was performed with antibody from the E-1 cell line which is designated "E-1 monoclonal antibody."

Polyclonal rabbit antibody to PAS IV was prepared by using the purified protein (see above) as immunogen. Purified PAS IV was separated by SDS PAGE and the monomeric form of the protein was excised from the gel slab. The pieces of polyacrylamide were mashed with a glass homogenizer and mixed with Freund's complete adjuvant in a 1:1 ratio (vol/vol). Approximately 100 μg of protein was injected once a month, and after at least three injections, antisera were collected by standard procedures. Affinity-purified antibodies were obtained from this serum by adsorption to and elution from PAS IV immobilized on Sepharose 4-B. Rabbit antisera (10 ml) was dialyzed overnight at 4°C against 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The dialyzed serum was then fractionated on a column of PAS IV bound to Sepharose 4-B, prepared by covalently linking 5 mg of purified antigen to 1 gram (dry wt) of cyanogen-bromide-activated Sepharose 4-B. After washing the column with the above Tris-HCl dialysis buffer, we eluted specifically bound antibodies with 3 M NaSCN and dialyzed them overnight at 4°C against 4 liters of the same buffer. The final yield of affinity-purified antibody was ~ 5 mg of protein, 1.5 mg/ml. Dilutions referred to in the text are from the stock solution of "undiluted antibody."

Partitioning of MFGM Proteins in Triton X-114 Solutions: Bovine MFGM (1 mg membrane protein/ml, final concentration) was extracted with Triton X-114 (1%, vol/vol) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EGTA, 1 mM 6-amino-*n*-caproic acid, aprotinin (650 iu/ml), and 0.5 mM PMSF. After being stirred for 20 min at 0°C, the mixture was incubated without stirring for an additional 20 min at 0°C and then centrifuged at 100,000 *g* for 1 h. The supernatant fraction was then warmed to 30°C and centrifuged at 2,000 *g* for 15 min. The aqueous and detergent phases were collected separately and washed either with Triton X-114 (1% [vol/vol], final concentration), or with a 10-fold excess of cold TBS, respectively. After a second condensation cycle, the two phases were made up to equal volumes with TBS. Methods were based on the procedures of Bordier (9).

Degradation of MFGM with Enzymes: Preparations of bovine MFGM (1 mg membrane protein/ml) were incubated with the proteinases trypsin, chymotrypsin, papain, pronase, and pepsin for 1 h at 37°C. Solutions used for these proteinases were as follows: 40 mM Tris-HCl, pH 7.8, containing 50 mM $CaCl_2$ (trypsin), 40 mM Tris-HCl, pH 8.1, containing 10 mM $CaCl_2$ (chymotrypsin), 5 mM cysteine, pH 6.2, containing 2 mM EDTA (papain), 60 mM Tris-HCl, pH 7.6, containing 0.39 M NaCl, 11 mM KCl, 5 mM $CaCl_2$, and 3.6 mM $MgSO_4$ (pronase), and 6 mM HCl (pepsin). After digestion, the samples were centrifuged at 100,000 *g* for 1 h. The treated membrane fractions were then resuspended in 10 mM Tris-HCl buffer, pH 7.4, and aliquots were prepared for SDS PAGE. MFGM was desialylated by treatment with neuraminidase (0.01 IU/mg membrane protein) for 1 h at 37°C in 50 mM sodium acetate buffer, pH 5.1.

SDS PAGE and Isoelectric Focusing: SDS PAGE and analytical and preparative isoelectric focusing were performed as previously described (55). Proteins were either stained with the periodic acid/Schiff reagent or Coomassie Blue (19, 55). Determination of the molecular weights of proteins by SDS PAGE was as described (70), using the following proteins as standards: myosin (200,000), β-galactosidase (116,000), phosphorylase *b* (92,500), BSA (66,500), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Immunoblotting Procedures: Protein samples were separated by SDS PAGE and electrophoretically transferred to nitrocellulose paper, essentially as described by Towbin et al. (72). The nitrocellulose paper was then rinsed sequentially with 0.1% (vol/vol) Triton X-100 in TBS (without proteinase inhibitors) for 1 h and overnight with 2% (wt/vol) BSA in 2% (wt/vol) BSA/TBS. Affinity-purified rabbit antibodies to PAS IV, diluted 1:200 in 2% (wt/vol) BSA/TBS, were then incubated with the paper for 1 h and the nitrocellulose was sequentially treated as follows: 0.5% (vol/vol) Triton X-100/TBS, 30 min; 0.5 M NaCl/TBS, 30 min; TBS, 30 min; 0.1% (wt/vol) BSA/TBS, 30 min; ^{125}I-labeled goat anti-rabbit IgG (~2 μCi) in 2% (wt/vol) BSA/TBS, 2 h; 0.1% (wt/vol) BSA/TBS, 30 min; 0.1% (vol/vol) Triton X-100/TBS, 30 min; 0.5% (vol/vol) Triton X-100/TBS, 30 min; 0.5 M NaCl/TBS, 30 min; and TBS, two changes, 30 min each. Preimmune serum was used in place of affinity-purified rabbit antibodies in control experiments.

Immunofluorescence Microscopy: For immunolocalization studies, tissue was obtained from the Beltsville Agricultural Research Center abattoir (Beltsville, MD), from Holstein cows at slaughter. Tissue was either frozen in 2-methylbutane cooled in liquid nitrogen and embedded in O.C.T. compound or fixed with "methacorn" (60% [vol/vol] methanol, 30% [vol/vol] chloroform, and 10% [vol/vol] acetic acid) and embedded in paraffin (74). Frozen tissue was sectioned with a cryostat/microtome into 5-μm-thick sections, air-dried, and fixed in acetone at −20°C for 7 min. Paraffin-embedded tissue was cut into 7-μm-thick sections and dewaxed in xylene and ethanol. Blood samples were obtained from the tail vein and lymphocytes were isolated according to the procedure of Böyum (10).

A similar staining protocol was used for all sections whether frozen or fixed in methacorn. Blood cells were stained in suspension using 50-μl aliquots of antibody contained in the wells of 96-well plastic plates. Samples were incubated with undiluted hybridoma culture supernatant or a 1:50 dilution of the affinity-purified rabbit antibodies to PAS IV in TBS that contained 1% (wt/vol) BSA. Preimmune serum or culture medium was used in control experiments. After 30 min at room temperature the slides were washed three times in TBS. Blood cells were also washed three times by repeated centrifugation at 1,500 *g* for 15 min and resuspension in TBS. Samples were then incubated with biotinylated horse anti-mouse IgG (4.3 mg/ml, stock solution) at a 1:100 dilution in TBS/1% (wt/vol) BSA for another 30 min. The samples were washed three more times in TBS and incubated with fluorescein isothiocyanate-conjugated avidin at a 1:100 dilution of the stock solution (5.0 mg/ml) in TBS/1% (wt/vol) BSA for 30 min. All slides were finally washed three times in TBS and once in distilled water, and the specimens were mounted in glycerol containing 5% (wt/vol) *n*-propyl gallate to prevent photobleaching of the fluorescein (26). Blood cell suspensions were directly mounted on microscope slides. Specimens were examined with a Leitz Dialux microscope equipped with an H filter for wide band blue light excitation.

Electron Microscopy: MFGM samples for electron microscopy were fixed in 2.5% (wt/vol) glutaldehyde in 50 mM sodium cacodylate buffer, pH 7.2 , containing 50 mM KCl, 2.5 mM $MgCl_2$, and 1.5 mM $CaCl_2$ and stained with 2% (wt/vol) OsO_4 for 1 h and 0.5% (wt/vol) uranyl acetate for 12 h. Specimens were dehydrated in a graded series of acetone solutions and embedded in Epon 812. Sections (silver grade) were stained with uranyl acetate and lead citrate.

Analytical Techniques: For amino acid analysis, purified PAS IV was hydrolyzed in 6 N HCl under nitrogen at 110°C for 24, 48, and 72 h. Amino acids were analyzed on a model JLC-6AH JOEL amino acid analyzer. Carbohydrates were analyzed by gas/liquid chromatography and mass spec-

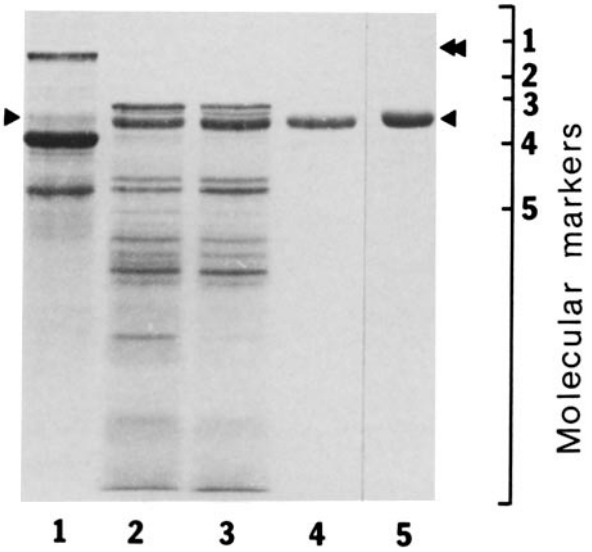

FIGURE 1 Analysis by SDS PAGE of fractions of PAS IV at various stages of purification. Samples were separated in 10% (wt/vol) polyacrylamide (1.5-mm thickness) as follows: lane *1*, unfractionated MFGM; lane *2*, membrane fraction following digestion with papain; lane *3*, papain-digested proteins solubilized in Triton X-100; lane *4*, soluble fraction after DEAE-cellulose chromatography; lane *5*, PAS IV after further purification by affinity chromatography on concanavalin A/Agarose. Proteins were stained with Coomassie Blue. M_r markers from top to bottom of the gel were as follows: *1*, myosin (200,000); *2*, β-galactosidase (116,000); *3*, phosphorylase *b* (92,500); *4*, BSA (66,500); and *5*, ovalbumin (45,000). PAS IV is denoted by single arrowheads and traces of a dimeric form of PAS IV by double arrowheads in lane *5*.

trometry as described by Sawardeker et al. (68). Sialic acid was analyzed by the method of Aminoff (1), after the samples were hydrolyzed in 0.025 N H_2SO_4 for 1 h at 80°C. Protein was analyzed by the method of Lowry et al. (49) or, in the presence of Triton X-100, as described by Peterson (63). In either case, BSA was used as a standard. Alkaline phosphatase was assayed as previously described using *p*-nitrophenyl phosphate as substrate (55).

RESULTS

Digestion of MFGM with Proteinases and Preparation of PAS IV

PAS IV was not degraded when preparations of bovine MFGM were extensively digested with the proteinases trypsin, chymotrypsin, or papain (see example for papain in Fig. 1, lane *2*). Incubation of membrane with pronase led to partial degradation of PAS IV, whereas digestion with pepsin, at low pH, resulted in the complete degradation of all MFGM proteins. After solubilization of PAS IV with detergents, the protein was degraded by all of the above enzymes and V-8 proteinase from *Staphylococcus aureus* (not shown).

PAS IV does not appear to be a component of the cytoplasmic coat of MFGM (21, 23, 77–79). Under conditions in which PAS IV remained associated with MFGM, this proteinaceous coat was completely removed by treatment with proteinases (e.g., papain, Fig. 2).

The resistance of membrane-bound PAS IV to proteinase digestion led to a purification procedure for this protein, the first step of which was digestion of the major proteins of MFGM with papain (Fig. 1, lanes *1* and *2*). Proteinase-resistant proteins were then solubilized in non-ionic detergent and PAS IV was purified from the soluble extracts by ion-exchange and lectin-affinity chromatography. Details of a representative purification procedure are given in Materials and Methods and summarized in Fig. 1.

Analysis of purified PAS IV by SDS PAGE showed a single major band of protein that stained with both the periodic acid/Schiff reagent and Coomassie Blue (single arrowheads, Fig. 1). Traces of a second component with an M_r exactly twice that of the principal component were also evident

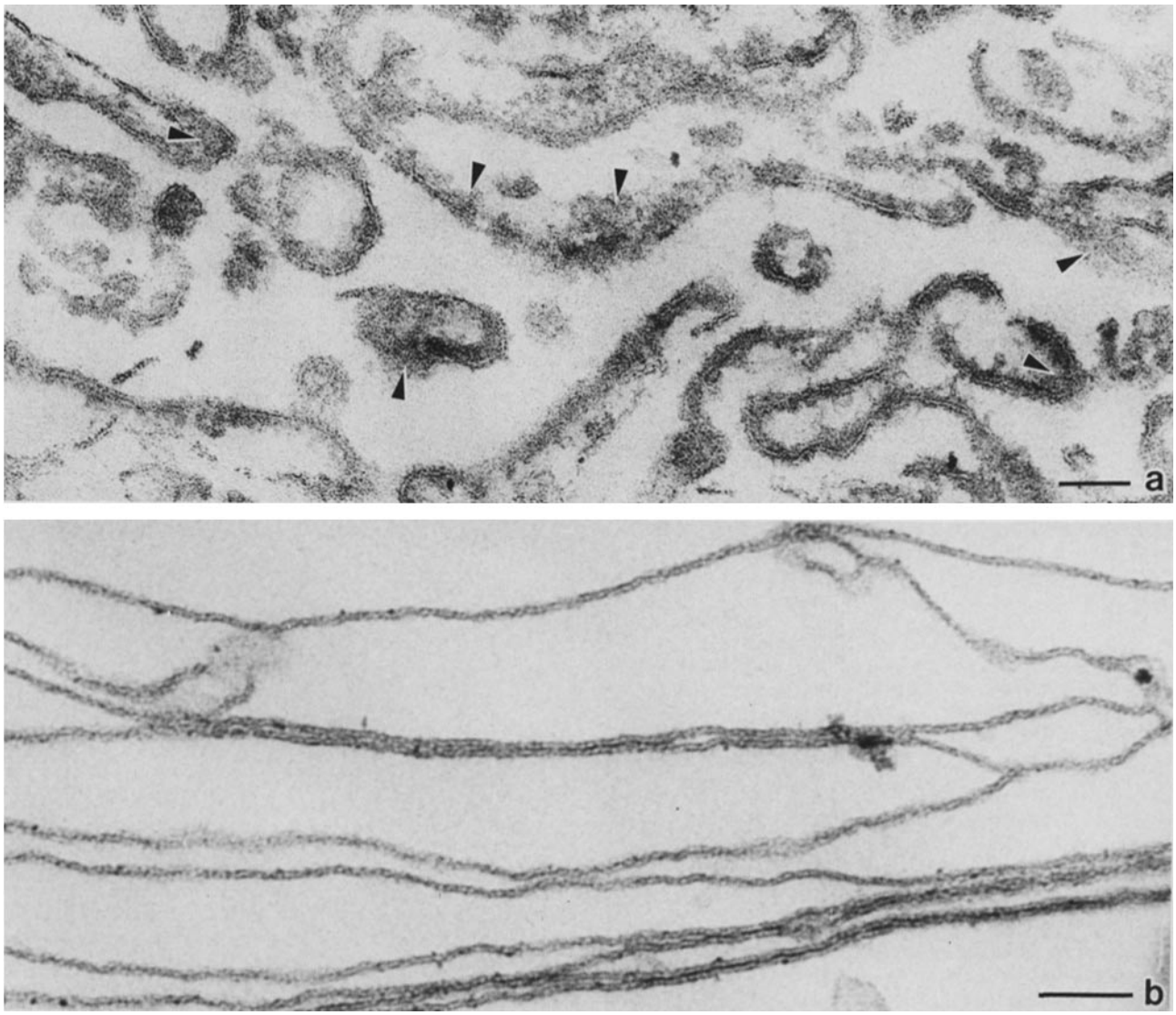

FIGURE 2 Electron microscopy of bovine MFGM before and after treatment with papain. MFGM (1 mg of membrane protein/ml) was incubated with papain (1.25 IU/mg of membrane protein), and samples of the digested membrane and untreated MFGM were processed for electron microscopy. (*a*) MFGM before treatment with papain. (*b*) MFGM after treatment with papain. Note the fuzzy 'cytoplasmic' coat material in *a* (arrowheads) is completely removed by papain treatment in *b*, leaving an uncoated bilayer membrane. Bars, 0.1 μm. (*a*) × 105,000; (*b*) × 135,000.

(double arrowheads, Fig. 1, lane *5*). Papain digests of the major and minor components were compared by SDS PAGE and immunoblotting techniques, using rabbit antibody to the PAS IV monomer. Almost identical patterns of immunologically reactive peptides were obtained, which indicates that the minor component of high M_r is most probably a dimeric form of PAS IV, which is resistant to disaggregation with SDS and thiol reducing agents (data not shown).

Physical and Chemical Characterization of PAS IV

The mobility of PAS IV during SDS PAGE was compared with a set of protein standards (see Materials and Methods), in 6, 8, 10, and 15% (wt/vol) polyacrylamide gels. Apparent molecular weights were estimated to be 78,500, 76,500, 76,000, and 75,500, respectively. The asymptotic minimum M_r for PAS IV was therefore estimated to be 76,000.

At least four variants of PAS IV were resolved by analytical isoelectric focusing, with apparent isoelectric points of 7.80, 7.95, 8.15, and 8.50. When PAS IV was treated with neuraminidase and subjected to preparative electrofocusing in flatbeds of Ultrodex (55), there was a shift of at least one pH unit, in the range of isoelectric points, towards the basic end of the pH gradient.

PAS IV appeared to have pronounced hydrophobic properties. Removal of detergent from preparations of the purified protein, by nonpolar adsorption chromatography, led to complete precipitation of PAS IV from solution. The protein remained firmly bound to MFGM after treatment of isolated preparations with Na_2CO_3 solutions at high pH (Fig. 3, lanes *1* and *2*). Previous work has shown that this procedure effectively removes loosely bound and peripheral membrane proteins (24, 35). PAS IV was also recovered from the detergent phase after soluble Triton X-114 extracts of bovine MFGM were warmed above the cloud point (Fig. 3, lanes *3* and *4*). In contrast, the major glycoprotein of high M_r (double arrowheads in Fig. 3, lanes *1* and *4*) was recovered from the aqueous supernatant. Other membrane glycoproteins were distributed between both the aqueous and detergent phases. Similar results were obtained with Triton X-114-solubilized proteins from human MFGM (Fig. 3, lanes *5* and *6*) and guinea pig MFGM (data submitted for publication).

The hydrophobic nature of PAS IV was confirmed by amino acid analysis of the purified protein (Table I). At least 50% of the amino acids have nonpolar residues and a further 20% have side chains with uncharged polar groups at neutral pH. The protein is also characterized by a low level of the sulphur-containing amino acids, cysteine and methionine, and a high percentage of the branched chain amino acids, valine, leucine, and isoleucine. The hydrophobicity index of PAS IV was estimated to be 1,174, which implies that the protein has a pronounced hydrophobic character (see Discussion). Approximately 5.3% of the glycoprotein is composed of carbohydrate on a weight basis. The principal sugars detected were mannose, glucose, galactose, and sialic acid (Table II). Since the quantity of material available for analysis was near the limits of sensitivity, the recorded values are only approximate.

Determination of the Distribution of PAS IV in Mammary Tissue and Milk Using Immunoblotting Procedures

The distribution of PAS IV in various fractions of milk and

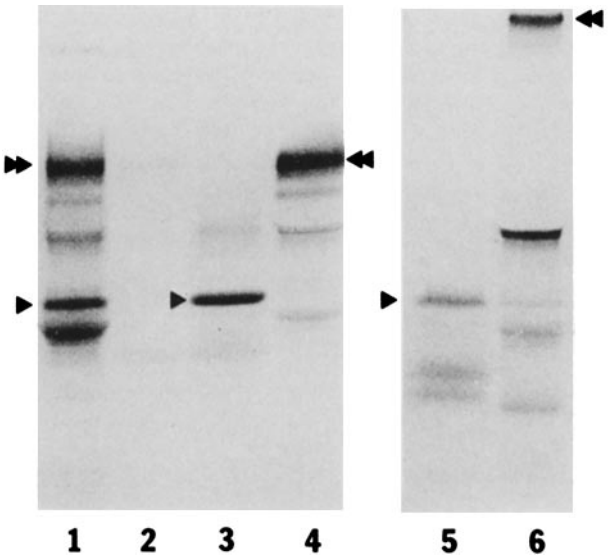

FIGURE 3 Analysis by SDS PAGE of MFGM proteins after treatment with Na_2CO_3 solutions and after partitioning in Triton X-114. MFGM was extracted with 0.1 M Na_2CO_3 as described (24, 35) and samples of the insoluble residue (lane *1*) and the Na_2CO_3 supernatant (lane *2*) were separated in 6% (wt/vol) polyacrylamide (3-mm thickness). Bovine and human MFGM were also extracted in Triton X-114 at 0°C and the soluble extracts were warmed to 30°C. After phase separation, the aqueous and detergent layers were collected and further treated as described in Materials and Methods. Samples, separated by SDS PAGE, are as follows: lane *3*, bovine MFGM, detergent phase; lane *4*, bovine MFGM, aqueous phase; lane *5*, human MFGM, detergent phase; lane *6*, human MFGM, aqueous phase. All samples were stained with the periodic acid/Schiff reagent. PAS IV in bovine and human MFGM is indicated by single arrowheads (lanes *1*, *3*, and *5*). Major glycoproteins of high M_r and with pronounced hydrophilic properties in bovine and human MFGM are indicated by double arrowheads (lanes *1*, *4*, and *6*).

TABLE I

Amino Acid Composition of PAS IV

Amino acid	Mole percent	Residues per 76,000 M_r
Nonpolar		
Alanine	5.8	49.5
Glycine	6.7	67.8
Isoleucine	8.2	47.5
Leucine	9.5	55.0
Methionine	1.0	5.1
Phenylalanine	6.6	30.4
Proline	5.2	34.3
Tryptophan	ND	—
Valine	9.5	61.7
Polar (with uncharged polar groups)		
Cysteine	1.8	11.3
Serine*	7.3	52.8
Threonine*	8.4	53.6
Tyrosine	3.8	15.9
Polar (with charged groups)		
Aspartic acid (+ asparagine)	7.7	44.0
Glutamic acid (+ glutamine)	3.4	17.6
Arginine	2.7	11.8
Histidine	5.0	24.5
Lysine	7.6	39.5

ND, not determined.

* Values for serine and threonine were calculated by extrapolating to zero time; values obtained from samples hydrolyzed for 24, 48, and 72 h.

mammary tissue was determined by SDS PAGE and immunoblotting techniques. Samples of MFGM, crude microsomal membranes, and Na_2CO_3-washed plasmalemma-enriched membranes from lactating mammary tissue and a sample of the soluble whey fraction of milk were separated by SDS PAGE. The separated proteins were electrophoretically transferred to nitrocellulose paper and immunoreactive proteins were detected by using affinity-purified polyclonal antibody to PAS IV and ^{125}I-labeled goat anti-rabbit IgG. Proteins of the same M_r as PAS IV were detected in MFGM, crude microsomes, and the plasmalemma-enriched fraction (Fig. 4, lanes *1*, *3*, *4*, and *6–8*). An additional prominent immunoreactive protein of approximate M_r 80,000–85,000 was detected in the plasmalemma-enriched fraction (asterisk in Fig. 4, lane *6*). Traces of a second immunoreactive protein in MFGM and the plasmalemma-enriched fraction of exactly double the M_r of PAS IV (dots in Fig. 4, lanes *4* and *6*) were also noted. This second component was assumed to be dimeric PAS IV, present in the SDS-solubilized extracts (Fig. 1). Cross-reaction with this protein was not due to the presence of trace impurities in our PAS IV preparations used for raising antibodies, because we used monomeric PAS IV, separated by SDS PAGE as immunogen (see Materials and Methods).

TABLE II

Sugar Composition of PAS IV

Sugar	μg/mg protein	mole/mole protein*
Galactose	20.2	8.6
Mannose	18.6	7.9
Glucose	[23.2]	—
N-Acetylglucosamine‡	?	?
N-Acetylgalactosamine	0	0
Sialic acid	14.7	3.65

* Assuming an M_r of 76,000.

‡ A peak of material at the correct retention time for *N*-acetylglucosamine, but with an incorrect mass spectrum, was detected.

In contrast to the above results, immunologically similar proteins to PAS IV were not detected in the soluble whey fraction of milk (Fig. 4, lane *5*). Prominent glycoproteins in bovine whey of M_r ~70,000–80,000 include secretory component, lactoferrin, and lactoperoxidase (13, 29, 30). We confirmed that PAS IV is not related to secretory component by immunodiffusion in Agarose gels and immunoblotting techniques with purified bovine secretory component and specific antibody to this protein. Nor was the amino acid composition of PAS IV (Table I) found to be similar to secretory component (30), lactoferrin, or lactoperoxidase (29).

Proteins of approximately the same M_r as bovine PAS IV were also present in human and guinea pig MFGM (Fig. 4, lanes *9–11*). Both the human and guinea pig proteins cross-reacted weakly with the antibody to bovine PAS IV, which indicates that a similar protein is present in the milk of all three species (Fig. 4, lanes *12–14*).

These immunoblots established the specificity of our affinity-purified polyclonal antibody preparations and confirmed that PAS IV is a distinct constituent of MFGM. The observation that an immunoreactive protein of slightly higher M_r

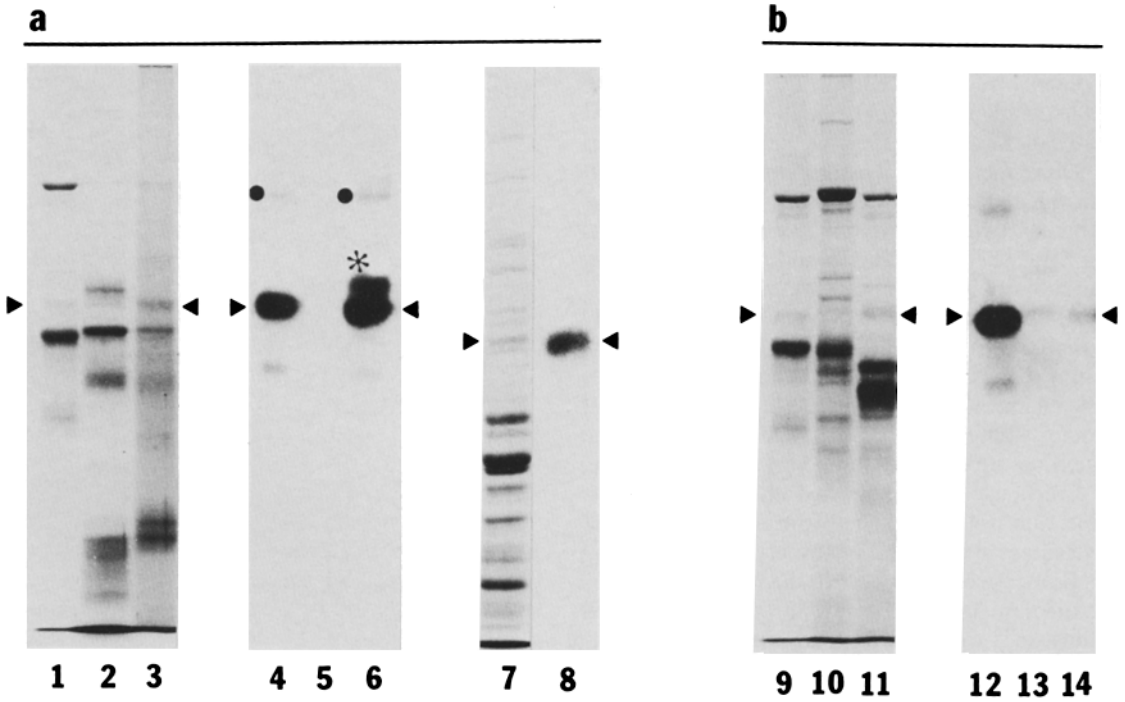

FIGURE 4 Distribution of PAS IV in mammary tissue and milk, determined by SDS PAGE and immunoblotting techniques. Samples were separated by SDS PAGE and the proteins were electrophoretically transferred to nitrocellulose paper. PAS IV-like proteins were localized by using affinity-purified polyclonal antibody. Duplicate control gels were stained with Coomassie Blue. (*a*) Lanes *1* and *4*, bovine MFGM; lanes *2* and *5*, soluble whey fraction; lane *3* and *6*, Na_2CO_3-washed plasmalemma-enriched fraction; lanes *7* and *8*, crude microsomal fraction. Lanes *1–3* and *7* are Coomassie Blue–stained gels and lanes *4–6* and *8*, the corresponding immunoblots. The M_r of PAS IV in bovine MFGM is denoted by a single arrowhead and cross-reactive proteins discussed in the text by an asterisk and dots. (*b*) Lanes *9* and *12*, bovine MFGM; lanes *10* and *13*, human MFGM and lanes *11* and *14*, guinea pig MFGM. Lanes *9–11* are Coomassie Blue–stained gels and lanes *12–14*, the corresponding immunoblots. The M_r of PAS IV in bovine MFGM is denoted by a single arrowhead.

is present in plasmalemma-enriched fractions isolated from mammary tissue (Fig. 4, lane *6*) raises the possibility that PAS IV is subjected to limited proteolysis during or following secretion.

Determination of the Distribution of PAS IV in Various Tissues Using Immunofluorescence Microscopy

PAS IV was localized in various tissues using immunofluorescence microscopy and both affinity-purified rabbit antibodies and monoclonal antibody E-1. Immunological determinants on PAS IV were detected with the affinity-purified rabbit antibodies using methacorn-fixed, paraffin-embedded specimens, and with the monoclonal antibody using acetone-fixed, frozen tissues. Neither antibody bound to PAS IV when conditions were reversed. Evidently both antibody preparations recognize conformational determinants on the antigen that are destroyed by different fixation and embedding procedures.

In lactating mammary tissue, PAS IV was detected with the polyclonal rabbit antibodies on the apical surfaces of secretory epithelial cells (double arrowheads, Fig. 5*a*). In view of the known origin of MFGM from the apical surfaces of secretory cells (2, 21, 61), this result was unsurprising. However, significant immunofluorescence was also detected on cells underlying the secretory epithelium (single arrowheads, Fig. 5, *a* and *b*) with both rabbit antibody (Fig. 5*a*) and monoclonal antibody E-1 (Fig. 5*b*). With the monoclonal antibody, immunofluorescence staining of these basal cells was more pronounced than staining of the apical surfaces of secretory epithelial cells. These immunoreactive cells were identified as capillary endothelial cells by using antibody to factor VIII antigen as a cell-specific marker (39, 41). Adjacent frozen sections of lactating mammary tissue were incubated with either antibody to factor VIII antigen or monoclonal antibody E-1. The same cells were apparently stained with either antibody (Fig. 5, *c* and *d*). In contrast, myoepithelial cells identified with antibody to prekeratin (22), were unstained with either of our PAS IV antibodies (not shown).

The endothelial location of PAS IV was confirmed by determining the distribution of this glycoprotein in other tissues. Prominent and specific immunofluorescence of capillary endothelial cells was seen with both polyclonal and monoclonal antibodies to PAS IV in heart and the muscularis of small intestine, tissues in which endothelial cells are readily identified (Fig. 6, *a* and *b*). Other organs and tissues in which endothelial cells were specifically decorated with PAS IV antibody were liver (Fig. 6*c*), spleen, salivary gland, pancreas, and the mucosae of small intestine. Monoclonal antibody E-1 appeared consistently specific for endothelial cells and cross-reacted in some tissues, e.g., liver, pancreas, and salivary gland, to which the rabbit antibody apparently did not bind (summarized in Table III). The rabbit antibody also weakly stained epithelial cells in some tissues. Occasional staining was detected on the apical surfaces of pancreatic acinar cells and the epithelial cells of intestinal crypts (Table III).

Apart from mammary gland, the only tissue examined (Table III) that showed significant staining of epithelial cells with both antibodies was lung. Apical surfaces of lung bronchioles were prominently and specifically stained (Fig. 6*d*). Interestingly, the extensive capillary bed of lung showed no reaction with either polyclonal or monoclonal antibody. The capillaries of brain and kidney were also unreactive.

In the vascular system, where PAS IV appeared to be restricted to the endothelial cells of capillaries, no significant staining of large blood vessels was detected. Peripheral lymphocytes and erythrocytes collected from individual blood samples of ten cows were also unstained by either antibody.

DISCUSSION

The presence of a relatively basic glycoprotein in MFGM, with an apparent M_r of 75,000–80,000, was recognized previously by SDS PAGE and isoelectric focusing (46, 51, 52, 55). This glycoprotein comprises ~5% of bovine MFGM on a weight basis (55), and our polyclonal rabbit antibodies show some cross-reaction with a similar glycoprotein in the human and guinea pig membranes. PAS IV remains associated with MFGM when membrane preparations are treated with 0.1 M Na_2CO_3 solutions at pH 11.5, conditions previously shown to remove a large proportion of loosely adsorbed and peripheral membrane proteins (24, 35). By several other criteria PAS IV is an integral membrane glycoprotein. Of the major proteins and glycoproteins routinely identified by SDS PAGE, PAS IV is the only component that is recovered in almost 100% yield from the detergent phase when total membrane extracts are partitioned in Triton X-114 solutions. In the membrane-bound form, the glycoprotein is resistant to proteolysis under conditions that completely remove the cytoplasmic protein coat material routinely seen in electron micrographs of MFGM. However, when solubilized by various detergents, PAS IV is readily degraded by a variety of proteinases. These results imply that PAS IV is closely associated with the membrane lipid bilayer and is not a peripheral protein of the cytoplasmic coat or loosely adsorbed to the external membrane surface. The resistance of the membrane-bound form of this glycoprotein to exogenous proteinases has been noted previously, but without extensive comment (47, 52).

The hydrophobic properties of PAS IV were confirmed by amino acid analysis of purified preparations. Amino acids with nonpolar residues, especially the branched chain acids, comprise a large proportion of the total. The hydrophobicity index (8, 59) of PAS IV was estimated to be 1,174, which compares favorably with other well-characterized integral membrane glycoproteins, e.g., 1,120 and 1,185 for bovine rhodopsin and bacteriorhodopsin, respectively (8, 16). This index, which is obtained from the molar free energies for the transfer of individual amino acids from aqueous solution to organic solvent, is correlated with the overall hydrophobic character of proteins (59). Whether PAS IV spans the lipid bilayer, however, cannot be determined from these data. Topological labeling studies and amino acid sequence work will be required to answer this question.

The carbohydrate composition of PAS IV appears typical of glycoproteins with complex-type and also possibly high mannose oligosaccharide chains, N-linked to asparagine residues (36, 66). The apparent absence of *N*-acetylglucosamine is probably a reflection of the relative insensitivity of the analytical procedures for amino sugars. Quite high levels of glucose were detected. Since glucose is not considered to be a common constituent of terminally processed glycoproteins, the presence of this sugar in PAS IV preparations may be the result of contamination, possibly from the cellulose ion-exchange columns used for purification of the protein.

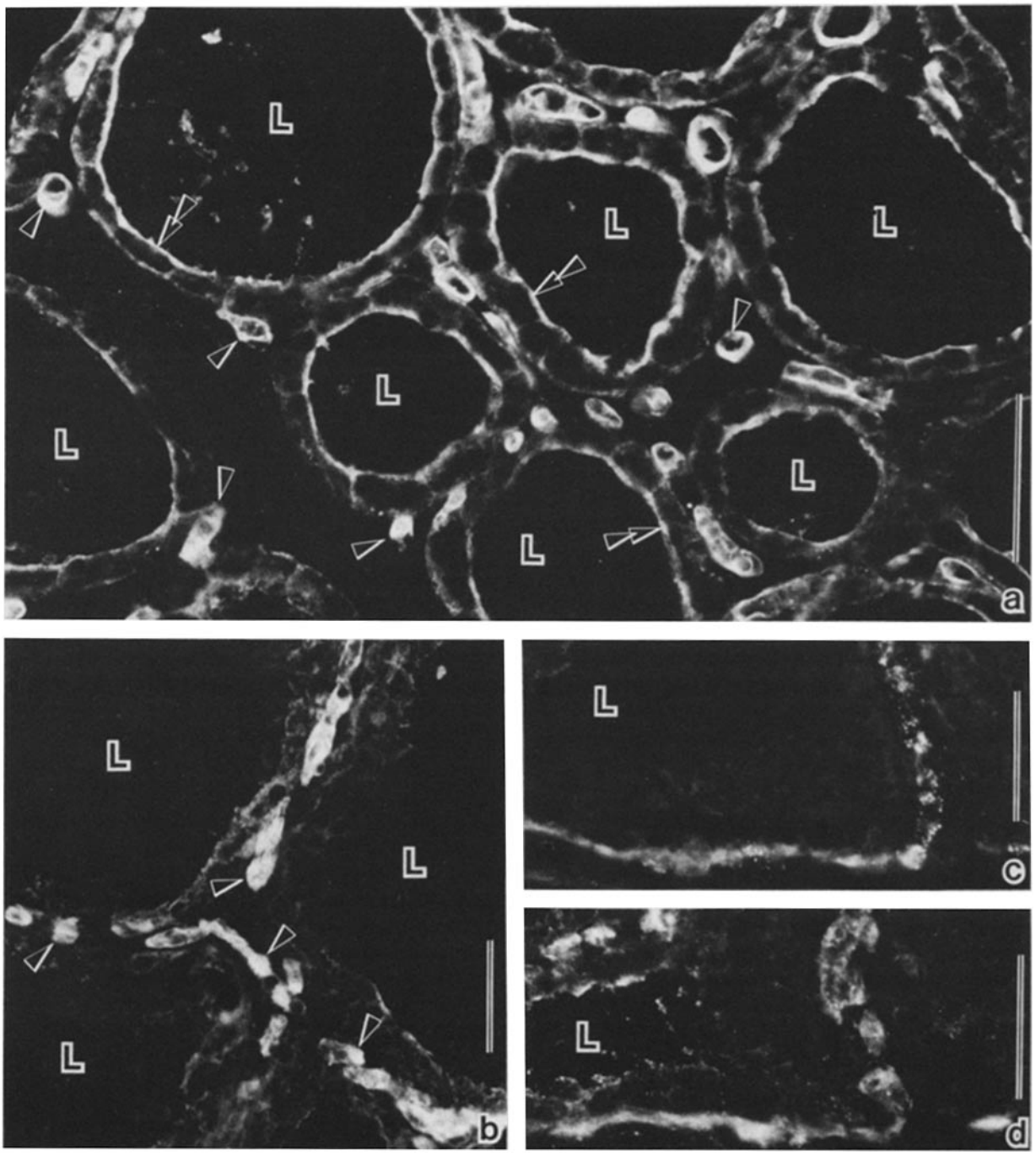

FIGURE 5 Localization of PAS IV and factor VIII antigen in lactating bovine mammary tissue by immunofluorescence microscopy. (a) Distribution of PAS IV determined in paraffin-embedded tissue using affinity-purified polyclonal antibody. Both capillary endothelial cells (single arrowheads) and the apical surfaces of secretory-epithelial cells (double arrowheads) are positive. (*b*) Distribution of PAS IV determined in acetone-fixed, frozen tissue using monoclonal antibody E-1. A staining pattern similar to that in *a* is seen (apical surfaces, less well stained). (c and *d*) Adjacent sections of acetone-fixed frozen tissue stained with either antibody to factor VIII antigen, a marker of capillary endothelial cells (c), or monoclonal antibody E-1 to PAS IV (*d*). The same cells appear to be stained with either antibody. *L*, alveolar lumen. Bars, 50 μm. (a) × 525; (*b*) × 350; (c) × 400; (*d*) × 450.

Sialic acid constitutes ~4 mol/mol of protein and variations in the level of this component may give rise to the isoelectric variants identified. Considering the amino acid composition, the desialylated protein is surprisingly basic, with isoelectric points over pH 9.0. Lysine and arginine together only approximately equal the number of glutamate and aspartate residues. This suggests that the amino acids with acidic side chains are present in the native protein as the amides, glutamine and asparagine, respectively. From practical considerations, this cannot be determined from the amino acid analysis presented in Table I.

The apparent M_r of purified PAS IV was 76,000, the same as the antigen detected in unfractionated MFGM or crude microsomal membranes isolated from lactating bovine mammary tissue. Since we used exogenous proteinases during the purification of PAS IV, this immunoblotting data established

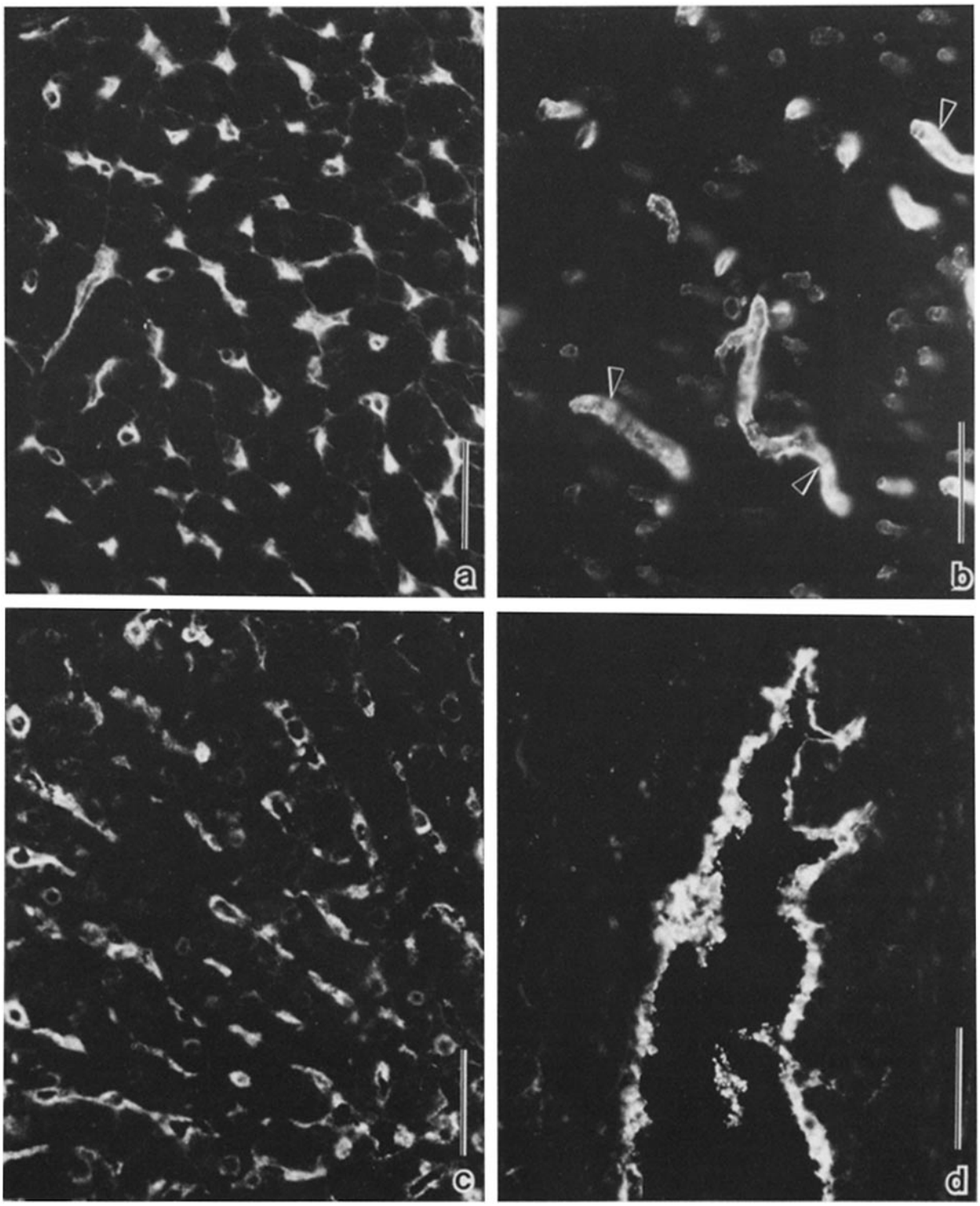

FIGURE 6 Localization of PAS IV in bovine heart, intestinal smooth muscle, liver, and lung by immunofluorescence microscopy. (*a*) Distribution of PAS IV determined in acetone-fixed, frozen heart tissue using monoclonal antibody E-1. Capillary endothelial cells throughout the section are stained. (*b*) Distribution of PAS IV determined in a paraffin-embedded section of smooth muscle from the small intestine using affinity-purified polyclonal antibody. Capillary endothelial cells are stained. Specimen shows capillaries sectioned in both the longitudinal (single arrowheads) and transverse planes. (*c*) Distribution of PAS IV in acetone-fixed, frozen liver using monoclonal antibody E-1, showing specific staining of sinusoidal cells. (*d*) Distribution of PAS IV determined in a paraffin-embedded section of lung tissue using affinity-purified antibody. Only the apical surfaces of epithelial cells of bronchioles are stained. Capillary endothelial cells in the lung did not stain with either monoclonal or polyclonal antibodies. Bar, 50 μm. (*a* and *c*) × 350; (*b* and *d*) × 400.

TABLE III

Summary of Distribution of PAS IV Determined by Immunofluorescence Microscopy

Tissue	Staining patterns	
	Monoclonal antibody E-1, frozen tissues	Affinity-purified polyclonal antibody, paraffin-embedded tissues
Mammary gland	Endothelia and apical surfaces of secretory epithelial cells	Same
Heart	Endothelia	Same
Spleen	Endothelia	Same
Intestinal smooth muscle	Endothelia	Same
Liver	Endothelia	Negative
Salivary gland	Endothelia	Negative
Pancreas	Endothelia	Occasional weak staining of the apical surfaces of acinar epithelial cells. Endothelia negative.
Mucosa of small intestine	Endothelia	Occasional weak staining of epithelial cells of the crypts. Endothelia positive.
Lung	Apical surfaces of the bronchiolar epithelial cells. Endothelia negative.	Same
Kidney	Particulate staining within cells of the distal convoluted tubules. Endothelia negative.	Negative
Brain	Negative	Same

the important point that our purified preparations did not consist of an artifactually produced peptide with an M_r coincident with that of the MFGM form of PAS IV. However, the observation that Na_2CO_3-washed, plasmalemma-enriched fractions from mammary tissue also contain an immunoreactive protein of slightly higher M_r implies that the native form of PAS IV may have an M_r from 80,000 to 85,000. The protein of M_r = 76,000 in MFGM may therefore be the product of limited proteolysis, either during or after secretion.

In mammary secretory epithelium, PAS IV is present in high concentrations at the apical pole of the cells. This was expected because MFGM is known to be derived largely from apical plasma membrane during the secretion of milk triacylglycerols (2, 21, 61). Whether this glycoprotein is also expressed on basolateral membranes remains uncertain. Weak but apparently specific immunofluorescence was often seen on all surface domains of the secretory cells, in contrast to antibodies specific for other MFGM constituents which appear to only decorate apical surfaces (references 4, 20, 21, and 31 and V. G. Johnson, D. E. Greenwalt and I. H. Mather, unpublished observations).

The most significant observation in this present study was the localization of PAS IV in capillary endothelial cells of many tissues, including the mammary gland. Unlike xanthine oxidase, which is also located in both capillary cells and the MFGM (11, 40), PAS IV is a glycoprotein and firmly bound to membranes. From the immunoblotting data described in this study and other work (40, 54), it is obvious that PAS IV and xanthine oxidase are unrelated. Nor is this glycoprotein related to secretory component, lactoferrin, or lactoperoxidase, all proteins present in bovine whey and with molecular weights similar to that of PAS IV (13, 29, 30).

We considered the possibility that PAS IV is one of the other well-characterized markers of endothelial cells previously described in the literature. Endothelial cells are known to express a number of antigens including a serum albumin-binding glycoprotein (67), complement $C1_q$ receptors (3), factor VIII/von Willebrand factor (39, 41), angiotensin-converting enzyme (5, 15), thrombospondin (57), thrombomodulin (18), plasminogen activators (48), and lipoprotein lipase (38, 53, 69). By the criteria of molecular size, chemical composition, or tissue distribution, PAS IV appears to be distinct from any of these antigens. This glycoprotein also appears to be different from less well characterized endothelial cell antigens defined by either monoclonal antibodies (17, 42, 62) or labeling techniques (73). Nor are the properties of PAS IV similar to the class I or class II antigens of the major histocompatibility complex (58) which are also expressed on capillary endothelial cells (25, 60, 64, 65) and, in the case of the HLA-DR and Ia antigens, on human MFGM (76) and rodent mammary cells (45), respectively. The apparent absence of PAS IV from the surface of peripheral blood cells also argues against this possibility.

PAS IV therefore appears to be a distinct membrane-bound antigen which is expressed in capillary endothelial cells in certain tissues and on the apical surfaces of the secretory epithelium of mammary cells and the bronchiolar epithelium of lung. The dual location of PAS IV in both exposed external epithelial surfaces in mammary gland and lung, and in capillary endothelial cells in many tissues, might imply that this glycoprotein has some protective function in the overall defense of the host against disease. Several of the previously recognized antigens of capillary endothelial cells are components of the immune system (3, 60, 64, 65). The function and identity of PAS IV, however, remains unknown.

Expression of PAS IV in capillary endothelial cells appears to vary between tissues. Capillaries of mammary gland, heart, liver, salivary gland, spleen, pancreas, or intestine contain variable levels of the antigen. However, the endothelial cells of kidney, brain, lung, or large blood vessels are apparently unreactive with either monoclonal or polyclonal antibody. These observations are consistent with the general hypothesis that endothelial cells from different tissues express different levels and complements of proteins and that these differences reflect the distinctive origins of capillary cells in embryonic tissues during development (5).

There is a current need for markers of capillary cells in tissues. Many of the known endothelial proteins are not

specific for capillary cells, are of uncertain specificity, or are present in relatively low quantities and are not readily available for study. The widely used capillary marker factor VIII/von Willebrand factor (39, 41) is a soluble protein present in megakaryocytes and platelets as well as associated with Weibel/Palade bodies in endothelial cells and in the extracellular matrix (33). To our knowledge, the only other practical marker that is commercially available is the lectin from *Ulex europaeus* (32) which binds to certain fucosyl residues. But this lectin binds to at least three different proteins in human umbilical vein endothelial cells (34) and is unlikely to be specific for capillary cells.

Specific membrane protein markers for endothelial cells, such as PAS IV, could be used for studies on the origin and development of organs 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. Since an immunologically similar protein to PAS IV is present in human breast milk, this glycoprotein may prove to be useful as a marker of capillary cells in human tissues.

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