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Specific antibodies to PAS IV, a glycoprotein of bovine milk-fat-globule membrane, bind to a similar protein in cardiac endothelial cells and epithelial cells of lung bronchioles

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We recently described the tissue distribution of PAS IV (periodic acid/Schiff-positive Band IV), a hydrophobic glycoprotein isolated from bovine milk-fat-globule membrane [Greenwalt & Mather (1985) *J. Cell Biol.* **100**, 397–408]. By using immunofluorescence techniques, PAS IV was detected in mammary epithelial cells, the bronchiolar epithelium of lung, and the capillary endothelium of several tissues, including heart, salivary gland, pancreas, spleen and intestine. In the present paper we describe the specificity of the antibodies used for these studies. Two monoclonal antibodies, E-1 and E-3, were shown by solid-phase immunoassay and immunoaffinity chromatography to be specific for PAS IV (of M_r 76000) in milk-fat-globule membrane and recognize a glycoprotein of slightly higher M_r (85000) in heart. Affinity-purified rabbit antibodies to PAS IV were also shown to recognize components of M_r 76000 and 85000 in fat-globule membrane and heart respectively, by using immunoblotting procedures after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Additionally, an immunoreactive protein in lung of M_r 85000 was detected. Despite these differences in molecular size, the fat-globule membrane and heart forms of PAS IV were shown to be very similar by peptide-mapping techniques. The possible significance of the expression of similar forms of PAS IV in both epithelial and capillary endothelial cells is briefly discussed.

We recently described the purification and characterization of a glycoprotein, PAS IV, present in bovine milk associated with the fat-globule membrane (Greenwalt & Mather, 1985). By using immunofluorescence techniques and affinity-purified polyclonal antibodies, PAS IV was detected in the apical membranes of secretory-epithelial cells in mammary tissue at the resolution of the optical microscope. In view of the derivation of milk-fat-globule membrane from the apical surfaces of mammary secretory cells, this result was not unexpected [for reviews on the origin of this membrane, see Anderson & Cawston (1975), Patton & Keenan (1975) and McPherson & Kitchen (1983)]. Several other fat-globule membrane components are similarly concentrated in apical membranes, including mucin-like glycoproteins of high M_r and butyrophilin in certain species (Arklie *et al.*, 1981; Franke *et al.*, 1981; Foster *et al.*, 1982; Heid *et al.*, 1983; Johnson & Mather, 1985) and glycoproteins of M_r approx. 100000 and 55000 in the cow and the guinea pig respectively (Johnson & Mather, 1985; C.S. Kaetzel & I. H. Mather, unpublished work). None of these proteins appears to be expressed in other cell types in mammary tissue, and they are therefore useful epithelial-cell markers. In contrast with these results, PAS IV was also detected in significant amounts in capillary endothelial cells in mammary tissue, and in certain other organs and tissues appeared to be largely restricted to this cell type (Greenwalt & Mather, 1985). In one notable exception, intense immunofluorescence was observed in the bronchiolar epithelial cells of the lung,

Abbreviations used: SDS, sodium dodecyl sulphate; PAS IV, periodic acid/Schiff-positive Band IV.

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and the capillary cells of lung were unreactive with either polyclonal or monoclonal antibodies.

The tissue distribution of this glycoprotein, however, was determined by using the single technique of immunofluorescence microscopy, and the results were based on the assumption that the antibodies employed were absolutely specific. In this present paper we describe the specificity of the monoclonal antibodies used in these studies and show, by immunoblotting techniques, immunoaffinity chromatography and peptide-mapping procedures, that the antibodies used in our previous studies recognize closely related, if not identical, forms of PAS IV in epithelial and capillary endothelial cells.

Materials and methods

Materials

Acrylamide, bisacrylamide, *NNN'N'*-tetramethylethylenediamine, agarose, Triton X-114, Triton X-100, silver-stain reagents and nitrocellulose membranes were purchased from Bio-Rad Laboratories (New York, NY, U.S.A.). Aminopterin, ammonium persulphate, bovine serum albumin (fraction V), hypoxanthine, *p*-nitrophenyl phosphate, n-propyl gallate, SDS, thymidine, Tris, papain (Type IV) and trypsin (Type XI) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Freund's adjuvant (complete), foetal-calf serum, glutamine, penicillin, streptomycin, RPMI 1640 culture medium and Dulbecco's modified Eagle's medium were all obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.). *Staphylococcus aureus* V8 proteinase, alkaline-phosphatase-conjugated rabbit anti-(mouse IgG) and rabbit antibodies to mouse immuno-globulin heavy chains, α, γ_1, γ_{2a}, γ_{2b}, γ_3 and μ, and immunoglobulin light chains, κ and λ, were purchased from Miles Laboratories (Elkhart, IN, U.S.A.), and biotinylated horse anti-(mouse IgG) and fluorescein-conjugated avidin were obtained from Vector Laboratories (Burlingame, CA, U.S.A.). Pristane was purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.) and CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). ^{125}I-labelled goat anti-(rabbit IgG) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Methods

Preparation of membrane fractions from bovine tissues and milk. Fat-globule membrane was prepared from bovine milk as described by Mather (1978). Microsomal membrane fractions were prepared from tissues obtained from Holstein cows at slaughter at the Beltsville Agricultural Research Center abattoir (Beltsville, MD, U.S.A.). Tissues were minced with scissors and homogenized in 3 vol. (w/v) of Tris-buffered saline [10 mM-Tris/HCl, pH 7.4, containing 0.15 M-NaCl, 5 mM-6-aminohexanoic acid, 0.5 mM-phenylmethanesulphonyl fluoride and aprotinin (650 kallikrein inhibitor units/ ml)] in a Sorvall Omnimixer at maximum speed for 30 s at 4°C. Homogenates were centrifuged at 1000*g* for 10 min and the resulting supernatants were further centrifuged for 10 min at 10000*g*. The 10000*g* supernatants were then centrifuged at 95000*g* for 1 h and the final membrane sediments and supernatants designated 'microsomal membrane fractions' and 'post-microsomal supernatants' respectively.

Preparation of purified PAS IV. PAS IV was purified by a four-step procedure involving digestion of bovine milk-fat-globule membrane with papain, followed by solubilization with Triton X-100 and chromatography on DEAE-cellulose and concanavalin A–agarose, as previously described (Greenwalt & Mather, 1985).

Immunization of mice. BALB/c mice were immunized by intraperitoneal injections of 100 μg of purified PAS IV emulsified in Freund's complete adjuvant. At 3 and 6 weeks after the initial immunization, the mice were injected a second and third time with 100 μg of PAS IV. At 3 days after the final injection, the mice were bled from the tail and the collected serum tested for the presence of antibody to PAS IV by an enzyme-linked solid-phase immunoassay. In this assay, 96-well plastic plates (Dynatech Laboratories, Alexandria, VA, U.S.A.) were coated with purified PAS IV (0.25 μg of protein/well) and mouse antibody bound to the immobilized antigen detected with alkaline phosphatase-conjugated rabbit anti-(mouse IgG). Procedures for this assay were essentially as described by Mather *et al.* (1982).

Hybridization techniques and production of monoclonal antibodies. Spleen cells (2×10^8) from two mice were fused with SP2/0 myeloma cells (2×10^7) as described by Kaetzel *et al.* (1984). Supernatants from positive clones were tested by enzyme-linked solid-phase immunoassay, and two hybridomas (E-1 and E-3) were subcloned four and three times respectively. These two hybridoma cell lines were subsequently grown as solid subcutaneous tumours and as ascites in pristane-primed mice.

Preparation of affinity-purified polyclonal antibodies to PAS IV. Polyclonal rabbit antibody to PAS IV was prepared as previously described (Greenwalt & Mather, 1985). Briefly, purified PAS IV was separated by SDS/polyacrylamide-gel electrophoresis and slices of polyacrylamide con-

taining the protein were mixed with Freund's complete adjuvant and used as the immunogen. A 100μg portion of protein was injected once a month for 3 months. Affinity-purified antibodies were obtained from the collected rabbit serum by adsorption to, and elution from, PAS IV immobilized on Sepharose 4B.

Electrophoresis. Samples were separated by SDS/polyacrylamide-gel electrophoresis in 7.5% (w/v)-acrylamide slab gels as described by Laemmli (1970). Proteins were stained with Coomassie Blue as previously described (Mather *et al.*, 1980), or with the Bio-Rad silver stain, based on the methods of Merril *et al.* (1979).

Immunoblotting procedures. Protein samples were separated by SDS/polyacrylamide-gel electrophoresis and electrophoretically transferred to nitrocellulose paper, essentially as described by Towbin *et al.* (1979). The nitrocellulose paper was then rinsed sequentially with 0.1% (v/v) Triton X-100 in Tris-buffered saline (without proteinase inhibitors) for 1h, and overnight with Tris-buffered saline containing 2% (w/v) bovine serum albumin. Affinity-purified rabbit antibodies to PAS IV, diluted 1:200 in Tris-buffered saline (7.5μg of protein/ml) were then incubated with the paper for 1h and unbound antibody removed by washing sequentially with Tris-buffered saline containing 0.5% (v/v) Triton X-100 (for 30min), 0.5M-NaCl (for 30min), no additions (for 30min), and 0.1% (w/v) bovine serum albumin (for 30min). The specifically bound rabbit antibodies were then detected by incubating the paper for 2h with ^{125}I-labelled goat anti-(rabbit IgG) (approx. 2μCi) in Tris-buffered saline containing 2% (w/v) bovine serum albumin. Unbound antibody was removed by washing sequentially with Tris-buffered saline containing 0.1% (w/v) bovine serum albumin (for 30min), 0.1% (v/v) Triton X-100 (for 30min), 0.5% (v/v) Triton X-100 for 30min, 0.5M-NaCl (for 30min) and two 30min washes with no additions. The nitrocellulose membrane was then dried and exposed, at -80°C, to X-ray film (Kodak XAR-5) for appropriate times. Autoradiographs were developed by standard procedures. Pre-immune serum was used in the place of affinity-purified rabbit antibodies in control experiments.

Characterization of monoclonal antibodies. Determination of isotype. Samples of culture supernatant were tested against specific rabbit antibodies to mouse α-, γ_1-, γ_{2a}-, γ_{2b}-, γ_3- and μ-heavy chains and κ- and λ-light chains by immunodiffusion analysis in agarose gels (Ouchterlony & Nilsson, 1978). Both monoclonal antibodies, E-1 and E-3, were determined to be of IgG_1 (κ-light chain) isotype.

Immunoaffinity chromatography. Monoclonal antibodies E-1 and E-3 were purified from ascites fluid by precipitation with $(NH_4)_2SO_4$ and immunoaffinity chromatography on sheep anti-(mouse Ig) covalently linked to Sepharose 4B. The affinity-purified antibodies were then separately coupled to CNBr-activated Sepharose 4B and unbound antibody removed by washing extensively with 0.1M-sodium borate buffer, adjusted to pH8.0 with HCl, and 0.1M-sodium acetate buffer, adjusted to pH4.0 with acetic acid. The immunoadsorbents were equilibrated and stored at 4°C in Tris-buffered saline, pH7.5, containing 0.1% (w/v) NaN_3. To test the specificities of the immobilized antibodies, the immunoadsorbents were packed into small chromatography columns (5.0cm × 0.8cm) and equilibrated with 60mM-Tris/HCl, pH7.5, containing 0.5M-NaCl. Bovine fat-globule-membrane proteins, solubilized in Triton X-114 (Bordier, 1981), were then fractionated on these columns and specifically bound material eluted with 3M-NaSCN. After removal of the NaSCN by dialysis overnight against 50mM-Tris/HCl, pH7.5 at 4°C, the samples were analysed by electrophoresis in SDS/polyacrylamide gels.

Detection of PAS IV in bovine tissues by immunofluorescence microscopy. Tissue samples were obtained from Holstein cows immediately upon slaughter at the Beltsville Agricultural Research Center abattoir, and frozen in isopentane cooled in liquid N_2. Frozen sections (5μm thick) were dried in air for 1–2h at room temperature and fixed in acetone for 7.5min at -20°C. PAS IV was detected in tissue sections by sequential incubations with a 1:100-fold dilution of ascites fluid, followed by biotinylated horse anti-(mouse IgG) (43μg/ml), followed by avidin–fluorescein (50μg/ml). All dilutions were made in Tris-buffered saline, pH7.5, and slides were rinsed three times with this solution between each incubation. Serum from unimmunized mice at a 1:100-fold dilution in Tris-buffered saline, pH7.5, was used instead of ascites fluid in control slides. Specimens were mounted in glycerol containing 5% (w/v) n-propyl gallate (Giloh & Sedat, 1982).

Peptide-mapping procedures. Protein samples previously purified by immunoaffinity chromatography and SDS/polyacrylamide-gel electrophoresis were treated, in gel slices, with various proteinases, essentially as described by Cleveland *et al.* (1977). Gel slices contained in the wells of 4%-(w/v)-polyacrylamide stacking gels were incubated with papain (0.01 unit/well), *Staphylococcus* V8 proteinase (0.03 Kunitz unit/well), or trypsin (0.6 unit/well). The resulting peptides were then separated by electrophoresis in 10% (w/v) polyacrylamide separating gels, and the peptides stained with silver. Controls, in which only proteinase was used, produced no visible bands of protein in the stained gels.

Results

The antibodies used for the identification of PAS IV in various bovine tissues were an affinity-purified preparation of polyclonal rabbit antibodies and murine monoclonal antibodies E-1 and E-3. Specificity of the rabbit antibodies was determined by immunoblotting techniques, after separation of bovine milk-fat-globule membrane and cell fractions from lactating mammary tissue by SDS/polyacrylamide-gel electrophoresis and electrophoretic transfer of the separated proteins to nitrocellulose paper (Greenwalt & Mather, 1985). However, binding of monoclonal antibodies E-1 and E-3 to the separated mammary proteins could not be detected by using similar techniques. Evidently these antibodies recognize conformational determinants on PAS IV that are destroyed by either treatment with SDS or separation by electrophoresis in polyacrylamide gels. The specificity of both monoclonal antibodies was therefore determined by solid-phase immunoassay and immunoaffinity chromatography (Figs. 1 and 2). Immunotitration of sera from mice carrying cell lines E-1 and E-3 as solid subcutaneous tumours demonstrated binding of the monoclonal antibodies to purified PAS IV (Fig. 1). Antibody was detected in serum samples diluted at least 50000-fold in both cases. Specificity was confirmed by immunoaffinity chromatography. Bovine milk-fat-globule membrane proteins were solubilized in Triton X-114 solutions at 0°C and the mixtures allowed to undergo phase separation at room

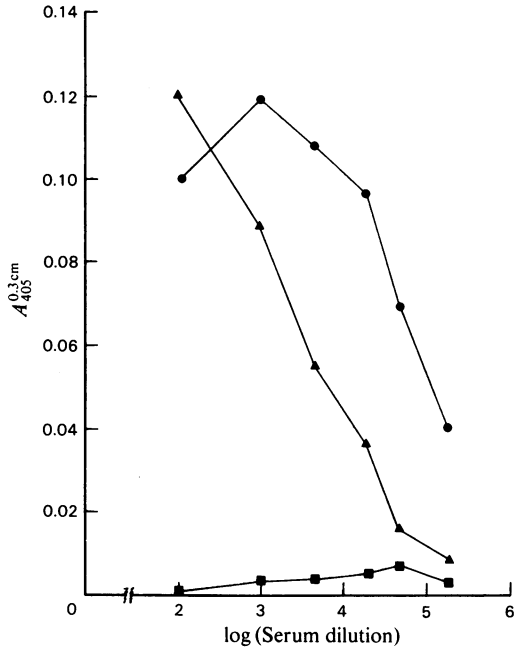

Fig. 1. *Immunotitration of sera from mice carrying antibody-secreting cell lines E-1 and E-3 as solid subcutaneous tumours*

Serum samples from mice bearing tumours of cell lines E-1 (●) and E-3 (▲) were diluted down to 1:256000, and various dilutions were tested by solid-phase enzyme-linked immunoassay using plastic plates coated with purified PAS IV (0.25μg of protein/well). Serum from normal mice was used as a control (■).

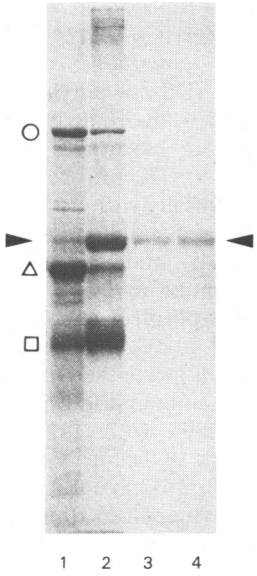

Fig. 2. *Immunoaffinity chromatography of detergent-solubilized milk-fat-globule-membrane proteins on E-1 and E-3 antibodies covalently linked to Sepharose 4B*

Bovine milk-fat-globule membrane (1.0mg of membrane protein/ml) was solubilized in Triton X-114 and the detergent phase of the extract equilibrated in 60mM-Tris/HCl, pH7.5, containing 0.5M-NaCl. The detergent-solubilized proteins were then fractionated on immunoadsorbents consisting of either monoclonal antibody E-1 or E-3 covalently linked to Sepharose 4B. Specifically bound protein was eluted with 3M-NaSCN and peak fractions were analysed by electrophoresis in SDS/(7.5%, w/v) polyacrylamide gels and compared with unfractionated and detergent-solubilized milk-fat-globule-membrane proteins. Gels were stained with Coomassie Blue. Samples were as follows: lane 1, milk-fat-globule membrane; lane 2, Triton X-114 detergent phase from extract of milk-fat-globule membrane; lane 3, eluate from E-1–Sepharose 4B; lane 4, eluate from E-3–Sepharose 4B. The solid arrowhead (►) to the left and right of the Figure indicates the position of PAS IV. Major proteins of bovine milk-fat-globule membrane and their respective M_r values are identified by symbols to the left of the Figure; ○, xanthine oxidase (155000); △, butyrophilin (67500); □, Band 16 (46000) (Mather & Keenan, 1975; Mather *et al.*, 1980).

temperature (Bordier, 1981). Because PAS IV is hydrophobic (Greenwalt & Mather, 1985), the bulk of this glycoprotein partitions into the detergent layer during phase separation (cf. lanes 1 and 2, Fig. 2). This detergent phase was therefore used as a source of soluble PAS IV for immunoaffinity chromatography. When such Triton X-114 extracts were fractionated on either E-1 or E-3 monoclonal antibody covalently bound to Sepharose 4B, PAS IV was the only protein specifically bound to, and eluted from, the immunoadsorbents (lane 3 and 4, Fig. 2). Specificity of the E-1 monoclonal antibody was also shown by the fractionation of detergent-solubilized heart proteins on the immobilized antibody (see below).

When either antibody E-1 or E-3 was used to detect PAS IV-like proteins in bovine tissues, pronounced immunofluorescence was observed in capillary endothelial cells. Antibody binding was detected in mammary gland, pancreas, salivary gland, spleen (examples for E1 antibody in Fig. 3), heart, the smooth muscle of intestine and liver (Greenwalt & Mather, 1985). No reaction of either antibody with the capillaries of lung, brain or kidney was observed. Binding of either monoclonal antibody to the secretory epithelial cells of mammary tissue was surprisingly weak considering the derivation of fat-globule membrane from these cells. The reason for this is unclear. Possibly fixation of the tissue in acetone selectively denatured the E-1 and E-3 epitopes of PAS IV in epithelial cells as opposed to capillary endothelial cells, because the antigen is present in different cellular environments. Polyclonal rabbit antibodies to PAS IV, which presumably bind to multiple determinants, strongly decorated capillary endothelial cells and the apical membranes of secretory cells in mammary tissue (Greenwalt & Mather, 1985). Both polyclonal and monoclonal antibodies also bound to the epithelial cells of lung bronchioles and secreted luminal material, although the alveolar epithelium was unreactive (Greenwalt & Mather, 1985).

By using the affinity-purified rabbit antibodies to PAS IV, cross-reactive proteins were detected by immunoblotting techniques in the microsomal membrane fractions of heart and lung (arrowheads in Fig. 4). Traces of an immunoreactive protein in

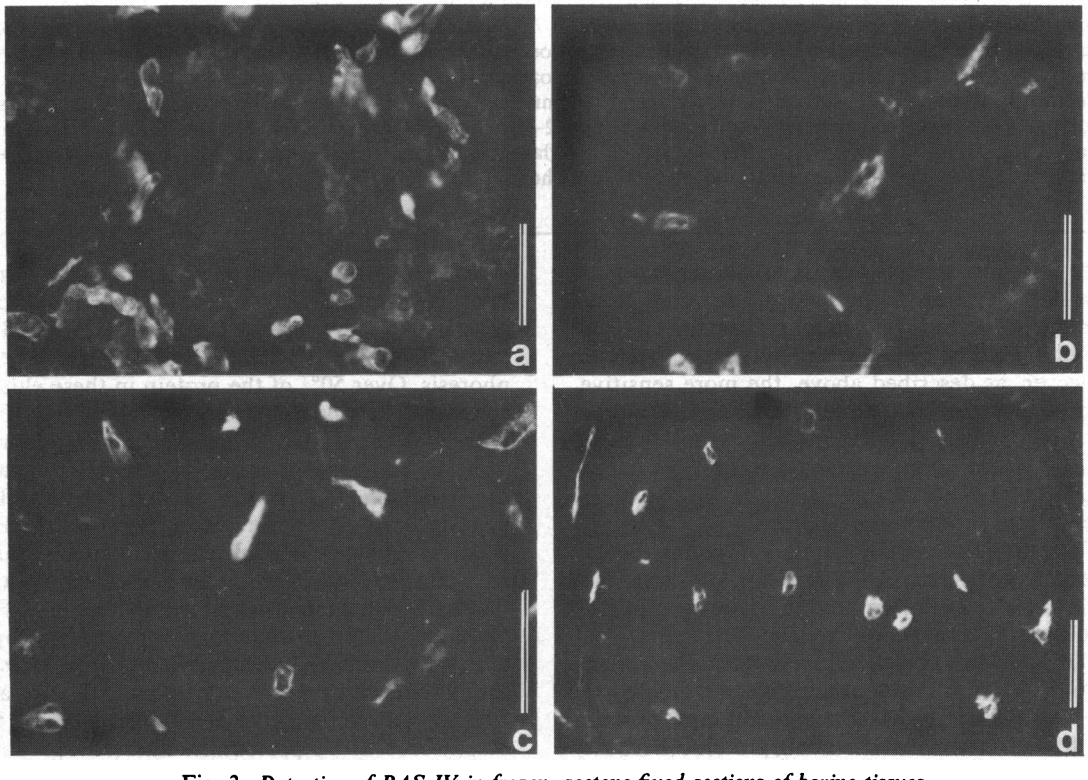

Fig. 3. *Detection of PAS IV in frozen, acetone-fixed sections of bovine tissues*
Sections were treated sequentially with E-1 monoclonal antibody, horse anti-(mouse IgG) conjugated with biotin and fluorescein–avidin as described in the Materials and methods section. Tissues were as follows: a, lactating mammary gland; b, pancreas; c, salivary gland; d, spleen. The bar represents 50 μm in all cases.

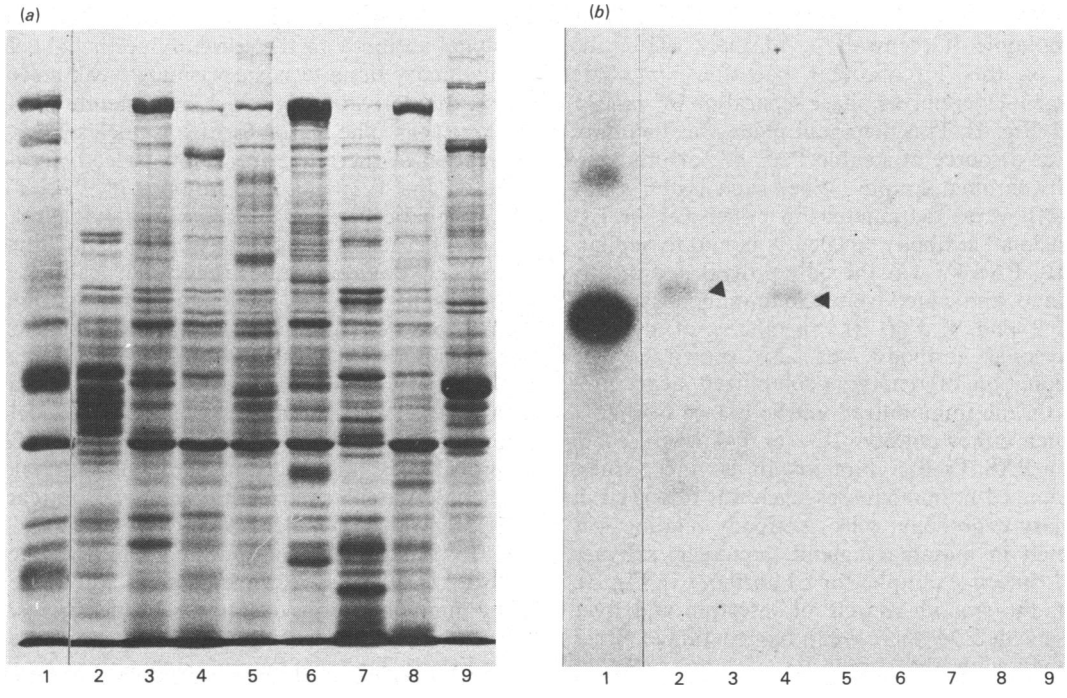

Fig. 4. *Distribution of PAS IV in bovine tissues determined by SDS/polyacrylamide-gel electrophoresis and immunoblotting techniques*

Samples of milk-fat-globule membrane and microsomal-membrane fractions (approx. 20μg of protein/sample) from bovine tissues were separated by electrophoresis in 7.5%-(w/v)-polyacrylamide gels containing SDS, and the separated proteins electrophoretically transferred to nitrocellulose paper. PAS IV was localized by using affinity-purified polyclonal rabbit antibody and ^{125}I-labelled goat anti-(rabbit IgG), followed by autoradiography. (*a*) Coomassie Blue-stained proteins; (*b*) corresponding immunoblot. Milk-fat-globule membrane in lane 1 was compared with microsomal membrane fractions in lanes 2–9 from the following tissues; 2, heart; 3, liver; 4, lung; 5, luminal 'scrapings' of intestine; 6, kidney; 7, salivary gland; 8, pancreas and 9, spleen. Major immunoreactive proteins in heart and lung are indicated by solid arrowheads (◀).

liver were also detected in some autoradiographs (not shown). Other tissues tested, including intestine, kidney, salivary gland, pancreas, spleen (Fig. 4) and brain (not shown) were apparently negative. However, as described above, the more sensitive immunofluorescence technique showed some cross-reaction of PAS IV antibody with all these tissues, except brain. In no case was any evidence obtained of PAS IV-like proteins in the post-microsomal supernatant fractions.

The immunologically similar proteins determined by immunoblotting in heart and lung had different M_r values from that of the protein in fat-globule membrane (compare lanes 1, 2 and 4, Fig. 4). In 7.5%-(w/v)-polyacrylamide gels, the apparent M_r values of these proteins were estimated to be 76000 for the fat-globule-membrane protein and 85000 for the heart and lung proteins. Because of these differences in apparent molecular size, the heart and fat-globule-membrane proteins were compared by peptide-mapping procedures. Triton X-100-solubilized microsomal membranes from heart were fractionated on E-1 monoclonal antibody covalently linked to Sepharose 4B. Specifically bound material was eluted with 3M-NaSCN and analysed by SDS/polyacrylamide-gel electrophoresis. Over 50% of the protein in these eluates consisted of a single component of M_r 85000. This protein was excised from the polyacrylamide slab gel and treated in gel slices, with papain, *Staphylococcus* V8 proteinase or trypsin. The peptide fragments were then separated by SDS/polyacrylamide-gel electrophoresis and compared with purified PAS IV, from fat-globule membrane, that had been treated in an identical manner (Fig. 5). Both the heart and mammary proteins appeared to be very similar. At least eleven polypeptides of similar size were present in the papain digests, and five similar components were identified in the *Staphylococcus*-V8-proteinase-digested preparations. Both proteins were relatively resistant to trypsin digestion under the conditions employed, and only one peptide of slightly lower M_r than that of the native proteins was detected. This was not

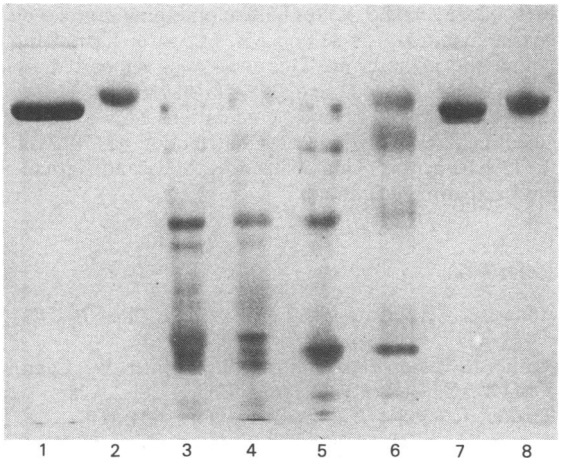

Fig. 5. *Comparison of PAS IV from milk-fat-globule membrane and heart by peptide-mapping procedures*
PAS IV purified from either milk-fat-globule membrane or heart, as described in the text, was separated by SDS/polyacrylamide-gel electrophoresis, and gel slices containing the separated proteins treated with papain, *Staphylococcus* V8 proteinase or trypsin. The proteolytically cleaved proteins were then compared by electrophoresis in 10%-(w/v)-polyacrylamide gels containing SDS, and the separated peptides located with silver stain. The mammary protein in lanes 1, 3, 5 and 7 is compared with the heart protein in lanes 2, 4, 6 and 8. Lanes 1 and 2, untreated purified proteins; lanes 3 and 4, papain-treated proteins; lanes 5 and 6, *Staphylococcus*-V8-proteinase-treated proteins; lanes 7 and 8, trypsin-treated proteins.

because the trypsin had been inactivated by SDS present in the polyacrylamide gels. Control experiments showed that xanthine oxidase under identical conditions was degraded by trypsin into at least 28 peptides. Despite the differences in M_r, the heart and mammary proteins therefore appeared to be very similar.

Discussion

PAS IV was originally identified as a component of bovine milk-fat-globule membrane by electrophoresis in SDS/polyacrylamide gels (Mather *et al.*, 1980). The protein contains mannose, galactose and sialic acid as principal sugars and has relatively basic isoelectric points, especially after removal of sialic acid residues (Greenwalt & Mather, 1985). When affinity-purified rabbit antibodies and monoclonal antibody E-1 were used in immunolocalization studies, pronounced staining of capillary endothelial cells was observed in many tissues. A tacit assumption in Greenwalt & Mather's (1985) study was that the antibodies were specific for PAS IV and that the immunofluorescence observed was due to the expression of PAS IV or PAS IV-like proteins in these tissues. The use of monoclonal antibodies, however, is no guarantee of specificity, because certain epitopes may be carried on several proteins that are otherwise unrelated (see, e.g., Nigg *et al.*, 1982).

Because of these uncertainties, the presence of PAS IV or PAS IV-like proteins in tissues other than the mammary gland was confirmed by immunoblotting, immunoaffinity chromatography and peptide-mapping techniques. By using these approaches, proteins similar to PAS IV were detected in microsomal membranes from heart and lung. Affinity-purified rabbit antibodies to the mammary form of PAS IV consistently bound to a component of M_r 85000 in both tissues (Fig. 4). PAS IV-like proteins were not detected by immunoblotting procedures in several tissues that bound antibody in frozen acetone-fixed sections (e.g. salivary gland, pancreas and spleen, Fig. 3). This apparent lack of reaction is probably due to the expression of PAS IV in amounts that are undetectable by immunoblotting techniques. Determination of the content of PAS IV in different tissues by an ultra-sensitive radioimmunoassay may help to resolve this question.

Isolation of PAS IV from heart by immunoaffinity chromatography on immobilized monoclonal antibody E-1, followed by peptide mapping with three proteinases, clearly showed that the capillary-endothelial form of PAS IV is similar to the epithelial (fat-globule membrane) form of this glycoprotein. The observed molecular sizes of PAS IV from heart and lung, however, were estimated to be several thousand daltons larger than that of the form of PAS IV associated with fat-globule membrane. The most likely explanation of this observation is that PAS IV associated with fat-globule membrane is degraded by proteinases, either during or after secretion, and that this glycoprotein is initially the same in all tissues. Mammary microsomal membranes isolated from lactating mammary tissue were previously shown to contain two immunoreactive proteins, one of M_r 76000, similar to that of PAS IV in fat-globule membrane, and the other of M_r 85000, similar to that of PAS IV in heart and lung (Greenwalt & Mather, 1985). However, the observed differences in molecular size may also reflect post-translational modifications, including variable glycosylation reactions of an initial translation product that is identical in all tissues. A more intriguing possibility is that PAS IV is truly polymorphic and exists in tissue-specific and cell-specific forms. Resolution of this question will require translation, *in vitro*, of mRNA species specific for PAS IV isolated from several tissues.

The expression of PAS IV in both exposed apical surfaces of epithelia in 'external' tissues such as lung and mammary gland and in capillary endothelial cells suggests that this glycoprotein may function as a component of the immune system. The size, composition and immunological properties of PAS IV, however, argue against it being one of several well-characterized proteins such as secretory component, one of the Ig isotypes, or the previously described components of capillary endothelial cells (discussed in detail by Greenwalt & Mather, 1985). A possible test of the hypothesis that PAS IV protects the host against disease would be to monitor the levels and distribution of this glycoprotein in milk during cases of bacterially caused mastitis. Such experiments are feasible because the mammary gland is an external organ and the secretion is readily available for analysis.

Interestingly, PAS IV is the second known example of a protein secreted in association with fat-globule membrane that is also expressed in capillary endothelial cells in the mammary gland and other tissues. The first well-documented example was xanthine oxidase (Jarasch *et al.*, 1981), a soluble cytoplasmic redox enzyme that is concentrated in milk-fat globules and becomes partly membrane-bound during or after secretion (Mather *et al.*, 1977, 1982; Bruder *et al.*, 1982). There is currently no reason to suspect, however, that PAS IV and xanthine oxidase are related either functionally or structurally. The tissue distributions of the two proteins are similar, but not identical (Jarasch *et al.*, 1981; Greenwalt & Mather, 1985), and they show no apparent immunological similarities (see, e.g., immunoblots in Jarasch *et al.*, 1981; Mather *et al.*, 1982; Greenwalt & Mather, 1985; and Fig. 2, the present study). The two proteins also differ markedly in physical properties, since PAS IV is hydrophobic and associated with membranes and the bulk of xanthine oxidase in cells and tissues is soluble. Although the functions of xanthine oxidase and PAS IV in mammary tissue remain unknown, it is interesting and possibly significant that these proteins are both expressed in capillary cells, and are the only known exceptions to the general observation that fat-globule-membrane proteins are specific to epithelial cells and highly concentrated in the apical pole of mammary secretory cells during lactation (Arklie *et al.*, 1981; Franke *et al.*, 1981; Jarasch *et al.*, 1981; Foster *et al.*, 1982; Greenwalt & Mather, 1985; Johnson & Mather, 1985).

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