PAS IV, an Integral Membrane Protein of Mammary Epithelial Cells, Is Related to Platelet and Endothelial Cell CD36 (GP IV)†

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ABSTRACT: PAS IV is a 78-kDa (bovine) to 80-kDa (human) integral membrane glycoprotein of unknown function which is found in mammary epithelial cells. We now report the purification of human PAS IV and native bovine PAS IV from the milk fat globule membrane (MFGM), a preparation of apical plasmalemma from epithelial cells of lactating mammary tissue. N-Terminal sequence analyses of human and bovine PAS IV revealed homology to the N-terminal sequence of the 88-kDa human endothelial and platelet glycoprotein CD36. The similarity of MFGM PAS IV to platelet CD36 was further established by immunoblots of purified platelet CD36 and MFGM PAS IV with MFGM PAS IV specific antiserum. The removal of N-linked oligosaccharides from PAS IV and CD36 by treatment with endoglycosidase F reduced the apparent M_r of both proteins to approximately 57 000. These data suggest that PAS IV and CD36 are similar if not identical polypeptides that undergo cell type specific glycosylation.

The milk fat globule membrane (MFGM)[1] is a homogeneous membrane preparation derived primarily if not completely from the apical plasmalemma of mammary secretory epithelial cells during the secretion of milk triacylglycerols (Mather, 1987; Keenan et al., 1988). Morphological and enzyme marker studies indicate that the MFGM consists of a unique fraction of the apical plasmalemma in which certain proteins have been selectively concentrated (Peixoto de Menezes & Pinto da Silva, 1978; Mather & Keenan, 1983). Bovine MFGM contains seven periodic acid/Schiff-positive proteins after separation on a SDS–polyacrylamide gel, and these are numbered I–VII according to the accepted nomenclature (Mather et al., 1980). The protein constituents of the MFGM have been described for several different mammals, and most have been purified to homogeneity (Mangino & Brunner, 1977; Franke et al., 1981; Sullivan et al., 1982; Shimizu & Yamauchi, 1982; Greenwalt & Mather, 1985; Johnson et al., 1988). Butyrophilin, an integral membrane protein of 67 kDa, and xanthine oxidase are the major proteins of the MFGM (Franke et al., 1981; Mather, 1987). Although greatly concentrated in the MFGM, the in vivo functions of these and other MFGM proteins are unknown. The only MFGM protein to be cloned is PAS-0, a mucin-like glycoprotein of greater than 400 kDa (Gendler et al., 1987).

Bovine PAS IV, a hydrophobic integral membrane glycoprotein of 78 kDa, constitutes 2–5% of the MFGM protein. Bovine PAS IV has been purified and localized to secretory epithelial cells of lactating mammary tissue and the capillary endothelium of the mammary gland, heart, and other tissues (Greenwalt & Mather, 1985; Greenwalt et al., 1985). In this study, we demonstrate that human and bovine PAS IV are related to the endothelial cell and platelet glycoprotein CD36 [also referred to as GP IV (Shaw, 1987)], one of several major proteins of the platelet cell surface (Clemetson & McGregor, 1987). CD36 has recently been shown to bind to thrombospondin, a large adhesive protein released by platelets upon activation (Asch et al., 1987; Silverstein et al., 1989; McGregor et al., 1989), to collagen fibrils (Tandon et al., 1989b), and to erythrocytes infected with the mature *Plasmodium falciparum* parasite (Barnwell et al., 1985, 1989; Ockenhouse et al., 1989; Oquendo et al., 1989). The N-terminal sequences of human and bovine PAS IV and CD36 are homologous, and all four proteins react with antiserum to human PAS IV. In addition, we show that human and bovine MFGM PAS IV, bovine endothelial cell CD36, and human platelet CD36, which have molecular masses of 80, 78, 85, and 88 kDa, respectively, all have M_r values of approximately 57 000 after deglycosylation. This suggests that cell type specific processing gives rise to differently glycosylated forms of CD36.

Experimental Procedures

Materials

Aprotinin, glycine, SDS, Tris, bovine serum albumin, PMSF, Triton X-114, Triton X-100, nitro blue tetrazolium, amino-*n*-caproic acid, and SDS–PAGE molecular weight standards were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide was purchased from Fisher Scientific (Fair Lawn, NJ). BCIP was purchased from U.S. Biochemical Corp. (Cleveland, OH), bicinchoninic acid was obtained from the Pierce Chemical Co. (Rockford, IL), and Ponceau S was purchased from Helena Laboratories (Beaumont, TX). Gel Bond and Gel Gard 2500 films were purchased from FMC Corp. (Rockland, ME) and Hoechst Celanese (Charlotte,

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[1] Abbreviations: MFGM, milk fat globule membrane; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbant assay; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ACD, acid citrate dextrose; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; BCA, bicinchoninic acid; PTH, phenylthiohydantoin; CBS, citrate-buffered saline; TBS, Tris-buffered saline; TBST, Tris-buffered saline–Triton; PBS, phosphate-buffered saline; DEAE, diethylaminoethyl; kDa, kilodaltons; CM, carboxymethyl.

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NC), respectively. Tetramethylethylenediamine, bis(acrylamide), alkaline phosphatase conjugated goat anti-rabbit IgG (H+L), Coomassie Brilliant Blue R-250, and nitrocellulose membrane were obtained from Bio-Rad Laboratories (Richmond, CA). CM-Sepharose, DEAE-Sepharose, and cyanogen bromide activated Sepharose 4-B were purchased from Pharmacia (Piscataway, NJ). Endoglycosidase F was purchased from New England Nuclear (Boston, MA) and lysyl endopeptidase was obtained from Wako Chemicals USA, Inc. (Dallas, TX). Purified human platelet CD36 was a gift of John McGregor and Larry Leung (Stanford Medical Center). Rabbit antiserum to human platelet GPIIb-IIIa was a gift of Leo Lin (Cetus Corp.).

Methods

Preparation of Membranes. Bovine MFGM was prepared by diluting fresh unwashed cream 1:1 with 0.1 M citrate buffer, pH 7.0, containing 0.15 M NaCl (CBS), followed by stirring in a Waring blender at 30% speed at 4 °C until butter formed. The aqueous buttermilk was filtered through four layers of cheesecloth and centrifuged for 1 h at 100000*g*. The membrane pellet was then resuspended in 20 mM Tris, pH 7.4, containing 0.15 M NaCl (TBS) and recentrifuged. Fresh human milk was obtained at a local milk bank and centrifuged at 1000*g* for 10 min at 4 °C. The floating cream layer was resuspended in 10 volumes of CBS at 37 °C and recentrifuged. Washed human cream was stored over liquid nitrogen. Human MFGM was prepared by diluting thawed cream 1:2 with TBS at 37 °C, warming to 30 °C in a water bath, and then cooling to 4 °C. After being stirred in a Waring blender as described above, the mixture was filtered through cheesecloth and the butter fraction warmed to 37 °C in 1 volume of TBS to release trapped membrane fragments. The combined butter wash and buttermilk fractions were then processed as described above. Bovine and human platelet membranes were prepared from platelets isolated from blood collected in ACD. After centrifugation at 1600*g* for 6 min, the supernatant was recentrifuged at 4000*g* for 10 min. The platelet pellet was resuspended in citrate buffer, sonicated, and centrifuged at 10000*g*. The supernatant was then centrifuged at 100000*g* for 1 h to obtain the final platelet membrane preparation.

Purification of Bovine MFGM PAS IV. Human MFGM was extracted in 1% Triton X-114 at a final protein concentration of 5 mg/mL in TBS containing 0.5 mM PMSF, 1% aprotinin, 1 mM aminocaproic acid, and 1 mM EGTA. After 30 min of stirring at 4 °C, the mixture was centrifuged at 100000*g* for 2 h. The supernatant was subjected to phase partitioning (Bordier, 1981) with the following modifications. The supernatant was warmed to 30 °C and then centrifuged at 1000*g* for 10 min. The detergent phase was diluted 10-fold with TBS, cooled to 4 °C in an ice bath, and repartitioned as described above. The resultant detergent phase was then diluted 1:5 with 10 mM Tris, pH 8.0, and dialyzed overnight against 10 mM Tris containing 0.1 Triton X-100. The sample was then chromatographed sequentially on carboxymethyl-Sepharose ion exchange columns at pH values of 8, 7, and 6. Protein that did not bind at pH 8 was dialyzed against pH 7 Tris buffer and rechromatographed on the same column reequilibrated at pH 7. This step was repeated at pH 6 in citrate buffer. Each column was eluted with 50, 100, and 500 mM NaCl prior to reequilibration. In each case, bovine PAS IV was eluted with 0.1 M NaCl.

Purification of Human MFGM PAS IV. Human MFGM was solubilized as described above at a final protein concentration of 1 mg/mL. Detergent-phase proteins were fractionated on DEAE-Sepharose anion exchange resin equilibrated in 10 mM Tris, pH 7.4, containing 0.5% Triton X-100. Human PAS IV was eluted with 75 mM NaCl. Fractions enriched in PAS IV were combined and subjected to SDS–PAGE. Pure PAS IV was electroeluted from an 8% polyacrylamide gel with an Elutrap apparatus as described by the manufacturer (Schleicher & Schuell, Inc., Keene, NH) and dialyzed against TBS containing 0.5% Triton X-100.

Gel Electrophoresis and Immunoblotting. Proteins were separated by SDS–PAGE in 0.75-mm minigel units as described by Laemmli (1965). The molecular weight standards were as follows: myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), BSA (66000), ovalbumin (45000), and carbonic anhydrase (29000). The relative concentrations of individual proteins in SDS–PAGE gels were obtained by densitometry after the gels had been dried between layers of Gel Bond and Gel Gard films. The dried gels, affixed to the Gel Bond, were analyzed in a Beckman Appraise densitometer at 600 nm. For immunoblotting, samples were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979). After transfer, the protein bands were stained with Ponceau S and their positions marked with a pencil. The stain was removed with a 2% BSA/TBS solution and the nitrocellulose was incubated with affinity-purified human MFGM PAS IV specific antibodies diluted 1:200 in TBS containing 0.1% BSA. The nitrocellulose was washed with a series of high-salt and detergent solutions as previously described (Greenwalt & Mather, 1985) and then incubated with 1:1000 alkaline phosphatase conjugated goat anti-rabbit IgG and rinsed a second time. The reactive protein bands were visualized with BCIP as the phosphatase substrate. Control blots utilized 1:200 control sera.

Antisera Production. Polyclonal rabbit antisera to bovine and human PAS IV were obtained after injections of the electroeluted protein. The protein was mixed with Freund's (bovine PAS IV) or RIBI's (human PAS IV), and 300 μg (bovine PAS IV) or 150 μg (human PAS IV) was injected into peripheral lymph nodes. Boosts of 150 μg (bovine PAS IV) or 75 μg (human PAS IV) were given every 3 weeks, and antisera were collected 10 days after each boost. Immunoglobulins were isolated by precipitation with disodium sulfate (18% w/v) and purified on PAS IV affinity columns produced by the covalent linkage of either bovine or human PAS IV to cyanogen bromide activated Sepharose 4-B. PAS IV specific Ig was eluted with 3 M NaSCN.

Sequence Analysis. Unmodified bovine and human MFGM PAS IV were subjected to N-terminal sequence analysis on an Applied Systems 470A gas-phase sequencer with an on-line Model 120A PTH amino acid analyzer (Hewick et al., 1981). Initial yields were calculated to be 57–62% with a repeptitive yield of 90–93%. PAS IV peptides were generated by enzymatic digestion of 120 μg of pure bovine MFGM PAS IV with lysyl endopeptidase (2% w/w) overnight at 40 °C. The resultant peptides were purified by C-4 reverse-phase HPLC with a 2-h gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Sequence comparisons were obtained by searching the Protein Identification Resource data base (9138 sequences) with the Eugene software package (Baylor College of Medicine, Houston, TX). The hydrophobic moment of the amino-terminal peptide was calculated with the algorithm of Eisenberg et al. (1984).

Endoglycosidase Reactions. Bovine PAS IV, bovine CD36 (Greenwalt et al., 1990), human PAS IV, or human platelet CD36 (1 μg/tube) was combined with 20 μl of 0.1 M phosphate, pH 6.1, 0.6 μL of β-mercaptoethanol, 0.6 μL of 10% SDS, 3.0 μL of 10% Triton X-100, and 0.4 unit of endo-β-

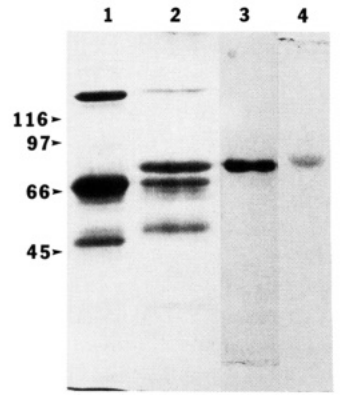

FIGURE 1: Purification of bovine and human PAS IV. Bovine MFGM proteins (lane 1), detergent-phase proteins of the bovine MFGM Triton X-114 extract (lane 2), purified bovine PAS IV (lane 3), and purified human MFGM PAS IV (lane 4) were separated on SDS–polyacrylamide (8%) gels under reducing conditions and then stained with Coomassie. Molecular mass standards (in kDa) are marked to the left of the gel.

Table I: Purification of Bovine PAS IV

purification step	total protein (mg)	purity (%)	PAS IV (mg)	yield (%)
milk fat globule membrane	1220	5[a]	61	100
Triton X-114 detergent phase	98	22	21.6	35 (100)[b]
CM-Sepharose ion exchange chromatography				
pH 8 elution	0.3	67	0.2	
pH 7 elution	1.9	93	1.8	
pH 6 elution (pure PAS IV)	17.5	100	17.5	29 (80)

[a] Calculated after densitometry of SDS–PAGE gels. [b] Calculations based on the observation that all of PAS IV is recovered in the Triton X-114 detergent phase.

N-acetylglucosaminidase (endo F) in a final volume of 30 μL and overlayed with mineral oil. The mixture was then incubated at 37 °C for 0–6 h. The reaction was terminated by dilution of 20 μL of the reaction mixture with 20 μL of SDS–PAGE sample buffer and heating in a boiling water bath for 1 min. Six-hour control incubations in which endo F was absent did not alter the apparent M_r of the substrates.

Protein Determinations. Protein concentrations were analyzed with the BCA reagent and BSA as a standard (Smith et al., 1985). When Triton X-114 was present, all samples and standards were adjusted to a final concentration of 0.1% SDS prior to the addition of the BCA reagent.

Results

Purification of Native Bovine PAS IV. Bovine MFGM PAS IV was purified from the MFGM by a combination of detergent partitioning and ion exchange chromatography. After the initial extraction of MFGM with the nonionic detergent Triton X-114, PAS IV partitioned exclusively into the detergent-rich phase upon heat (37 °C) induced phase transition (Figure 1). The enrichment of PAS IV at this stage is 4–11-fold, depending on the accuracy of the original estimate of MFGM PAS IV content. Quantification of the PAS IV content of bovine MFGM by densitometry of Coomassie-stained polyacrylamide gels gives a value of 5% (Table I). This value may be an overestimate, however, as the MFGM SDS–PAGE profile is dominated by a major band of the protein butyrophilin at 67 kDa (Figure 1, lane 1). The intensity of the butyrophilin band may exceed the linear range of the densitometer and result in an underestimation of butyrophilin and an overestimation of minor bands such as PAS IV. The observation that PAS IV partitions completely into the detergent phase of Triton X-114 extracts (Greenwalt & Mather, 1985) and the recovery of only 22 mg of PAS IV instead of the theoretical 61 mg support this conclusion (Table I). Native bovine PAS IV was eluted from CM-Sepharose at pH values of 8, 7, and 6. The purity of PAS IV eluted at these pH values was 67%, 93%, and 100% respectively, with 89% of the eluted PAS IV obtained as a pure protein at pH 6 (Table I).

Table II: Purification of Human MFGM PAS IV

purification step	total protein (mg)	PAS IV (mg)[a]	yield (%)
Triton X-114 extract	204.4	3.0	100
Triton X-114 detergent phase	20.4	3.0	99
DEAE-Sepharose fraction	7.1	1.6	53
electroeluted protein	0.8	0.8	26

[a] The amounts of PAS IV were calculated after densitometry of SDS–PAGE gels.

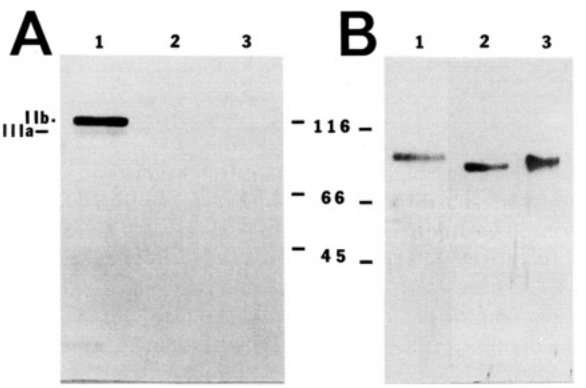

FIGURE 2: Immunoblot analysis of milk fat globule and platelet membranes with anti-PAS IV and anti-platelet GP IIb-IIIa antisera. Human platelet membranes (lanes 1) and bovine (lanes 2) and human (lanes 3) MFGM were boiled in Laemmli sample buffer, and solubilized proteins (10 μg/well) were separated by SDS–8% PAGE under reducing conditions. The separated proteins were transferred to nitrocellulose, blocked in 2% BSA/TBS, and incubated in (A) antiserum to human platelet GP IIb-IIIa or (B) affinity-purified antibody to human PAS IV. Bound antibodies were visualized after sequential incubation in 1:1000 alkaline phosphatase conjugated anti-rabbit Ig and the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate. Molecular mass markers in kDa are marked at the side of each blot. Platelet proteins GP IIb-α (124 kDa) and GP IIIa (108 kDa) are labeled.

Purification of Human MFGM PAS IV. Human PAS IV, like the bovine protein, was extracted from the MFGM by Triton X-114 and was selectively concentrated in the detergent-rich phase after phase partitioning (Table II). Chromatography of the detergent-rich phase on a DEAE-Sepharose column provided only a 50% increase in purity and necessitated a final step in which PAS IV was eluted from a SDS–polyacrylamide gel (Figure 1; Table II). The final yield of human PAS IV was 0.8 mg/204 mg of solubilized MFGM with a recovery of 26%. Purified human PAS IV was approximately 2 kDa larger than bovine PAS IV (80 kDa vs 78 kDa).

Immunological Cross-Reactivity of PAS IV and CD36. Immunoblot analysis of human platelet membranes with antiserum to platelet membrane glycoprotein IIb-IIIa detected bands of 124 and 108 kDa (Figure 2A, lane 1). These proteins were not detected in either bovine or human MFGM, thereby demonstrating the absence of platelet membrane contamina-

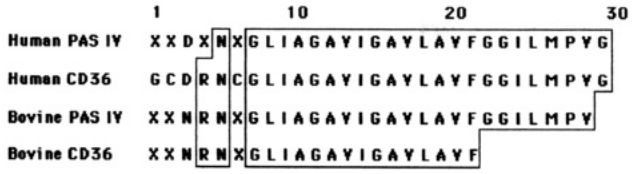

FIGURE 3: Comparison of the N-terminal amino acid sequences of bovine and human PAS IV and of bovine and human CD36. Regions of identity are boxed.

Table III: Determination of the Sequence of Bovine PAS IV Peptide 65b

cycle	amino acid	pmol
1	Val	85
2	Phe	83
3	Asn	72
4	Gly	65
5	Lys	71
6	Asp	69
7	Asp	67
8	Ile	61
9	Ser	ND[a]
10	Lys	13

[a] Not determined.

tion in the MFGM preparations (Figure 2A, lanes 2 and 3). Analysis of these same three membrane preparations with affinity-purified antibodies to human PAS IV revealed reactive bands of 88 kDa in human platelet membranes and of 78 and 80 kDa in bovine and human MFGM, respectively (Figure 2B). In addition, anti-human PAS IV cross-reacted with authentic purified human platelet CD36 and purified bovine endothelial cell CD36 (see Figure 5, lanes 7 and 3, respectively). Affinity-purified antibody to bovine MFGM PAS IV did not cross-react with the 88-kDa human platelet CD36 (data not shown).

N-Terminal Sequence Analysis. N-Terminal amino acid sequence analysis of bovine and human MFGM PAS IV produced sequences of 28 and 29 residues, respectively (Figure 3). A search of the Protein Identification Resource data base did not reveal any sequences similar to the N-terminal sequences of PAS IV. The sequences were nearly identical however to the recently published N-terminal sequences of human platelet CD36 (Tandon et al., 1989a; Oquendo et al., 1989) and bovine CD36 (Greenwalt et al., 1990). Residues 7–29 constitute a hydrophobic region that is conserved in all four proteins. Calculation of hydrophobicity and hydrophobic moment values of 0.87 and 0.25, respectively, suggests that this sequence comprises an amphiphilic region analagous to the membrane-spanning regions of integral membrane proteins that contain multiple membrane-spanning helices (Eisenberg et al., 1984). The presence of an asparagine in place of aspartic acid at position 3 was a consistent difference between the human and bovine proteins. The identification of human PAS IV residues 1, 2, 4, and 6 and bovine PAS IV residues 1, 2, and 6 could not be satisfactorily determined.

Internal Sequence Analysis. Digestion of bovine PAS IV with a lysine-specific endopeptidase resulted in the production of a number of PAS IV fragments, which were purified by reverse-phase HPLC (Figure 4). Sequence analysis of peptide 65b revealed a 10-residue sequence identical with human CD36 residues 214–223 except for the substitution of an aspartic acid residue for asparagine at position 220 (Table III, cycle 7). This substitution removes a potential glycosylation site (Asn-X-Ser) at residues 220–222.

Deglycosylation of PAS IV. A time course study of the deglycosylation of bovine PAS IV with endoglycosidase F

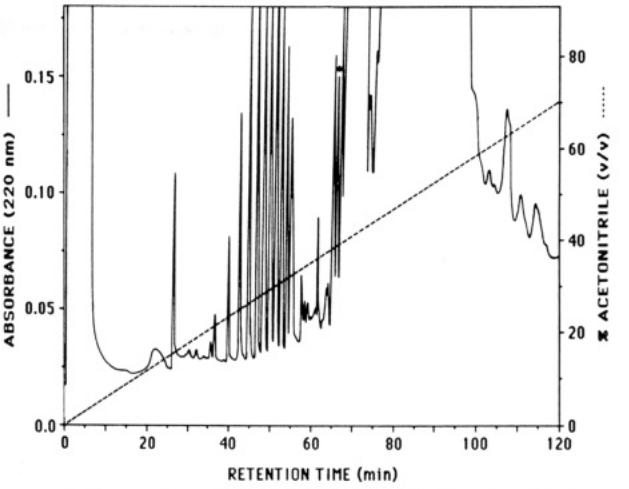

FIGURE 4: Separation of bovine MFGM PAS IV proteolytic fragments. Pure bovine PAS IV was cleaved with lysyl endopeptidase as described under Methods, and the resulting fragments were purified by chromatography on a C-4 reverse-phase HPLC column. Peptide 65b, which eluted at approximately 66 min, is marked with an asterisk. The dashed line represents the acetonitrile gradient used in elution of the peptides.

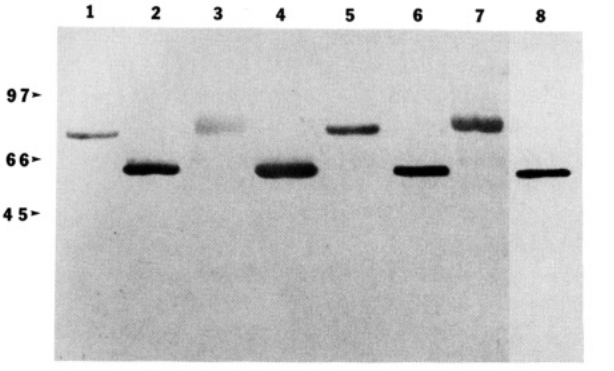

FIGURE 5: Removal of asparagine-linked oligosaccharides from PAS IV and CD36 by endoglycosidase F. One microgram each of bovine PAS IV (lanes 1 and 2), bovine CD36 (lanes 3 and 4), human PAS IV (lanes 5 and 6), and human CD36 (lanes 7 and 8) was treated with either buffer (lanes 1, 3, 5, and 7) as controls or 0.4 unit of endoglycosidase F (lanes 2, 4, 6, and 8) for 6 h at 37 °C in 0.1 M phosphate buffer, pH 6.1, in the presence of 0.2% SDS, 1% Triton X-100, and 2% 2-mercaptoethanol. The reaction was terminated by boiling in SDS–PAGE buffer, and the proteins were separated on SDS–8% PAGE gels under reducing conditions. Proteins were transferred to nitrocellulose and detected by immunoblot analysis with affinity-purified antibody to human PAS IV. Molecular mass standards (in kDa) are marked to the left of the gel.

demonstrated a linear decrease in the apparent M_r of PAS IV with an end point at approximately 57 000 after 3 h of incubation. Treatment of bovine and human PAS IV and bovine and human CD36 with endoglycosidase F revealed that all four proteins had the same apparent M_r after removal of N-linked oligosaccharides (Figure 5). Unlike PAS IV, both bovine and human CD36 migrated on SDS–PAGE gels as broad diffuse bands before deglycosylation and as relatively narrow bands after deglycosylation. All four proteins stained more intensely in immunoblots with anti-human PAS IV after removal of N-linked oligosaccharides, indicating increased reactivity of the antibodies with the deglycosylated polypeptides.

Discussion

The milk fat globule membrane glycoprotein PAS IV has been purified from both human and bovine milk. The protocol used for the purification of native bovine PAS IV is an im-

provement over that first reported in that (1) limited proteolysis of the membrane is not required and (2) the final yield of pure protein is approximately 3-fold higher than that reported in the original protocol (Greenwalt & Mather, 1985). The yield of human PAS IV is less, due in part to the presence of smaller amounts of PAS IV in human MFGM.

The most interesting aspect of this study is the demonstration that mammary epithelial cell PAS IV is related to the glycoprotein CD36 (GP IV or GP IIIb) of platelets and endothelial cells. Polyclonal antibody to human MFGM PAS IV cross-reacts with an 88-kDa protein in human platelets, purified human platelet CD36, and purified bovine endothelial cell CD36. In addition, N-terminal analyses of human and bovine PAS IV were identical with the published N-terminal sequences of human CD36 (Tandon et al., 1989a; Oquendo et al., 1989) and bovine CD36 (Greenwalt et al., 1990) respectively. The possibility that PAS IV is present in the MFGM as a result of contamination of that membrane preparation with platelets was ruled out by experiments which demonstrated the absence of GP IIb-IIIa, the major platelet membrane protein, from MFGM preparations.

The mobility of the mammary epithelial cell and platelet/endothelial cell proteins on SDS–PAGE gels demonstrates that they are not identical. The apparent M_r values for bovine and human PAS IV are 78 000 and 80 000, respectively. These values are 7–8 kDa smaller than the values for bovine and human CD36. Enzymatic removal of N-linked oligosaccharides from PAS IV and CD36 demonstrated that the deglycosylated proteins from both species had M_r values of approximately 57 000. The difference between this value and the cDNA-derived M_r of CD36 of approximately 53 000 may be due to inherent inaccuracy in the calculation of M_r values from SDS–polyacrylamide gels. It is also possible however that the proteins contain O-linked oligosaccharides which would not have been cleaved by the action of endoglycosidase F. Carbohydrates characteristic of O-linked oligosaccharides have been reported for human platelet CD36 (Tandon et al., 1989a) but are absent from bovine MFGM PAS IV (Greenwalt & Mather, 1985).

The size differences between mammary epithelial cell PAS IV and platelet CD36 could be the result of cell type specific glycosylation of identical core polypeptides. Human platelet CD36 is highly sialylated and has an isoelectric point of 4.5–5.5 (Asch et al., 1987). Neuraminidase treatment of human platelet CD36 has been reported to decreased its M_r to 85 000 (Kieffer at al., 1989). Neuraminidase treatment of human and bovine MFGM PAS IV however has little effect on their mobility in SDS–PAGE gels (Greenwalt, unpublished observations). The smaller size of bovine PAS IV and CD36 relative to their human counterparts may be due to a smaller number of N-linked oligosaccharides. The cDNA-derived sequence of human CD36 contains 10 Asn-X-Ser/Thr glycosylation sites (Oquendo et al., 1989). At least one of these glycosylation sequences is absent in bovine PAS IV due to the substitution of aspartic acid for asparagine at position 220. The absence of one 2.5-kDa N-linked oligosaccharide could explain the 2–3-kDa difference between the human and bovine proteins. Final analysis of the relationships of the mammary epithelial cell and platelet core polypeptides and their species-specific differences must await isolation and sequencing of the various cDNAs. At present there is no conclusive evidence for or against PAS IV/CD36 sequence identity.

When PAS IV was first characterized, it was noted that antiserum to MFGM PAS IV recognized a component of the capillary endothelial cells of a variety of bovine tissues. The endothelial cell protein was subsequently shown to be larger than the mammary epithelial cell protein but similar to PAS IV when analyzed by peptide mapping (Greenwalt et al., 1985). It is now apparent that the endothelial cell protein is CD36. While recent work by several laboratories has determined that endothelial and platelet CD36 bind thrombospondin and collagen and are involved in platelet aggregation (Asch et al., 1987; Silverstein et al., 1989; McGregor et al., 1989; Tandon et al., 1989b), the function of mammary epithelial cell PAS IV is unknown. Immunolocalization studies have demonstrated low levels of PAS IV on the basal membranes of differentiated mammary epithelial cells. Thrombospondin has been demonstrated to be a component of the extracellular matrix of a number of cell types (Jaffe et al., 1983; Sage et al., 1983; Wilkner et al., 1987), and it is possible that PAS IV could interact with mammary epithelial cell extracellular matrix thrombospondin. The vast majority of PAS IV however is located on the apical surface of the mammary secretory cells and in the milk fat globule membrane. Dawes et al. (1987) have recently reported very high levels of thrombospondin in human colostrum. The presence of both soluble thrombospondin and a thrombospondin receptor-like protein (PAS IV) in milk is intriguing and suggests a possible interaction between these two proteins.

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Purification and Characterization of the *Tetrahymena pyriformis* P–C Bond Forming Enzyme Phosphoenolpyruvate Phosphomutase†

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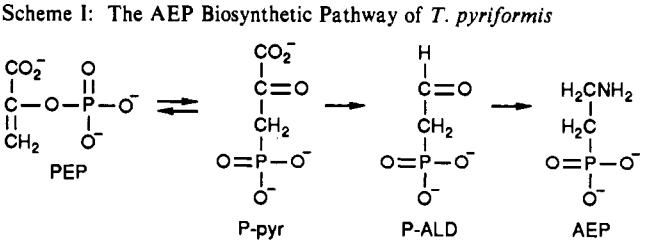
ABSTRACT: In this paper the purification and characterization of the *Tetrahymena pyriformis* enzyme phosphoenolpyruvate phosphomutase are described. PEP phosphomutase was first fractionated from *T. pyriformis* cellular extract by using 70% ammonium sulfate. Chromatography of the crude protein fraction on a DEAE-cellulose column followed by phenyl-Sepharose column chromatography and then Bio-Gel P-200 column chromatography afforded pure PEP phosphomutase in an approximate overall yield of 70 units/150 g of cells. The maximum turnover number observed for PEP phosphomutase catalysis of the phosphonopyruvate → PEP reaction is 38 s^{-1} (25 °C). The enzyme was shown to be a homodimer of 38 000-dalton subunits and to require a divalent metal ion for activity. Mg^{2+} (relative V_m = 1), Co^{2+} (rel V_m = 0.5), Zn^{2+} (rel V_m = 0.4), and Mn^{2+} (rel V_m = 0.3) each satisfied the cofactor requirement. Binding of the physiological cofactor, Mg^{2+} (K_i = 0.3 mM at pH 7.5), and phosphonopyruvate (K_m = 2 μM at pH 7.5) was found to be ordered, with cofactor binding preceding substrate binding. Within the pH range of 5–9 catalysis (V_m) was found to be pH independent, while phosphonopyruvate binding dropped at acidic and basic pH.

Following the first discovery of a P–C bond containing natural product that took place over 30 years ago (Horiguchi & Kandatsu, 1959) the presence of phosphonates and phosphinates in a variety of biological systems has been firmly established [for reviews on this topic see Hori et al. (1984), Hilderbrand (1983), and Mastelerz (1984)]. To this day, however, we know very little about how these reduced phosphorus compounds are synthesized, how they are degraded, and how they serve the organisms in which they are found.

Recently, our laboratory set out to investigate the modes of biosynthesis and biodegradation of this interesting class of natural products. In an earlier paper (Barry et al., 1988) we reported on the steps of the biosynthetic pathway which lead, in *Tetrahymena pyriformis*, to the most ubiquitous of the phosphonate natural products, (2-aminoethyl)phosphonate (AEP).[1] We had found, true to an early hypothesis (Warren,

Scheme I: The AEP Biosynthetic Pathway of *T. pyriformis*



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