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The Sialoglycoprotein Subunits of Human Placental Brush Border Membranes Characterized by Two-Dimensional Electrophoresis

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A brush border membrane enriched fraction was isolated from human, full-term placenta. This membrane fraction exhibited large membrane fragments with microvilli projecting from the basal membrane in electron micrographs and was enriched tenfold in alkaline phosphatase, a brush border enzyme marker. The sialoglycoproteins associated with this membrane fraction were tritiated by mild periodate oxidation of sialic acid and reduction with tritiated $NaBH_4$. The membranes were solubilized in 8 M urea, 2% Triton X-100, and the tritiated glycoprotein subunits were reduced with β-mercaptoethanol and characterized by 2-dimensional polyacrylamide gel electrophoresis using a method similar to that described by O'Farrell and Bhakdi, Knüferman, and Wallach. The tritiated subunits were detected in the gels by autofluorography. The 2-dimensional subunit "maps" resolved at least 17 major sialoglycoprotein subunits whereas only 10 major periodate-Schiff reagent staining components were resolved by 1-dimensional SDS polyacrylamide gel electrophoresis. Placental alkaline phosphatase (PAP) was identified on the subunit maps by inclusion of ^{32}P-labeled PAP in the tritiated membrane sample. The ^{32}P-labeled PAP corresponded to a major tritiated sialoglycoprotein subunit, which was heterogeneous with respect to charge as demonstrated by 3 closely running spots of the same molecular weight.

Key words: placenta, brush border, sialoglycoprotein, alkaline phosphatase, two-dimensional electrophoresis

The brush border membrane of the syncitial trophoblast of human placenta is in direct contact with the maternal circulation and, therefore, represents the first placental barrier through which numerous solutes are transportable. The human placenta has been demonstrated to actively transport L-amino acids, creatine, Na^+, and K^+, vitamin B_{12}, and acetylcholine (1), and to passively transport sugars, creatinine, p-aminohippurate, urea, tetraethyl ammonium, norepinephrine, and antipyrine (1). Consequently, the brush border membrane of this organ must possess the capabilities for facilitated diffusion and, perhaps, active transport.

The ability to saturate the placental amino acid transport systems and demonstrate

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Michaelis-Menten kinetics for transport processes (2, 3) indicate the presence of carrier systems in the placental membranes. Furthermore, the specificity which many transport systems exhibit suggests the presence of carriers at the cell surface which can discriminate between transported and nontransported molecules (4).

Although the mechanisms for solute transport by placental tissue has been investigated by kinetic methods, the molecular components which participate in these mechanisms have not been identified. Since one subunit of the Na^+,K^+-stimulated ATPase has been demonstrated to be a glycoprotein (5) and most proteins on the external cell surface appear to be glycosylated (6, 7), the cell surface glycoproteins of the human placental brush border membranes are prime candidates for transport mediating membrane components. In this paper, we report the characterization of the sialoglycoprotein subunits of the isolated placental brush border by a 2-dimensional electrophoretic technique utilizing isoelectric focusing in the first dimension and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the second. Apparent isoelectric point (pI) and apparent subunit molecular weight (M_r) were calculated from the relative positions of the subunits in two-dimensional maps. The utility of the mapping of membrane subunits was demonstrated by the identification of placental alkaline phosphatase in the maps by covalent labeling of the enzyme with [^{32}P]orthophosphate. Using the physical characterization of the glycoprotein membrane components which the subunit maps provide, further studies may associate specific subunits with transport processes or other cellular functions.

MATERIALS

Substrates for enzyme assays, sodium meta-periodate and E. coli alkaline phosphatase, type III, were obtained from Sigma Chemical Co. Reagents for polyacrylamide gels were from Bio-Rad Laboratories and Canalco, and ampholytes were from LKB Produkter AB. Materials for scintillation spectrophotometry and autofluorography, and scintillation grade Triton X-100 were from New England Nuclear; tritiated sodium borohydride (6 Ci/mmole) was from Schwartz-Mann or Amersham-Searle; and ultrapure urea was obtained from Schwartz-Mann. Other chemicals used were J. T. Baker "analyzed reagent" grade unless otherwise specified.

METHODS

Membrane Preparations

The microvillus membranes of full-term human placentas were prepared by the methods of Carlson, Wada, and Sussman (8) with the following modifications. Minced placental tissue, washed free of maternal blood, was suspended in 5 volumes of isotonic dextrose and stirred in a Waring blender at 4°C for 2.5 min, using a rheostat to reduce the rpm to the lowest possible setting. The connective tissue and major blood vessels remained intact, but soft villus tissue was disrupted. When free nuclei were observed in the suspension by phase contrast microscopy, the undisrupted tissue was filtered off with plastic window screen (1/16 inch mesh), and the purification was carried on from this step as previously described, substituting 5 mM NaEDTA, pH 7.4, for 4 mM $NaHCO_3$, pH 8.1, 1 mM $MgCl_2$ as the buffer in sucrose solutions. The microvillus membranes obtained from this method were found to be of the same purity as those obtained from the earlier methods. The advantage of the modification is the omission of a tedious step in which placental villus tissue is scraped from blood vessels and connective tissue.

Tritiation of Placental Brush Border Membrane Fractions

Placental brush border membranes were labeled with tritium by a modification of the method of Blumenfeld, Gallop, and Liao (9) which utilized oxidation of sialic acid by mild periodic acid oxidation and reduction by $[^3H]NaBH_4$. The conditions for oxidation were that of Van Lenten and Ashwell (10) which were highly specific for sialic acid.

Membranes were suspended in 0.9 ml of cold 0.1 M sodium acetate, 0.15 M NaCl, pH 5.6, and the oxidation was initiated by addition of 0.1 ml of 50 mM sodium meta-periodate. The oxidation was allowed to proceed at 0°C in the dark for 10 min and was then terminated by dilution with 2 volumes of cold 50 mM sodium phosphate, 0.15 mM NaCl, pH 7.4 (PBS) and pelleting the placental membranes at 12,000 × g for 10 min. The membranes were washed twice with 3 ml of PBS. The oxidized membranes were suspended in 1 ml of PBS, pH 7.4, and reduced with 1 -2 mCi of $[^3H]NaBH_4$ (6–10 Ci/mmole) for 30 min at 0°C. Unlabeled $NaBH_4$, approximately 1 mg, was added and reduction was allowed to proceed for 10 min at 0°C. The membranes were then diluted with 2 volumes of PBS, pelleted, and washed with 3 ml of PBS. The tritiated membranes were stored frozen at −20°C.

Labeling of Placental Alkaline Phosphatase

A modification of Milstein's procedure (11) to covalently bind $^{32}PO_4$ to purified E. coli alkaline phosphatase was used to ^{32}P-label placental alkaline phosphatase. The procedure was previously described (8) utilizing an incubation of $^{32}PO_4$ with alkaline phosphatase at pH 5.0, acid denaturation, and acetone precipitation of labeled enzyme. The precipitate was redissolved in 8 M urea, 5% β-mercaptoethanol, 2% Triton X-100, 5 mM $NaPO_4$, pH 8.0, for electrophoretic analysis.

Standards of placental alkaline phosphatase were purified to homogeneity according to the method of Sussman et al. (12). Standards of PAP were tritiated by mild periodate oxidation and reduction by $[^3H]NaBH_4$ according to the method of Van Lenten and Ashwell (10).

Two-Dimensional Electrophoresis

Solubilization of membrane glycoproteins. The tritiated membrane preparations were extracted with 8 M urea, 2% Triton X-100, 5 mM $NaPO_4$, pH 8.0 (saturated with PMSF), at room temperature for 5 min according to Bhakdi, Knüferman, and Wallach (13), chilled to 4°C on an ice bath, and centrifuged at 140,000 × g for 60 min at 4°C. Under these conditions 50–60% of the acid precipitable tritium extracted into the supernatant from placental membranes. The supernatant was treated with 5% β-mercaptoethanol for 10 min at room temperature and utilized for electrophoretic analysis immediately or after concentration by dialysis against a dessicant at 4°C.

The first dimension: isoelectric focusing. A 2-dimensional electrophoretic system very similar to that described by O'Farrell (14) was utilized to resolve the membrane glycoproteins. The first dimension was isoelectric focusing (IEF) in polyacrylamide gels containing 8 M urea, 0.5% (vol/vol) Triton X-100, 4% acrylamide (10% crosslinker), 10% glycerol, 1% 3–10 pH Ampholine (LKB Produkter) which were cast in 3 × 100 mm rods. The cathode buffer was 1% NaOH, and the anode buffer was 1% H_3PO_4. The gels were prefocused for 1 h at 150 V. The samples were applied in 100 μl of extraction buffer containing a trace of bromphenol blue. The samples were run in at 150 V for 2 h and focused at 400 V for at least 14 h at 18°C, constant voltage. After termination

of the focusing, the pH gradient was measured in one gel using a membrane electrode (Brinkman Instruments). The other gels were soaked at room temperature in equilibration buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) with gentle agitation for 1 h. Equilibrated gels were either run in the second dimension immediately or stored at −20°C.

IEF gels were sliced into 1-mm slices and counted for ^{3}H and ^{32}P by scintillation spectrophotometry. Each gel slice was dissolved in 0.5 ml of 30% H_2O_2, 0.7% perchloric acid at 37°C for 48 h, and counted with 5 ml of scintillation cocktail (1 vol Triton X-100:2 vol 5 gm Omnifluor dissolved in 1 liter of toluene).

The second dimension: sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The second dimension electrophoresis was run in 0.1% sodium dodecylsulfate (SDS), 8% polyacrylamide gel slabs (1.5 mm thick × 14 cm wide × 10 cm high) according to Laemmli (15). The equilibrated, isoelectric focusing gel rod was sealed to the top of the stacking gel with 0.75% agar solution as described by O'Farrell (14). The sample was electrophoresed into the stacking gel for 20 min at 15 mA/slab with 2% (wt/vol) SDS in the top buffer. The top buffer was replaced with 0.1% SDS buffer containing a trace of bromphenol blue, and the run was continued at 20 mA/slab until the tracking dye reached the bottom of the slab. The gel was then fixed in 50% methanol for 2 h and impregnated with PPO for autofluorography according to Bonner and Lasky (16). The PPO impregnated, vacuum dried gels were autofluorographed on Kodak Royal RP X-Omat medical x-ray film at −76°C to detect tritiated glycoprotein subunits. Globular protein standards were used to estimate subunit molecular weights of the glycoprotein spots by the method of Weber and Osborn (17).

RESULTS

The Brush Border Membrane Preparation

The membranes prepared from human, full-term placenta exhibited ultrastructural and enzyme marker characteristics compatible with a brush border membrane. Electronmicrographs demonstrated microvilli projecting from the basal membrane sheets and may represent the terminal web of the brush border (Fig. 1). A brush border enzyme marker, alkaline phosphatase, was enriched tenfold over the tissue homogenate, while other enzyme markers for contaminating organelles remained constant or were decreased (8).

Tritiation of Sialoglycoproteins

The glycoproteins of the placental brush border membranes were heavily labeled by mild periodic acid oxidation and $[^{3}H]NaBH_4$ reduction under conditions reportedly specific for sialic acid (10). The lack of tritium incorporation into a nonglycosylated membrane protein, the 45,000 M_T "actin-like" protein, indicated specific tritiation of glycoproteins. Furthermore, control studies with purified transferrin as a model sialoglycoprotein demonstrated that 85% of the incorporated tritium was on sialic acid which was labile to neuraminidase digestion.

Extraction of 50–60% of the acid precipitable tritiated glycoproteins from the membranes was accomplished by 8 M urea, 2% Triton X-100. Triton X-100 alone would only extract 25% of the acid precipitable tritium.

Two-Dimensional Electrophoresis

The tritiated, extracted glycoprotein subunits were focused in IEF polyacrylamide

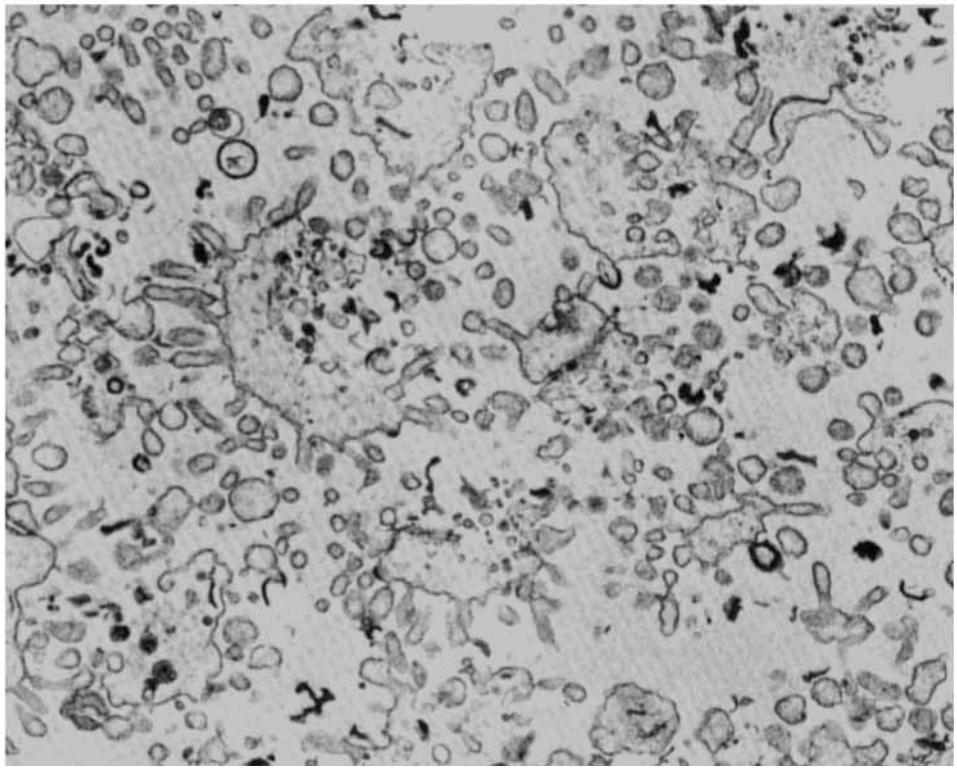

Fig. 1. Electron micrograph of isolated placental brush border membranes. Magnification 24,000 × shows representative field of placental brush border membrane preparation.

gels containing 0.5% Triton X-100 and 8 M urea. Lowering of the Triton X-100 gel concentration to 0.1% caused incomplete focusing of the subunits which may be explained by nonspecific aggregation. Temperature also was critical in attaining complete focusing. At 4°C, focusing of subunits was incomplete in 14 h, while at 18°C focusing was complete in 14 h. In the second dimensional SDS-polyacrylamide gels, the best definition and resolution of spots was obtained when 2% SDS buffer was used as top tank buffer for the first 20 min of electrophoresis. The higher SDS concentration of the leading ion front may be necessary to remove excess Triton X-100 associated with the glycoprotein subunits.

The modified 2-dimensional electrophoretic technique produced subunit maps resolving the labeled glycoproteins of the placental brush border membranes into 17 major subunits and at least 33 total subunits (Fig. 2a), ranging in M_r from 262,000 to 40,000. Using a one-dimensional SDS-polyacrylamide gel system, only 10 major PAS positive bands were detected in the same placental brush border preparation (8).

A control experiment in which placental brush border membranes were reduced with [^{3}H] $NaBH_4$ prior to meta-periodate oxidation indicated that the labeling of spots identified as sialoglycoproteins was dependent on oxidation. Unoxidized membranes incorporated only 2% of the tritium incorporated into oxidized membranes as measured by TCA precipitable counts from Triton-urea membrane extracts. Equivalent exposures of 2-dimensional gels indicated no detectable labeling of sialoglycoprotein spots from unoxidized membranes,

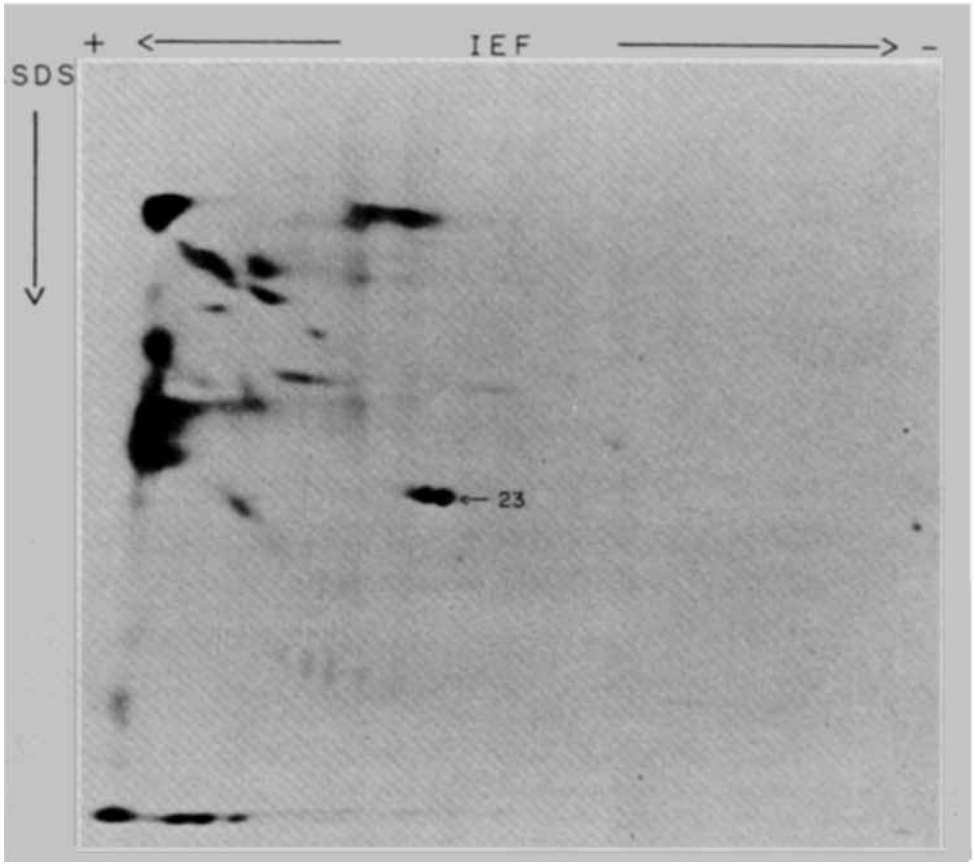

Fig. 2a. Two-dimensional electrophoretic map of placental brush border sialoglycoprotein subunits. 1.4×10^6 dpm of tritiated membrane glycoprotein was focused in a 3–10 pH gradient IEF gel rod for the first dimension and electrophoresed in a 8% acrylamide, 0.1% SDS gel slab for the second dimension. The dried, PPO impregnated gel was autofluorographed at −76°C for 18 h.

which are heavily labeled in gels containing equal amounts of protein from oxidized membranes.

The pIs of the subunits resolved in the subunit map were estimated from the coordinates (R_f) measured for the center of each spot, using a pH vs R_f standard curve which was determined for each run (Fig. 2b). The glycoprotein subunits ranged in pI from pH 7.2 to pH 4.6 with the majority in the 6–4.6 range (Table I). Most of the glycoprotein subunits were heterogeneous with respect to charge which was demonstrated by close running groups of spots with the same M_r or single spots with a broad band of pI distribution (Fig. 2).

Identification of a Specific Sialoglycoprotein in Subunit Maps

A specific brush border membrane component, placental alkaline phosphatase (PAP), was identified in the subunit maps by coelectrophoresis of ^{32}P-labeled PAP with tritiated membrane subunits. The ^{32}P-labeled PAP corresponded in the maps with a major sialoglycoprotein subunit of the placental brush border membrane (Fig. 3). The membrane

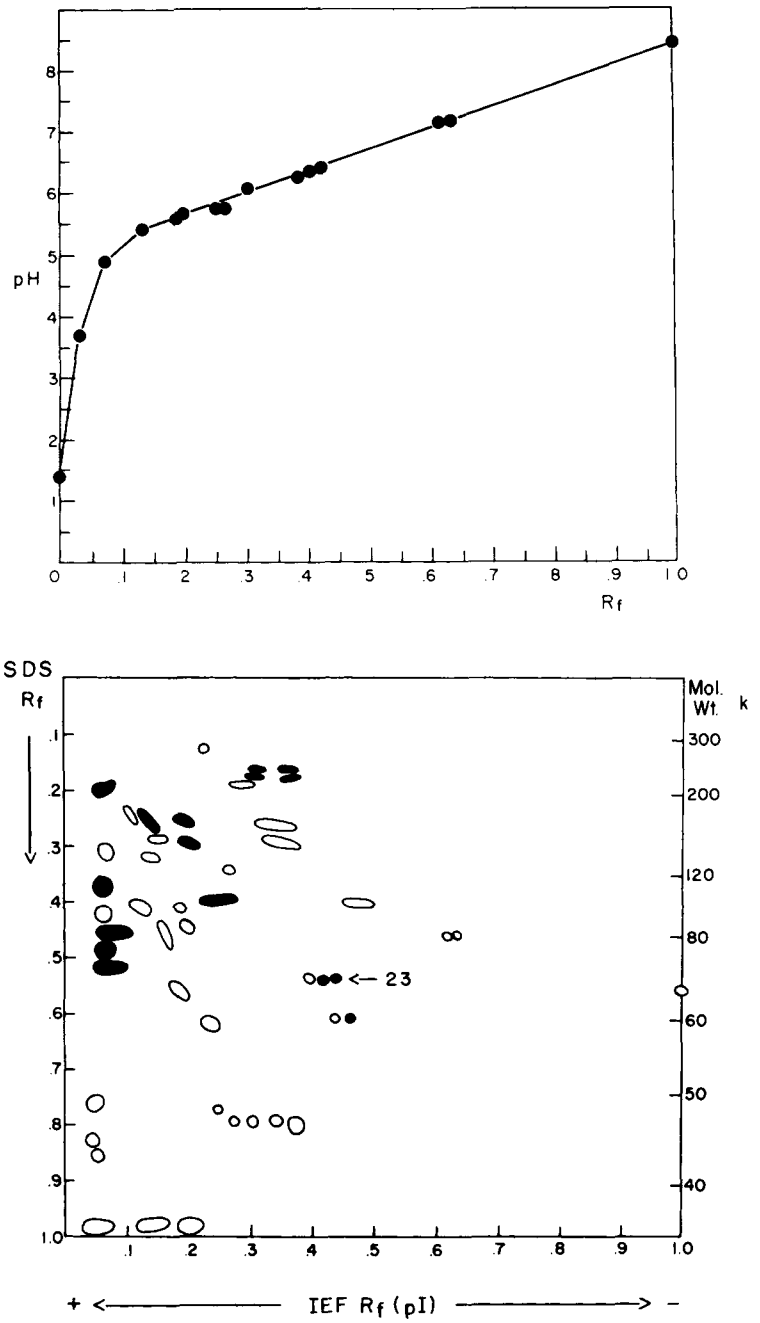

Fig. 2b. Coordinates of sialoglycoprotein subunits in 2-dimensional electrophoretic maps. The top figure shows the pH gradient obtained in the first dimensional IEF gel. The bottom figure illustrates the sialoglycoprotein subunits plotted with respect to R_f in the 2 dimensions. The molecular weight (M_T^-) scale and pI scale were extrapolated from R_f vs M_T^- or pH standard curves. This figure was obtained by tracing the spots directly from the autofluorogram, and the R_f values were assigned from the axis formed at the bottom by the leading ion front of the SDS dimension, and the axis formed at the right side by labeled material which does not penetrate the IEF gel. The left limiting boundary of the map was determined by slicing and counting IEF gels to find the R_f of the tritiated material closest to the anode.

TABLE I. Coordinates of Placental Brush Border Sialoglycoprotein Subunits in 2-Dimensional Maps

Spots		R_f X(pI)	R_f Y($M_{\bar{T}}$)	Apparent pI	Apparent $M_{\bar{T}}$ (K)
1		0.224	0.140	5.72	262.0
2		0.308	0.170	6.09	235.0
3		0.359	0.190	6.19	218.0
4	a	0.060	0.200	4.60	210.0
	b	0.283		5.93	210.0
5		0.368	0.240	5.88	181.0
6	a	0.131	0.258	5.45	170.0
	b	0.194		5.68	170.0
7		0.368	0.272	5.88	161.0
8		0.200	0.294	5.68	148.0
9		0.104	0.250	5.18	175.0
10		0.160	0.286	5.50	153.0
11		0.060	0.307	4.60	142.0
12		0.136	0.318	5.40	136.0
13		0.263	0.344	5.86	123.0
14		0.060	0.381	4.60	107.0
15	a	0.252	0.400	5.82	100.0
	b	0.469		6.57	100.0
16	a	0.131	0.420	5.36	94.3
	b	0.187		5.60	94.3
17		0.197	0.444	5.63	85.2
18		0.202	0.422	5.65	92.4
19		0.162	0.461	5.51	80.0
20		0.060	0.459	4.60	77.8
21		0.060	0.488	4.60	72.5
22	a	0.616	0.463	7.10	74.6
	b	0.631		7.19	74.6
23	a	0.389	0.550	6.22	66.2
	b	0.404		6.30	66.2
	c	0.424		6.39	66.2
24		0.177	0.572	5.60	64.2
25		0.227	0.633	5.73	59.0
26	a	0.429	0.622	6.43	59.9
	b	0.449		6.50	59.9
27		0.066	0.769	4.60	49.0
28	a	0.242	0.794	5.79	47.0
	b	0.272		5.89	47.0
	c	0.303		6.00	47.0
	d	0.338		6.12	47.0
	e	0.369		6.22	47.0
29		0.505	0.844	6.70	44.1
30		0.060	0.871	4.60	42.5
31		0.060	1.00	4.60	< 40
32		0.146	1.00	5.45	< 40
33		0.207	1.00	5.66	< 40

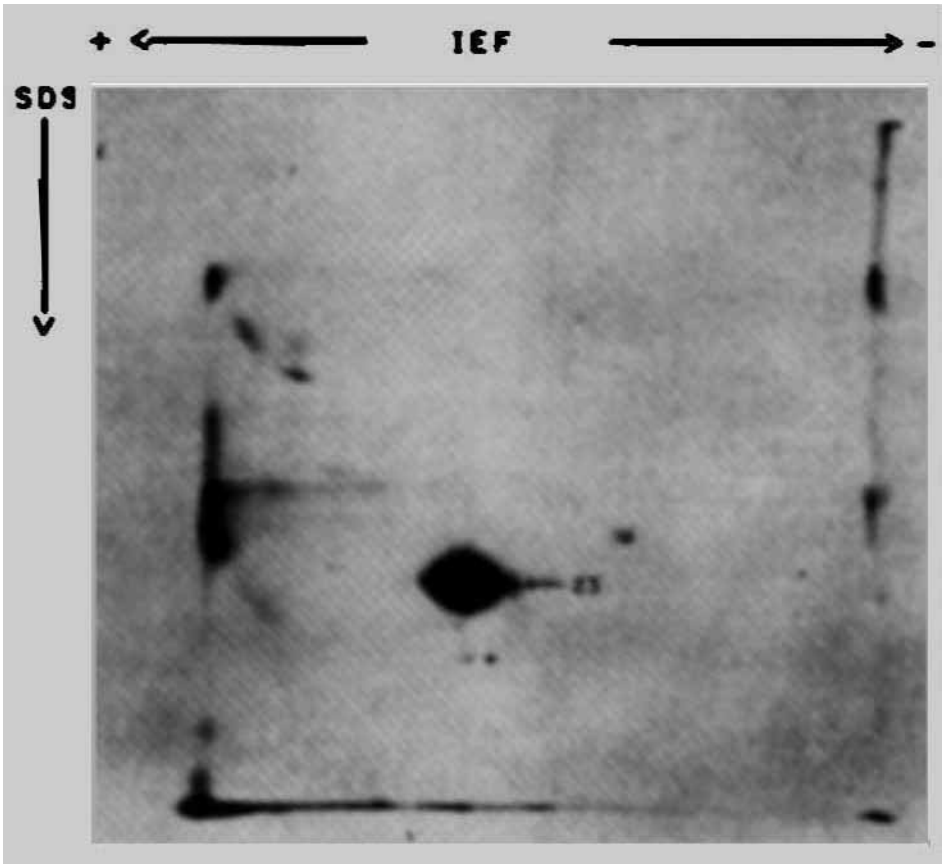

Fig. 3. Identification of placental alkaline phosphatase in 2-dimensional electrophoretic maps. 1.4 × 10^6 dpm of tritiated membrane glycoprotein and 0.2 μg of ^{32}P-labeled purified PAP were run together in a 2-dimensional gel as described for Fig. 2. The dried, PPO impregnated gel was autofluorographed at −76°C for 6 h.

component corresponding to PAP was heterogeneous with respect to charge, giving 3 closely running spots with the same M_r, 66,200 (spot 23, Table I).

A rigorous measurement of the pI of PAP subunits was attained from 1-dimensional IEF gels run on purified PAP. In these experiments a mixture of unlabeled PAP subunits with tritiated PAP subunits showed correspondence between the Coomassie blue stain for protein and counts of tritium. This indicated these subunits had the same pI. However, the active site phosphorylation of PAP caused a 0.1 pH unit, acid shift in pI of the subunits as shown in Fig. 4a using ^{32}P-labeling of the enzyme. IEF gels run on purified E. coli alkaline phosphatase indicated that a similar, 0.1 pH unit, acid shift in pI was present for the phosphorylated subunit relative to unlabeled material in the same gel (Fig. 4b). The occurrence of 2 forms of the unlabeled E. coli phosphatase resolved in the IEF gels was probably due to deamidation of the nascent polypeptide which occurs with long term storage of the purified enzyme (unpublished observation).

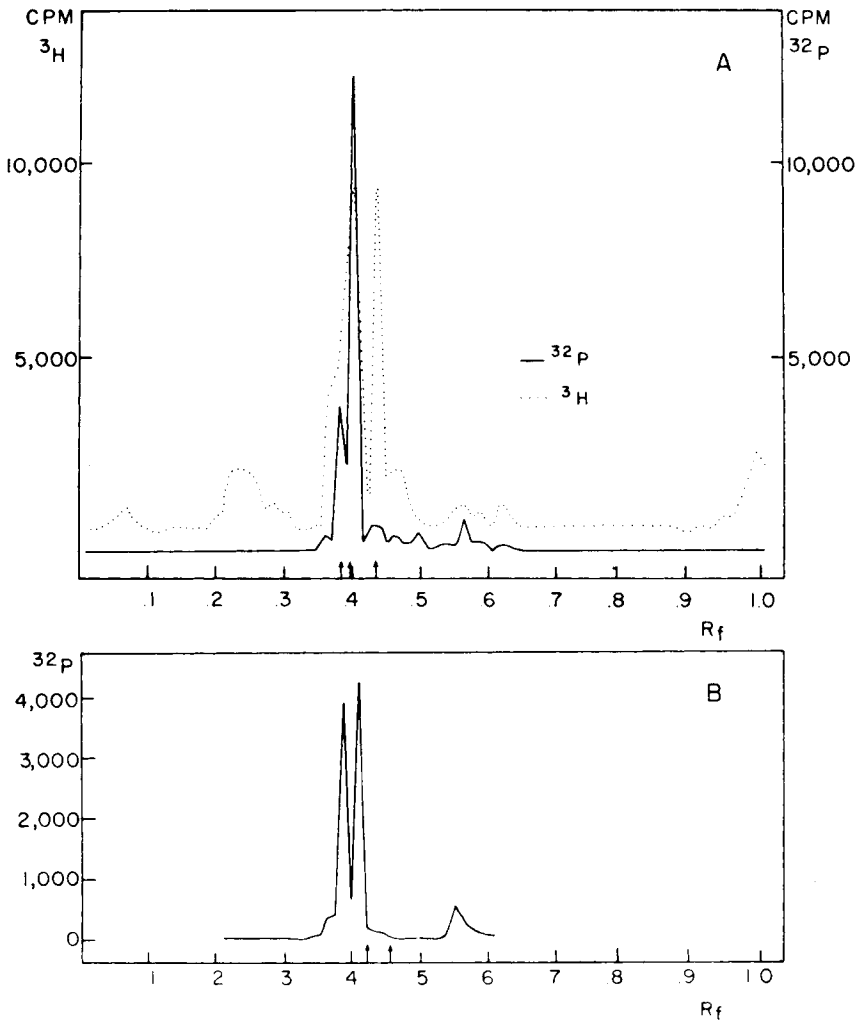

Fig. 4. The effect of phosphorylation on the pI of alkaline phosphatase subunits. A) 1 μg of purified PAP tritiated by the method of Van Lenten and Ashwell (10) and 1 μg of ^{32}P-labeled PAP were focused together in an IEF gel. The gel was fixed in 50% methanol for 2 h, stained with Coomassie blue (.02% Coomassie blue, 5% trichloroacetic acid, 5% sulfosalicylic acid, 18% methanol) for 2 h, destained in 25% ethanol, 5% acetic acid, and stored in 7% acetic acid. Gel was frozen, sliced into 1-mm sections, and solubilized for double label counting of ^{3}H and ^{32}P. The pH gradient in IEF gels were measured on a duplicate gel.

Calculated pI (3 sets of gels)

R_f	0.375	0.397	0.433
Tritiated PAP		6.32 ± 0.11	6.43 ± 0.08
Phosphorylated PAP	6.23 ± 0.07	6.34 ± 0.08	
pI Shift	0.09	0.08	

B) 2 μg of unlabeled E. coli alkaline phosphatase and 0.2 μg of ^{32}P-labeled E. coli enzyme were co-focused in IEF gels, stained, sliced, and counted for ^{32}P. Positions of protein bands are denoted by arrows.

Calculated pI (single set of gels)

R_f	0.389	0.413	0.423	0.456
Unlabeled E. coli AP			6.38	6.50
Phosphorylated E. coli AP	6.30	6.36		
pI shift	0.08	0.14		

DISCUSSION

The tritiation of placental brush border membranes by a method specific for sialic acid and 2-dimensional electrophoresis of the extracted, labeled membrane components has enabled the characterization of a complex mixture of brush border membrane sialoglycoprotein subunits. Using the parameters of pI and M_T, it was possible to assign coordinates to each subunit resolved. The method was found to be very reproduceable as evidenced by the consistent reproduction of the same pattern of spots for placental membranes from run to run, with duplicate runs yielding identical patterns.

The accuracy of the M_T assignments from the 2-dimensional maps are similar to those from 1-dimensional SDS-polyacrylamide gel electrophoresis which were reported to be accurate to a standard deviation of 10% (17). It should also be noted that the M_T for the sialoglycoprotein subunits may not be an accurate estimate of the true subunit molecular weight, due to the anomalous migration in SDS gels of heavily glycosylated subunits (19, 20). The assignment of pI from the focusing dimension of the 2-dimensional maps was found to be more variable than the M_T assignment. Reproduceability was dependent on complete focusing of subunits, uniformity of IEF gel preparation, and careful pH measurements on the focused gels. The assigned pIs must also be considered to be an apparent value obtained under the particular experimental conditions. For example, native PAP has a mean pI of 4.4 in sucrose gradient IEF (21), while in the present study PAP subunits were found to have a mean pI of 6.30 in IEF polyacrylamide gels containing 8 M urea, 0.5% Triton X-100.

The careful examination of the pI of purified PAP in 1-dimensional IEF gels indicated that tritiation of sialic acid did not alter the pI of the enzyme subunits while phosphorylation caused an acid shift similar to the acid shift observed for the phosphorylated E. coli alkaline phosphatase.

The E. coli enzyme is not glycosylated and is a dimer coded by a single cistron (22, 23), making this enzyme an ideal model for the effect of phosphorylation on pI. The acid shift in pI was expected, since phosphorylation involves the addition of a negatively charged moiety to an uncharged serine residue at the active site of the molecule (18). Based on the evidence that E. coli (24), bovine kidney (25), and intestinal (26) alkaline phosphatases are phosphorylated at a single site per subunit, phosphorylation of PAP would add 1.48 electron charges per subunit.* Since the differences in pI between the multiple molecular forms of unlabeled PAP are similar to the acid shift caused by phosphorylation, the heterogeneity of PAP is due to single charge differences. Peripheral differences, such as deamidation (27) or sialic acid content of the carbohydrate moiety (28) would explain the microheterogeneity of PAP and the other sialoglycoprotein subunits identified in the 2-dimensional, subunit maps. It has been previously shown for the alkaline phosphatase of pig kidney that varying degrees of sialylation was the cause of microheterogeneity, and neuraminidase reduced the different forms of the enzyme to a single, desialylated enzyme (30).

Using PAP as an example, we have shown that 2-dimensional electrophoresis can be used in conjunction with affinity labeling to resolve and identify sialoglycoproteins of the human placental brush border membrane. The identification of other membrane components such as transport carrier molecules and membrane hormone receptors may also be possible utilizing this same approach.

*The addition of 1.48 electron charges by phosphorylation is based on pKa of 1.60 and 6.62 for a model organophosphate, ethyl phosphate (29) at pH 6.3.

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