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Characterization of Antigenic Sialoglycoprotein Subunits of the Placental Brush Border Membranes: Comparison With Liver and Kidney Membrane Subunits by Two-Dimensional Electrophoresis

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The sialoglycoprotein subunits of human placental brush border membranes were labeled by sequential treatment with periodate and (^{3}H)-sodium borohydride, which trititates sialic acid, and by lactoperoxidase-catalyzed (^{125}I) iodination of tyrosine residues. The labeled subunits were characterized with respect to their affinity for antisera raised against Triton X-100 extracts of placental brush border membranes. The immunochemically reactive components were analyzed by two-dimensional electrophoresis according to a modification of the O'Farrell technique [20] enabling the assignment of estimated M_r and pI. Of the 33 ^{3}H-labeled brush border subunits present in Triton X-100-solubilized membrane preparations, 18 subunits reacted with antiplacental brush border antisera insolubilized on CNBr-activated Sepharose or in immunoprecipitates. Fourteen of these tritiated subunits were also labeled with ^{125}I, confirming that these are glycoproteins.

The plasma membranes of normal human liver and microsomes from kidney were examined for the placental brush border glycoprotein subunits by reaction with insolubilized antiplacental brush border antisera and two-dimensional electrophoresis of the reacting tritium-labeled subunits. Comparison of the two-dimensional electrophoretic maps of the immunochemically reacting glycoproteins from liver, kidney, and placenta resulted in the identification of seven placental subunits in common with liver and kidney on the basis of antigenic cross-reactivity, M_r, and pI. Four placental glycoproteins were not found in the other tissues and are potentially specific to the placenta. Three of the placental subunits were only seen in placenta and kidney. Three of the subunits ran at the dye front and could not be assigned molecular weights. One of the subunits was poorly labeled by tritiation of sialic acid and was not considered.

Key words: antigenic membrane glycoproteins, immunoprecipitation, two-dimensional electrophoresis

Abbreviations: PMSF) phenylmethylsulfonyl fluoride; SDS) sodium dodecyl sulfate; PPO) 2,5-diphenyl oxazole; APBB) antiplacental brush border; PAP) placental alkaline phosphatase; LAP) liver alkaline phosphatase; DPNH) reduced diphosphopyridine nucleotide; IEF) isoelectric focusing.

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The brush border membrane of the syncytial trophoblast (frequently referred to as the placental brush border membrane) is the cell surface of the human placenta which is in direct contact with the maternal circulation. This membrane is the initial barrier separating the fetal and maternal circulations, and across it occurs most of the transport of the solutes between fetus and mother. This membrane is believed to have properties which contribute to the invasive growth of the trophoblast during natural implantation or when placental tissue is experimentally transplanted to other vascularized tissues [1]. The similarity of the properties of the trophoblast cell to neoplastic cells with regard to their invasive growth, escape from immunosurveillance [2, 3], and cell surface charge properties [4, 5] has been recognized. Since integral glycoproteins are believed to be important determinants of cell membrane properties, we have been interested in characterizing the glycoproteins of the placental brush border membrane.

In previous studies, the placental brush border membrane of the human trophoblast has been isolated as a microvillus membrane-enriched fraction, and the protein and glycoprotein subunits have been characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [6]. A more definitive characterization of the sialoglycoprotein subunits of the placental brush border membrane was subsequently performed by selective labeling of the sialic acid residues by sequential treatment with periodate and (^{3}H)-$NaBH_4$ followed by two-dimensional polyacrylamide gel electrophoresis [7]. The purpose of the present study was to investigate whether the antigenic sialoglycoprotein subunits of placental brush border membrane are specific to the placental trophoblast. The experimental approach used to define placental specificity was to compare the integral glycoprotein subunits of liver plasma membranes and kidney membranes with those of the placental brush border membrane using an antiserum developed against the placental brush border glycoproteins. The antiserum was used for immunoaffinity chromatography and immunoprecipitation in combination with two-dimensional electrophoresis of immunoisolated glycoprotein to facilitate the identification of placental subunits.

Using the specificity of the immunochemical techniques in combination with two-dimensional electrophoresis, it was possible to identify a set of four glycoprotein subunits present only in the placental brush border membranes. A group of seven placental glycoproteins were found to be common to liver and kidney membranes.

METHODS

Membrane Preparations

Placenta plasma membrane. The placental brush border membrane of full-term placentas was prepared by the methods of Wada et al [7].

Liver plasma membrane. These were prepared from livers obtained from organ donor cadavers maintained by perfusion for up to 48 h prior to removal of heart and kidneys for transplant surgery. Liver membranes were prepared by the method of Aronson and Touster [8]. The liver was perfused with 9.0% (v/v) glycerol, 4 mM $CaCl_2$ to remove blood and stabilize nuclei. Fifty grams of tissue were scraped free of connective tissue and blood vessels and treated as previously described. The tissue was homogenized in 150 ml of 0.25 M sucrose, 5mM $NaHCO_3$, pH 8.1, saturated with 1 mM PMSF using one pass of a Teflon pestle Potter-Elvehjam homogenizer. The homogenate was filtered through plastic window screen (1/16 inch pore) and centrifuged at 1,000 g for 10 min. The resulting nuclear pellet was washed twice by homogenization in 1 volume of 0.25 M sucrose and centrifugation. Before the final centrifugation, the homogenizied pellet was filtered through Nitex nylon gauze (118-μ pore). The supernatants were pooled (S_1) and the final

nuclear pellet (N_1) was saved for subsequent steps. S_1 was spun at 33,000 g for 7.5 min (including acceleration and deceleration) to pellet mitochondria and lysosomes. The microsomal fraction (M) was then pelleted at 78,000 g for 100 min. M and N_1 were each suspended in 53% sucrose by dounce homogenization and overlayed with sucrose gradients in nitrocellulose centrifuge tubes (four gradients for N_1 and ten gradients for M). The gradient steps consisted of 42, 37, 34, and 24% sucrose layers. After an overnight spin (18 h), the plasma-enriched fraction was collected from the 24–34% sucrose interface by a hypodermic needle connected to a peristaltic pump. The membranes were diluted with one volume of 4 mM $NaHCO_3$, 1mM $MgCl_2$, pH 8.1, and pelleted at 140,000g for 60 min.

Kidney microsomal membrane. These were prepared from kidneys obtained within 6 h of death. The procedure used for the liver tissue was followed for the preparation of the kidney microsomal fraction (M).

Enzyme marker assays. The liver plasma membrane preparations were evaluated for enzymes used as markers for cellular organelles. Alkaline phosphatase and 5′-nucleotidase were used as plasma membrane markers [8–10], succinate-cytochrome C oxidoreductase as a mitochondrial marker [11], DPNH diaphorase as an endoplasmic reticulum marker [10], acid phosphatase as a lysosomal marker [9, 10], and catalase as a peroxisomal marker [12].

Protein was assayed by the method of Lowry [13] or by the method of Schaffner and Weissmann [14] when Triton X-100 or β-mercaptoethanol were present.

Labeling of Membrane Glycoproteins

Tritiation of placental, liver, and kidney membrane fractions. Placental brush border membranes, liver, and kidney membrane fractions were labeled with tritium by a modification of the method of Blumenfeld and co-workers [15], which utilized oxidation of sialic acid by mild periodic acid oxidation and reduction by (^{3}H)-$NaBH_4$. The conditions for oxidation were those of Van Lenten and Ashwell [16], 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 oxidation was initiated by addition of 0.1 ml of 50 mM sodium meta-periodate. The oxidation was allowed to proceed at 0° in the dark for 10 min and was then terminated by dilution with two volumes of cold 50 mM sodium phosphate 0.15 M NaCl, pH 7.4 (PBS) and pelleting of the placental membranes at 1,500g for 10 min and pelleting of the liver or kidney membranes at 140,000g for 10 min. The membranes were washed twice with 3 ml of PBS. The oxidized membranes were suspended in 1 ml of PBS and reduced with 1–2 mCi of (^{3}H)-$NaBH_4$ (6–10 Ci/mmole) in 25 μl of 0.01 M NaOH for 30 min at 0°. Unlabeled $NaBH_4$ (approximately 1 mg) was added and reduction was allowed to proceed for 10 min at 0°. The membranes were then diluted with two volumes of PBS, pelleted, and washed with 3.0 ml of PBS. The tritiated membranes were stored frozen at −20°.

Iodination of placental brush border proteins. Placental brush border membrane proteins were labeled by lactoperoxidase-catalyzed iodination of tyrosine residues with ^{125}I [17]. Brush border membranes were washed free of ethylenediaminetetracetic acid (EDTA) buffer by one wash with 5 mM $NaHCO_3$ (pH 8.1), 1 mM $MgCl_2$, and one wash with 0.9% NaCl. The membranes containing 5 mg of protein were suspended in 1 ml of PBS (pH 7.4), 1 mM PMSF, and 50 μg of lactoperoxidase (Sigma Chemical Co.), and 200 μCi of carrier-free (^{125}I) sodium iodide were added. The iodination reaction was initiated by the addition of 10 μl of 2.2 μM H_2O_2, and after 5 min at room temperature another

10 μl of H_2O_2 was added. Five minutes later, the reaction was stopped by the addition of 50 μl of 0.5 M NaI and 5 ml of ice-cold PBS. The labeled membranes were washed three times by centrifugation at 2,000g for 10 min at 4° and resuspension in cold PBS. The washed pellet was then extracted with 2 ml of 2% Triton X-100, 5 mM $NaPO_4$ (pH 8.0), 1 mM PMSF. This extract was used directly for immunoprecipitation, or solid urea was added to a concentration of 8 M for two-dimensional electrophoretic analysis.

Two-Dimensional Electrophoresis

The tritiated and iodinated membrane preparations were extracted with 8 M urea, 2% Triton X-100, 6mM $NaPO_4$, pH 8.0 (1 mM PMSF) at room temperature (24°) for 5 min according to Bhakdi et al [18], chilled at 0° on an ice bath, and centrifuged at 140,000g for 60 min at 4°. Under these conditions 50–60% of the acid precipitate tritium extracted into the supernatant from placental membranes and 80–100% from the liver 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°.

The two-dimensional electrophoresis was conducted as previously described [7]. The first dimension was isoelectric focusing in 8 M urea (Schwarz-Mann ultrapure), 0.5% (v/v) Triton X-100, 4% acrylamide (10% cross-linker), 10% glycerol, 1% Ampholines, pH 3.5–10 (LKB Produkter) polyacrylamide gels which were cast in 3-mm × 100-mm rods; and the second dimension was run in 0.1% sodium dodecyl sulfate (SDS), 8% polyacrylamide gel slabs, according to Laemmli [19]. The gels containing tritium-labeled material were then fixed in 50% methanol, 5% acetic acid, for at least 2 h, swollen in 7% acetic acid, and impregnated with PPO for autofluorography according to Bonner and Lasky [21, 22]. The PPO-impregnated, dried gels were autofluorographed on Kodak RP X-Omat x-ray film, at −76° to detect tritiated glycoprotein subunits. The gels containing iodinated material were fixed, swollen in 1% glycerol for 30 min, and dried down for autoradiography on x-ray film with or without the use of Dupont Lightening-Plus intensifying screens to enhance ^{125}I-localization on film.

Production of Antisera to Plancental Brush Border Membrane Components

Antisera to placental brush border membrane components was produced by subcutaneous injections in a young (1–2 years old) female goat. Five brush border membrane preparations were accumulated over four weeks and stored at −76°. The pooled membranes were washed by pelleting out of 5 mM sodium EDTA (pH 7.4) at 5,000g for 20 min. The membranes were extracted with 2% Triton X-100, 6 mM $NaPO_4$, pH 8.0 for 8 min at room temperature and centrifuged at 140,000g for 60 min at 4°. The supernatant was dialyzed over night at 4° against 0.9% w/v NaCl, 10 mM $NaPO_4$, 0.2% v/v glycerol, pH 7.4, with Biobeads SM-2 (Bio-Rad Laboratories) added to the dialysis buffer to remove excess Triton X-100. The final volume of dialyzed extract was 1.5 ml, which was homogenized with 3.5 ml of Freund's complete adjuvant (Gibco) and injected subcutaneously at four sites near the lymph nodes. The first series of injections contained 0.3 mg of placental brush border proteins, containing approximately 22 μg of placental alkaline phosphatase (PAP). A booster was administered four weeks later using the proteins extracted from five more placental brush border preparations. This injection contained 1.04 mg placental protein containing approximately 32 μg of PAP. A serum sample was drawn and assayed for anti-PAP activity two weeks later by a double antibody-immunoprecipitation assay [23]. The

goat was then bled from the femoral artery, yielding 500 ml of serum. Sodium azide was added to the serum as a bacteriostatic agent. The serum was filtered through glass wool and stored in small aliquots at −76°.

Characterization of Antisera

The antisera were found to have strong anti-PAP activity and no detectable anti-LAP activity by immunoprecipitation assay. This antiplacental brush border antiserum is referred to as APBB antiserum.

APBB antiserum was adsorbed with washed liver microsomes to remove nonspecific binding components and antibodies to components in common with liver. Liver microsomal fraction (M) was washed twice by homogenization with 5 mM $NaHCO_3$ (pH 8.1) and centrifugation at 140,000g for 60 min. The antiserum was adsorbed by homogenization of 2 ml of APBB antiserum with 2 ml of washed microsomes. After 40 min at 4°, the suspension was centrifuged at 140,000g for 60 min. The serum was adsorbed three times with 2 ml of packed liver microsomes.

Preparation of APBB Sepharose for immunoaffinity columns. The IgG fraction of the unadsorbed APBB antisera was isolated by three ammonium sulfate precipitations (50% v/v saturated solution of ammonium sulfate) and resolubilizations in deionized water. The final IgG fraction was redissolved in 10 mM ammonium bicarbonate, dialyzed against the same buffer, and lyophylized. Insolubilization of the APBB IgG was accomplished by linking the IgG to cyanogen bromide-activated Sepharose-4B [24]. Nonspecific goat IgG was isolated from goat serum (Gibco) and linked to Sepharose for use in control studies.

Immunoaffinity Chromatography and Immunoprecipitations

Immunoaffinity chromatography of placental brush border glycoproteins. Tritiated placental brush border membranes from a single placenta were extracted with 0.5 ml of 2% Triton X-100, 6 mM $NaPO_4$, pH 8.0, 1 mM PMSF. No urea was used in the extractions, since this was found to interfere with the immunoadsorption. After centrifugation at 140,000g for 60 min at 4°, the supernatant was used for the immunoaffinity chromatography experiment.

The supernatant containing the tritiated placental brush border sialoglycoproteins was mixed with 2.0 ml of APBB Sepharose in 0.5 M NaCl, 0.2 M Tris-HCl, pH 8.0, 0.2% Triton X-100 (1:1, Sepharose:buffer) at 4° for 2 h. The suspension was then allowed to warm to room temperature and poured into a Pasteur pipette column plugged with glass wool. The column was washed at room temperature with ten bed volumes of 0.1% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, and the adsorbed material was eluted with 8 M urea in wash buffer. The column void volume, wash, and elution were collected in 0.5 ml fractions and assayed for tritium and alkaline phosphatase. The eluted peak of alkaline phosphatase, which corresponds to the tritium peak, was pooled and concentrated at 4° in an Amicon Miniconcentrator A-15 (15,000-dalton membrane exclusion limit) to less than 100 μl. The sample was then reduced by 5% v/v β-mercaptoethanol, 10 min at room temperature, prior to two-dimensional electrophoretic analysis.

Controls using nonspecific goat IgG Sepharose were run identically and in parallel to immunoadsorption studies with APBB Sepharose.

Immunoaffinity chromatography of liver membrane glycoproteins. Tritium-labeled plasma membranes from liver were extracted by 2% Triton buffer without urea and adsorbed on APBB Sepharose, as previously described for the placental brush border

extracts. The bound membrane components were eluted with 8 M urea, and the elution fractions were assayed for the tritium label. The eluted tritium peak was pooled, concentrated, and analyzed by two-dimensional electrophoresis.

Immunoprecipitation of membrane glycoproteins. As an alternative to immunoaffinity columns which require large amounts of antisera, formalin-fixed Staphylococcus aureus was used to precipitate the antigen-antibody complexes formed between APBB antisera and labeled membrane glycoproteins. This procedure was necessary for the use of liver-absorbed APBB antiserum, since small amounts of this antiserum were available. The procedure of Kessler [25] was followed for the preparation of the S. aureus and the immunoprecipitation reactions. The only modification of this method was the use of 0.1% SDS in the immunoprecipitation reaction, to eliminate nonspecific precipitations. The immunoprecipitate was washed three times and the pellet was then extracted with 200 μl of 9 M urea in 2% Triton X-100 extraction buffer. The S. aureus was pelleted from the extract at room temperature at 2,000g for 10 min and the supernatant collected. The supernatant was concentrated to 100 μl and reduced with 5% v/v β-mercaptoethanol prior to two-dimensional electrophoretic analysis.

RESULTS

Membrane Preparations

Placenta. The preparation of brush border membrane from full-term human placentas was previously described [6, 7]. This preparation yielded large membrane fragments with microvilli projecting from basal membranes, and the plasma membrane enzyme markers, placental alkaline phosphatase, and 5′-nucleotidase were enriched 10-fold and 18-fold, which was consistent with previous results.

Liver. The human liver preparation was monitored by the enzyme markers, alkaline phosphatase, and 5′-nucleotidase, which showed 21.5-fold and 6.7-fold enrichment in specific activity of the membranes from the nuclear pellet (NF). Acid phosphatase was increased 2.3-fold and DPNH diaphorase was increased 2.4-fold. Succinate-cytochrome C oxidoreductase decreased in specific activity by a factor of 0.8 (Table I). Enzyme recovery data indicated 100% of the alkaline phosphatase was in 5.0% of the total cell protein for the final plasma membrane fraction prepared from the nuclear pellet (NF). The final plasma membrane fraction from the microsomal pellet (MF) gave 100% of the alkaline phosphatase in 13.4% of the total cell protein, indicating a higher degree of purity for the NF preparation.

Labeling of Membrane Glycoproteins

The carbohydrate moiety of the membrane glycoproteins was tritiated by mild oxidation with Na-meta-periodate and reduction with (^{3}H)-$NaBH_4$, which resulted in the incorporation of 90×10^6 dpm/mg membrane protein. This technique incorporates tritium predominantly into the sialic acid moieties of the erythrocyte ghost [15] and has been described as a technique for sialic acid labeling [15, 16, 26]. As previously described [7], the tritiation of the placental brush border membranes by this method was entirely dependent on oxidation by periodate prior to (^{3}H)-$NaBH_4$ reduction, which is consistent with labeling the sialic acid residues. As further evidence for the incorporation of the label into sialic acid, predigestion of these membranes with C perfringens neuraminidase (Worthington Biochemicals, 0.1 unit/ml at 37° for 30 min) inhibited label incorporation

by 40% as compared to membranes incubated without neuraminidase. The specificity of this labeling technique for carbohydrate was also demonstrated by the fact that the label was not incorporated into a major nonglycosylated membrane protein, the "actin-like" polypeptide [27].

Lactoperoxidase-catalyzed iodination of placental brush border proteins resulted in the incorporation of 5.5×10^6 dpm of ^{125}I per milligram of protein into trichloroacetic acid-precipitable membrane components. The incorporation of iodine into the membrane components of interest was entirely dependent on the addition of lactoperoxidase, which indicates that the iodination is the enzyme-catalyzed incorporation of label into tyrosine residues [17, 31, 32].

Immunochemical Characterization of the Placental Brush Border Membrane

Characterization of the immunogen used to develop the APBB antisera. Since the initial two-dimensional mapping technique [7] utilized 8 M urea for membrane extraction while urea was deleted for immunogen extraction, we determined which glycoproteins were present in the immunogen. Two-dimensional maps of brush border extracts with and without 8 M urea showed that all of the glycoproteins present in the initial two-dimensional maps were also present in the immunogen. The use of Triton X-100 extraction without urea appears to have achieved a partial purification of the brush border glycoproteins as evidenced by a 5–10-fold increase in alkaline phosphatase specific activity (units/mg protein) over the brush border membranes. This may be due to the selective extraction of integral membrane proteins by Triton X-100, leaving the filamentous matrix attributable to "spectrin-like" polypeptides intact, as has been demonstrated for erythrocyte ghosts [28]. The placental brush border membrane preparation also has been shown to possess a prominent network of microfilaments on its inner surface [6].

TABLE I. Purification of Liver Plasma Membrane

Specific activity (units/mg protein)[a]	Homogenate (H)	Sucrose gradient fractions[b]		Enrichment (final/homogenate)	
		NF	MF	NF	MF
Alkaline phosphatase ($\times 10^{-1}$) (average of two preps)	0.24	5.16	4.83	21.5	20.1
5'-Nucleotidase ($\times 10^{-2}$) (average of two preps)	0.96	6.40	28.2	6.66	29.3
Acid phosphatase ($\times 10^{-1}$) (one prep)	0.32	0.73	2.40	2.28	7.45
Succinate-cytochrome C oxidoreductase ($\times 10^{-4}$) (one prep)	0.94	0.88	0.76	0.76	0.81
DPNH-diaphorase (average of two preps)	0.72	1.70	3.69	2.35	5.13

[a]One unit of enzyme activity equals one μmole product per minute at 37°.
[b]NF, MF) Final plasma membrane containing fractions from the nuclear pellet and microsomal pellet, respectively.

Identification of tritium-labeled subunits. The immunoaffinity chromatography of the Triton X-100-extracted tritiated glycoproteins on APBB Sepharose was monitored by measuring the retention in the column of alkaline phosphatase activity and tritium label. That most of the enzyme activity was bound to the APBB Sepharose was indicated by recovery of only 7% of the applied activity in the void volume of the immunoaffinity columns. The bound enzyme activity was eluted with 8 M urea; however, the activity eluted could not be quantitated since the urea partially inactivates the enzyme (Fig. 1a). The control column of nonspecific goat IgG Sepharose did not bind alkaline phosphatase; this was indicated by the fact that 89% of the enzyme activity was in the void volume, and no activity was detected in the 8 M urea eluant (Fig. 1b). The pooled fractions containing the alkaline phosphatase peak eluted from the APBB Sepharose column contained 1.1×10^6 dpm (3.9% of the applied tritium) above the background found in the wash fractions: no radioactivity above background was detected in the eluant from the nonspecific IgG Sepharose column.

Two-dimensional mapping of the tritium-labeled placental glycoproteins in the studies using immunoaffinity columns demonstrated that 16 of the 33 labeled components present in the total membrane extracts (Fig. 2a,b) reacted with the APBB antiserum. The 8 M urea eluant from the control, nonspecific IgG Sepharose column gave no detectable components in two-dimensional maps, indicating the specificity of the binding of the subunits to the

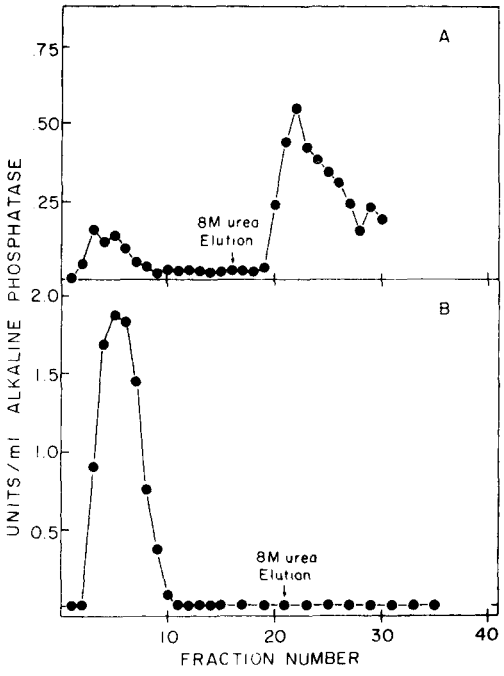


Fig. 1. Immunoadsorption of Triton X-100 solubilized placental brush border glycoproteins by APBB Sepharose. Tritiated placental brush border membranes from one placenta were extracted with 0.5 ml of 2% Triton buffer (no urea) and split into equal aliquots of 0.25 ml each containing 2.5 units of alkaline phosphatase. One aliquot was adsorbed to APBB Sepharose (A) and the other to control nonspecific IgG Sepharose (B) as described in Methods. Columns of A and B were poured, washed with buffer, and eluted with 8 M urea in buffer. The columns were monitored by alkaline phosphatase activity.

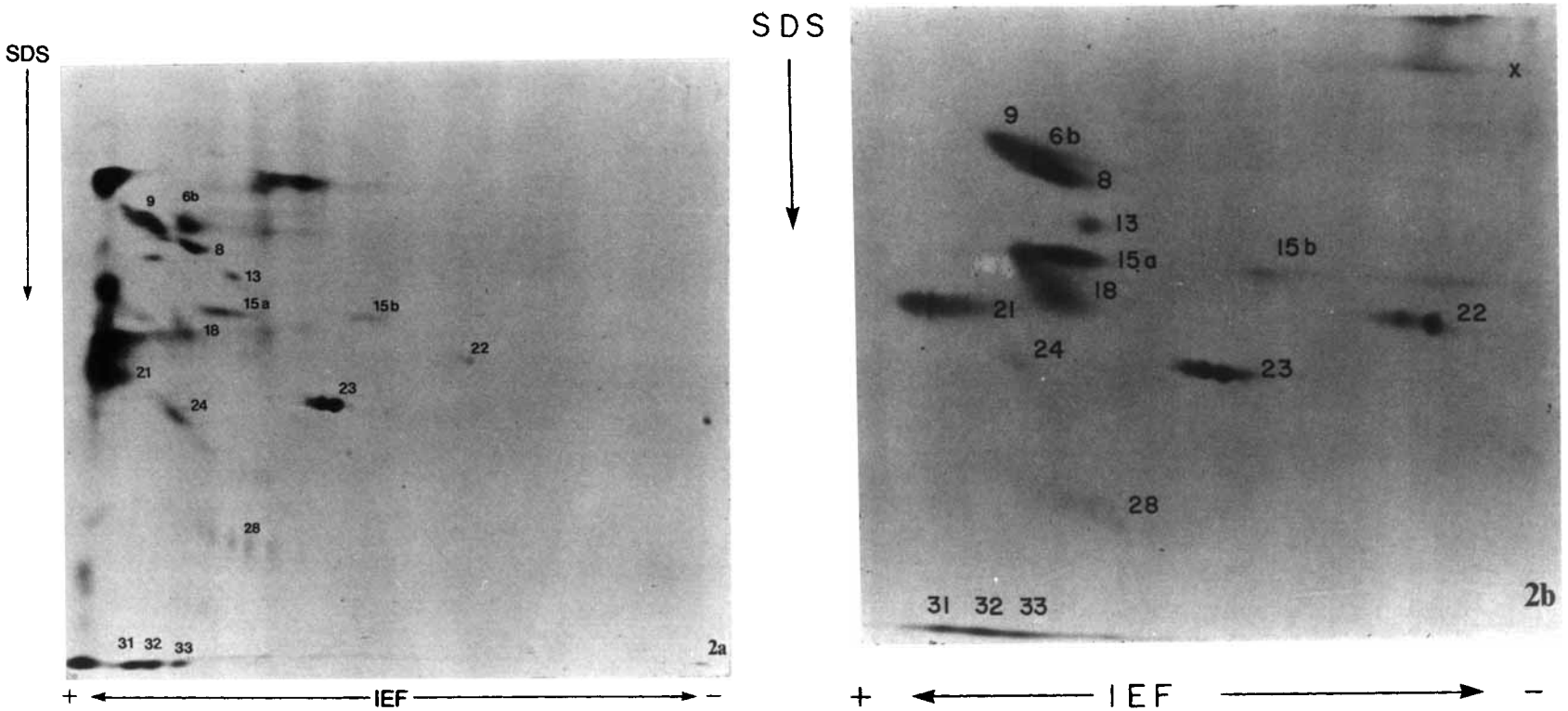


Fig. 2. a: Tritium-labeled placental brush border subunits; 1.4×10^6 dpm of acid-precipitable tritium extracted from labeled brush border membranes with 2% Triton X-100, 8 M urea buffer was mapped by two-dimensional electrophoresis in 3–10 pH, IEF gel rod, and an 8% acrylamide, 0.1% SDS gel slab. The gel was autofluorographed at −76° for 18 h. The numbers labeling spots on the map correspond to those from Table II, which presents the M_r and pI coordinates of the subunits. b: Tritium-labeled placental brush border subunits adsorbed to APBB Sepharose; 1.1×10^6 dpm of tritiated membrane glycoproteins eluted from APBB Sepharose from the same experiment as described in Fig. 1a was mapped by two-dimensional electrophoresis. The gel was autofluorographed for four days.

APBB Sepharose. The M_r and pI coordinates of these subunits were determined for the 16 antigenic placental glycoproteins by their correspondence to spots on the maps of total membrane extracts (Table II). The maps and coordinates of the total membrane extracts coincided with those previously reported for placental brush border membrane glycoproteins [7].

One of the major antigenic placental glycoproteins was PAP, which corresponds to spot No. 23 in Fig. 2b. This spot had previously been determined by ^{32}P-labeling of PAP and coelectrophoresis with tritium-labeled brush border glycoproteins in two-dimensional maps [7]. The ability to identify PAP by labeling the active center as well as the carbohydrate portion made it a good reference point in the evaluation of other spots in the maps.

Immunoprecipitation of placental brush border glycoproteins with APBB antisera using S. Aureus gave two-dimensional maps equivalent to the maps obtained by the immunoaffinity columns. Controls using normal goat serum in place of specific antiserum showed no precipitation of glycoprotein subunits from placental extracts, which indicated the specificity of the method.

Identification of iodinated subunits. Two-dimensional mapping of the iodinated whole placental brush border membrane protein (Fig. 3a) demonstrated that most of the very acidic, heavily tritiated placental brush border components running at the anodal end of the whole membrane two-dimensional maps in Fig. 2a were not iodinated. These acidic components which were tritiated but not iodinated were not antigenic as determined by their failure to bind to APBB Sepharose. The only subunit iodinated in this region of the maps was Spot No. 21, which also was antigenic.

TABLE II. Coordinates of Placental Brush Border Sialoglycoprotein Subunits in Two-Dimensional Maps

Spots	Rf		Apparent pI	Apparent M_r ($\times 10^3$)
	X(pI)	Y(M_r)		
X*†	–	–	8.15	308.0
6b*	0.194	0.258	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
13*	0.263	0.344	5.86	123.0
15*a	0.252	0.400	5.82	100.0
15*b	0.469	0.400	6.57	100.0
18*	0.202	0.422	5.65	92.4
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
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
31*	0.060	1.00	4.60	< 40.0
32*	0.146	1.00	5.45	< 40.0
33*	0.207	1.00	5.66	< 40.0

*Subunits adsorbed to APBB-Sepharose.
†X was only detectable after immunoadsorption to APBB-Sepharose.
‡7 was only detectable after immunoprecipitation with liver adsorbed APBB-antisera.

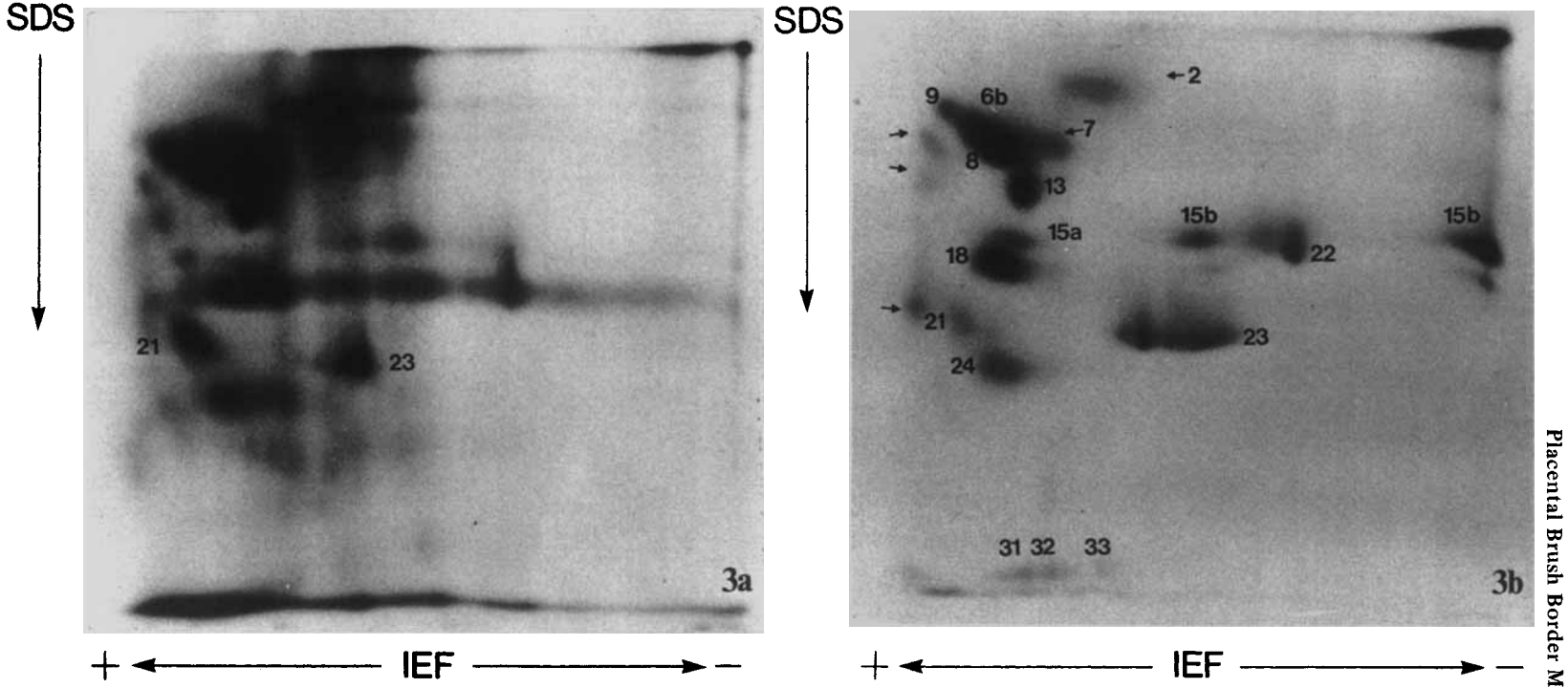


Fig. 3. a: Iodine-labeled placental brush border subunits; 1.1×10^5 cpm of acid-precipitable ^{125}I extracted from labeled brush border membranes with 2% Triton X-100 buffer (no urea) was mapped by two-dimensional electrophoresis in 3–10 pH, IEF gel rod, and 8% acrylamide SDS gel slab. The gels were autoradiographed for four days with intensifying screens at −76°. The numbers labeling these spots correspond to those of Table II. b: Iodine-labeled placental brush border subunits precipitated with APBB antisera; 4.4×10^5 cpm of acid-precipitable ^{125}I extracted from labeled membranes was immunoprecipitated with 20 μl of APBB antisera and 200 μl of S aureus as described in Methods. The washed precipitate was eluted with 200 μl of 9 M urea in extraction buffer, concentrated to 100 μl, and mapped by two-dimensional electrophoresis. The gel was autoradiographed with intensifying screen for four days. Arrows in left margin denote subunits undetected by periodate/(^3H)-$NaBH_4$ tritiation.

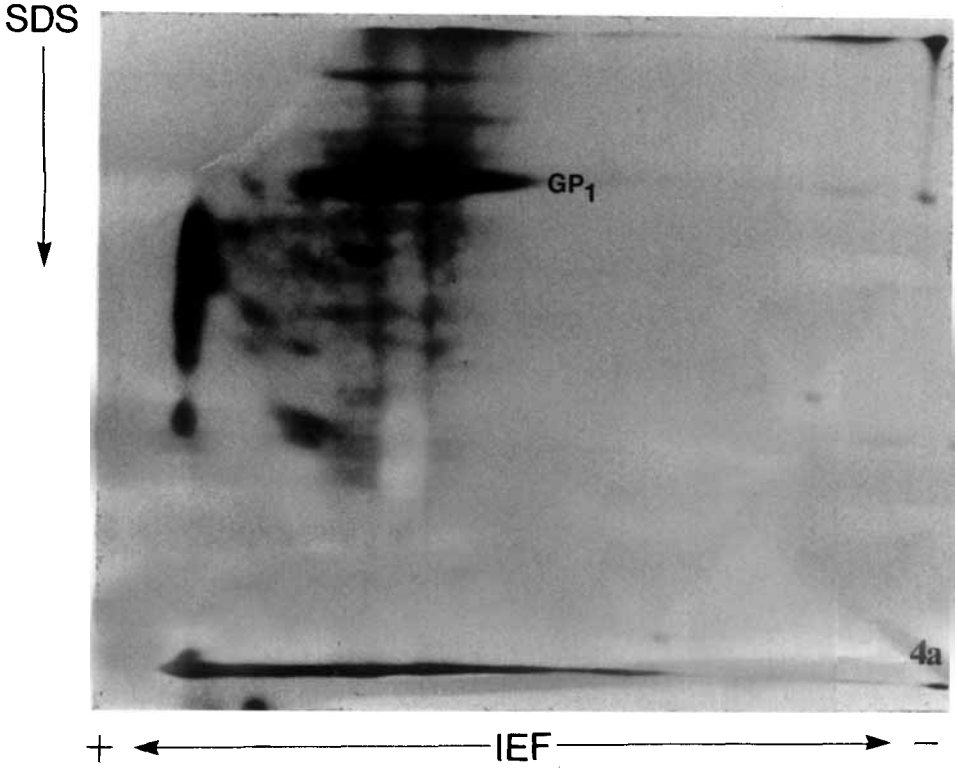
Two-dimensional maps of immunoprecipitated iodinated placental brush border membrane proteins demonstrated that most of the tritiated placental glycoproteins immunoprecipitated by APBB antisera are also iodinated by lactoperoxidase (Fig. 3b). This observation was interpreted as confirmation of the protein nature of these spots. There were two immunoprecipitated, tritiated components which were not iodinated: spots No. 28 and X. These may represent components with inaccessible tyrosines. Conversely, there were two subunits which were at the limits of detection by tritium labeling but easily detected by iodination (spots No. 7 and 2 in Fig. 3b). There were three subunits not previously observed in placental membranes by tritiation which were observed in iodinated membranes. These components were minor components in the maps of immunoprecipitated material and ran at the anodal pole of the two-dimensional maps.

Comparison of Placental Brush Border Subunits to Liver and Kidney Membrane Subunits

Liver membranes. The two-dimensional map of the solubilized liver plasma membrane preparation from the nuclear pellet (NF) and from the microsomal pellet (MF) were indistinguishable in pattern. The pattern of spots in the liver membrane maps (Fig. 4a) were clearly different from the maps of the placental brush border membranes (Fig. 2a). One major tritiated liver spot, designated GP_1, was found to have a broad pI distribution (5.48–6.60), and a $M_{\bar{r}}$ of 169 k. It was the principal sialoglycorpotein of the liver membrane containing at least 10% of the tritium, which was incorporated into sialic acid. This subunit may be analogous to a prominent sialoglycoprotein component containing 10% of the sialic acid present in rat liver plasma membranes [29, 30]. This rat liver component is called GP_1 and had a $M_{\bar{r}}$ of 125–130 k.

The predominant glycoproteins seen in the maps of the solubilized liver plasma membrane (Fig. 4a) were absent in the two-dimensional electrophoretic maps of the components eluted from the APBB Sepharose columns (Fig. 4b). The maps of the eluted components (1.7% of the applied tritium) contained seven glycoproteins which corresponded to the spots in the placental map (Fig. 2b): spots Nos. 6b, 8, 9, 13, 18, 21, and 24. These spots had not been apparent in the maps of the solubilized whole liver plasma membrane preparation. The correspondence of these seven glycoproteins with those in the placental brush border was established by the $M_{\bar{r}}$ and pI coordinates of the two-dimensional maps of the immunoadsorbed liver and placental preparations which were electrophoresed at the same time in the same apparatus. Three subunits, spots Nos. 31, 32, and 33, were not clearly present or absent in the liver membranes because they ran with a streak of undefined liver material at the dye front in the SDS dimension. Six placental subunits, spots Nos. 15a, 15b, 22, 23, 28, and X, were clearly not detectable in the liver (Table III).

Fig. 4. a: Liver plasma membrane tritiated subunits; 4.3×10^6 dpm of acid-precipitable tritium from labeled liver plasma membrane-enriched fraction NF was extracted with 2% Triton X-100, 8 M urea buffer and was mapped by two-dimensional electrophoresis. The tritium was visualized by autofluorography for 18 h at −76°. b: Liver plasma membrane tritiated subunits adsorbed to APBB Sepharose. Tritiated liver plasma membrane-enriched fraction MF from 1/5 of a liver preparation was extracted with 0.5 ml of 2% Triton X-100 (no urea). Half of this extract, containing 20×10^6 dpm of acid-precipitable tritium, was adsorbed to APBB Sepharose as described in Methods. Labeled glycoproteins eluted from the APBB Sepharose, containing 0.8×10^6 dpm, were mapped by two-dimensional electrophoresis. The gel was autofluorographed for 10 days at −76°. The numbers labeling these spots correspond to placental glycoprotein subunits listed in Table III. Spots labeled by M were not seen in placental brush border membranes and were enriched in liver microsomal membranes.


SDS
GP1
4a
+
IEF
−

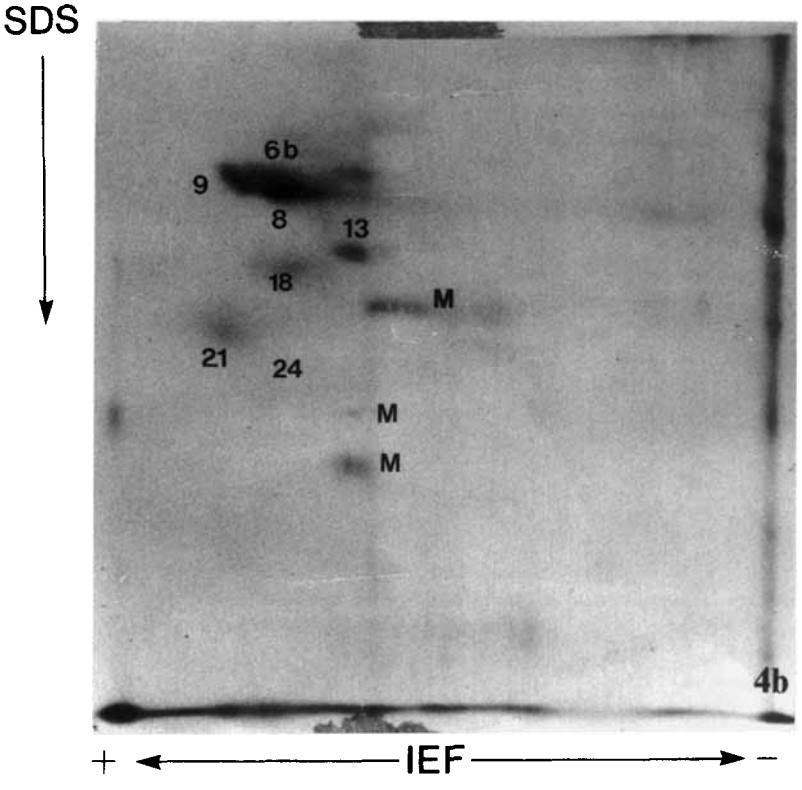

SDS
6b
9
8
13
18
M
21
24
M
M
4b
+
IEF
−

TABLE III. Placental Brush Border Glycoproteins Found in Liver and Kidney Membranes: Summary of Data

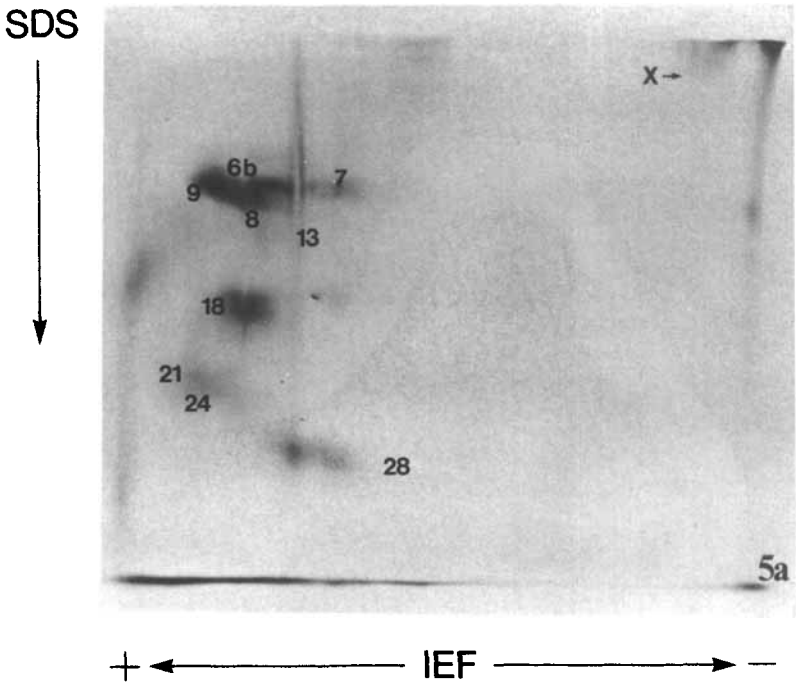
Placental brush border		Liver	Kidney
$M_{\bar{r}}(\times 10^3)$	Number	plasma membrane*	microsomes*
308	X	–	+
175–181	9	++	+
170	6b	++	+
161	7†	–	+
140	8	++	++
123	13	+	+
100	15a	–	–
100	15b	–	–
92.4	18	+	+
74.6	22	–	–
72.5	21	++	+
66.0	23	–	–
64.0	24	+	+
47.0	28	–	+
< 40.0	31	ND	ND
< 40.0	32	ND	ND
< 40.0	33	ND	ND

*Symbols: ++) present in higher quantities, +) present, –) absent, ND) not determined.
†7 was not a major glycoprotein in placental brush borders and was not previously considered in the 16 components binding to APBB antisera (Fig. 3b).

Kidney membranes. Immunoprecipitation of the kidney microsomal glycoproteins with APBB antisera gave two-dimensional maps which contained the seven glycoprotein subunits common to liver and placenta (Fig. 5a). Spots Nos. 7, X and 28, which were in the placenta but not the liver preparation, were present in the kidney maps, indicating that these subunits are not placental-specific. The potential placental specificity of spots Nos. 15a, 15b, 22, and 23 was supported by their absence from the kidney maps.

Immunoprecipitation studies using liver-adsorbed APBB antisera. The APBB antiserum was adsorbed with liver microsomes to increase its specificity for the placental components. Two-dimensional maps showed liver-adsorbed antisera did not immunoprecipitate any tritiated subunits from the liver microsomes, indicating adequate adsorption of the antisera.

Fig. 5. a: Kidney microsome tritiated subunits precipitated with APBB antisera; 17.4×10^6 dpm of acid-precipitable tritium extracted from labeled kidney microsomes with 2% Triton X-100 buffer (no urea) was immunoprecipitated with 20 μl of APBB antisera and 200 μl of S aureus. The washed precipitate was eluted and mapped by two-dimensional electrophoresis. The gel was autofluorographed for four days at −76°. The numbers labeling these maps correspond to those placental glycoprotein subunits listed in Table II. b: Kidney microsome tritiated subunits precipitated with liver-adsorbed APBB antisera; 17.4×10^6 dpm of tritiated kidney microsome Triton X-100 extract was immunoprecipitated with 100 μl of liver-adsorbed APBB antisera and 200 μl of S aureus. The washed precipitate was eluted and mapped by two-dimensional electrophoresis. The gel was autofluorographed for four days.
c: Placental brush border tritiated subunits precipitated with liver-adsorbed APBB antisera; 10×10^6 dpm of tritiated placental brush border Triton X-100 extract was immunoprecipitated with 100 μl of liver-adsorbed APBB antisera and 200 μl of S aureus. The washed precipitate was eluted and mapped by two-dimensional electrophoresis. The gel was autofluorographed for four days.


SDS
X
6b
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21
24
28
5a
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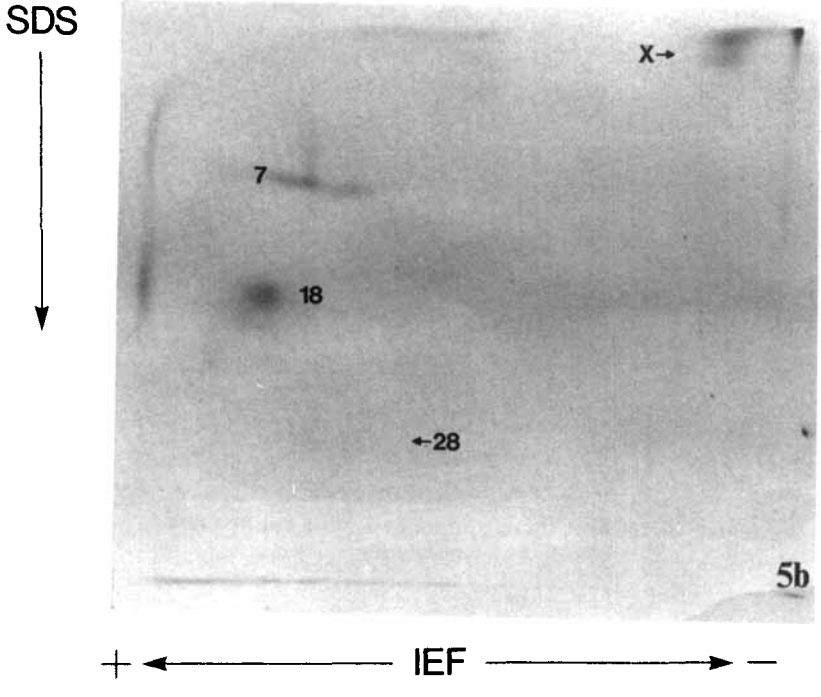
SDS
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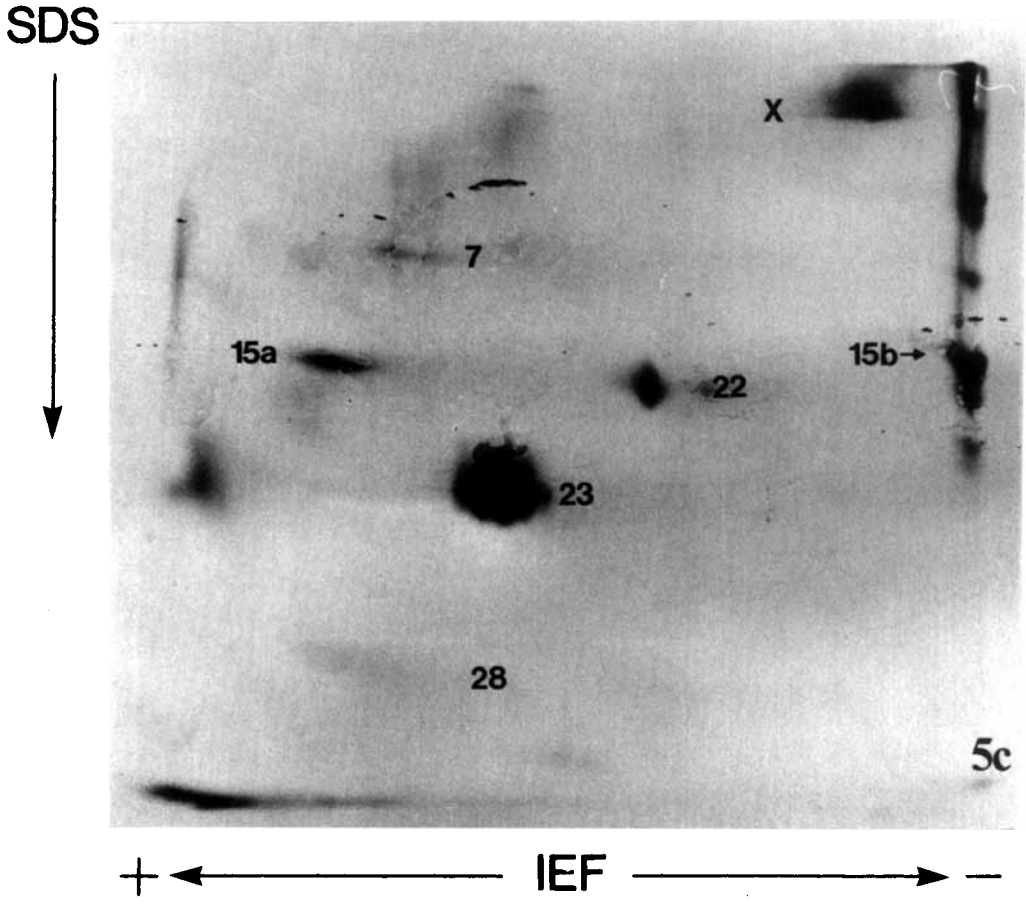

When the liver-adsorbed APBB antisera was reacted with solubilized placental membranes, there was no significant precipitation of subunits common to the liver. The prominent subunits which precipitated were 15a, 15b, 22, 23, 28, X, and 7 (a component not easily detected by tritium labeling) (Fig. 5c). Use of the adsorbed antisera brought out the presence of 7 by the removal of 8, 9, and 6b, which run very close to this subunit and are present in much larger amounts. The precipitation of these placental subunits by the adsorbed antisera indicated that these components must not be present in the liver microsome preparation or may be present in extremely low levels, since adsorption with the liver microsomes did not remove antibodies against these subunits. The potential placental specificity of 15a, 15b, 22, and 23 was also indicated by the fact that these were not immunoprecipitated from the kidney microsomal preparation. The precipitation of spots Nos. 7, 18, 28, and X, from the kidney microsomal preparation by the liver-adsorbed APBB antisera indicated that these subunits are not placenta-specific (Fig. 5b), a finding

that is in agreement with the results using unadsorbed antisera. It is not clear why spot No. 18 was not removed by liver adsorption, because this spot is present in liver plasma membrane maps and would be expected to be removed by liver adsorption. One possibility is that spot No. 18 is coprecipitating with one of the other subunits from the kidney membrane extracts.

DISCUSSION

In previous studies, specific tritiation of membrane sialic acid and two-dimensional electrophoresis of the extracted, labeled membrane components enabled the characterization of a complex mixture of placental brush border membrane sialoglycoprotein subunits [7]. The present investigation was undertaken as an approach to determine whether any of these subunits are specific to the placental brush border membrane.

Initial studies compared the solubilized glycoproteins extracted from placental brush border membranes and a liver plasma membrane preparation. The two-dimensional electrophoretic maps of the solubilized membranes were good for the comparison of the major glycoprotein patterns. These maps clearly showed different patterns for the placental and liver membranes. However, it was difficult to accurately locate and compare individual subunits due to the smearing of some subunits and the high background in certain areas of the maps. These results demonstrated the need for more accurate resolution of the individual subunits before comparative analysis of the membrane preparations could be made.

Subsequent studies were directed to characterize the antigenic glycoproteins of the placental brush border membrane. These studies were conducted using either immunoaffinity chromatography or immunoprecipitation with an antiserum raised against the solubilized glycoproteins from the placental brush border membrane (APBB antiserum). This antiserum made possible the identification of a set of 16 antigenic subunits of the 33 subunits present in the whole membrane extracts. The two-dimensional electrophoretic maps of the immunochemically isolated antigenic glycoproteins of the placental brush border membrane were well-defined in contrast to the maps of the solubilized whole membrane preparation. These maps were consistently obtained and could be accurately compared with maps of the immunochemically cross-reacting glycoproteins from liver membrane and kidney microsome preparations.

Of the 18 antigenic glycoproteins, a set of seven glycoprotein subunits common to placental brush border, liver plasma membrane, and kidney microsomes (spots Nos. 6b, 8, 9, 13, 18, 21, and 24) were identified on the basis of M_r, pI, and immunochemical cross-reactivity (Table III). The fact that these subunits were iodinated by lactoperoxidase demonstrated that these components are glycoproteins, rather than glycolipid or polysaccharide. Three of the antigenic glycoprotein subunits (spots Nos. 7, X and 28) were only seen in kidney and placental membranes. Some subunits (spots Nos. 31, 32, and 33) were running in the dye front of the electrophoretic maps and could not be compared. A subunit which was not easily detected by tritium labeling (spot No. 2, Fig 3b) was also not considered. The identification of glycoproteins that are common to the plasma membranes of different tissues suggests that these may be concerned with common or constitutive functions such as carriers for essential metabolities and ion transport [33].

A set of four antigenic glycoprotein subunits (spots Nos. 15a, 15b, 22, and 23) were not found in the liver or kidney membranes (Table III). The techniques of two-dimensional electrophoresis and autofluorography utilized for the detection of the tritium-labeled glycoproteins could not be specifically quantitated. However, an estimate of the lower limit of detection for a single tritiated subunit can be made on the basis of the studies of

Bonner and Laskey [21] and Laskey and Mills [22] on the quantitation of autofluorography. Using their lower limit of 500 dpm/24 h exposure, a subunit containing 0.05% of the tritium in 1×10^6 dpm sample is detectable. The sensitivity of the detection was increased in our studies by longer exposure times (up to 10 days). At this level of sensitivity of detection and with the use of immunochemical isolation, even low-level membrane constituents can be detected. The failure to identify the four placental glycoproteins in liver and kidney indicates their total absence or selective modification of these subunits, such as complete removal of sialic acid, which would prevent labeling.

As an additional test for the placental specificity of this set of glycoproteins, the APBB antiserum was adsorbed with human liver microsomes. The adsorption greatly decreased the affinity of the antisera for the seven common glycoprotein subunits, while not diminishing the high affinity for the putative placental specific subunits. These results support the earlier conclusion that the common set of subunits is present in liver membranes and the placenta-specific set of subunits is absent.

One of the putative placenta-specific glycoprotein subunits (spot No. 23) was previously shown to be placental alkaline phosphatase by coelectrophoresis in two-dimensional gels with purified, ^{32}P-labeled enzyme [7]. Placental alkaline phosphatase has been demonstrated to be specific to the placental trophoblast [23] and localized in the brush border membrane [6]. If the other three putative placenta-specific glycoproteins (spots Nos. 15a, 15b, and 22) are limited to the placenta, one could speculate that they contribute to the specialized properties of the trophoblast membrane. The definitive proof of placental specificity and elucidation of the biologic function of these placental brush border glycoproteins will require further study of the purified glycoproteins.

The definition of the common and specific placental glycoproteins as cell surface components is an important consideration which can be approached by cell surface labeling of cells in vitro. These experiments have been performed and confirm that the placental glycoproteins characterized in the present study are localized at the cell surface. The results of these experiments will be reported in a subsequent paper (Hamilton, Wada, and Sussman, manuscript in preparation, 1979).

NOTE ADDED IN PROOF

Further studies on the identity of glycoprotein subunit No. 22 have revealed this component to be transferrin, a serum protein, specifically bound to the placental brush border membrane. The studies, to be reported in a subsequent paper, indicated the membrane-bound transferrin to be associated with glycoprotein subunit No. 15b, which appears to be the placental brush border membrane receptor for maternal transferrin.

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