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Isolation of Highly Enriched Apical Plasma Membranes of the Placental Syncytiotrophoblast

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Abstract

The human placenta is a complex organ whose proper function is crucial for the development of the fetus. The placenta contains within its structure elements of the maternal and fetal circulatory systems. The interface with maternal blood is the lining of the placenta, that is a unique compartment known as the syncytiotrophoblast. This large syncytial structure is a single cell layer in thickness, and the apical plasma membrane of the syncytiotrophoblast interacts directly with maternal blood. Relatively little is known about the proteins that reside in this unique plasma membrane or how they may change in various placental diseases. Our goal was to develop methods for isolating highly enriched preparations of this apical plasma membrane compatible with high quality proteomics analysis and herein describe the properties of these isolated membranes.

Keywords

Placenta; plasma membrane; syncytiotrophoblast

Introduction

The human placenta is a vital organ mediating the efficient and selective transfer of solutes and gases between mother and fetus during gestation. It also produces hormones and growth factors that support pregnancy and serves as a barrier to the maternal immune system thereby protecting the developing fetus. Despite the anatomical complexity of the placenta proper, the arrangement of physiologically-relevant units for maternal-fetal exchange (the terminal villus) can be conceived quite simply: herein, fetal capillaries, invested in finger-like projections of stromal and placental cells, are situated in close proximity with the maternal bloodstream. Intervening between these dual circulatory systems is a syncytium of placental epithelial cells (syncytiotrophoblast, STB) in direct contact with maternal blood. The STB serves to establish a physical, metabolic, and immune barrier between mother and fetus [1].

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The apical portion of the STB contains microvilli (MV) forming a large surface area for absorption and secretion. Transport of small molecules including: glucose, amino acids, water, ions, vitamins, metabolic gases, and lipids as well as some macromolecules crucial to the developing fetus occurs at this site [2,3]. Transplacental transport mechanisms rely heavily on proteins of the apical plasma membrane (PM) of the STB. Moreover, disorders in placental structure and/or function underpin many severe complications of pregnancy, including pre-eclampsia (i.e., hypertension of pregnancy), intrauterine growth restriction, and alloimmunization disease [4]. Despite the obvious importance of the apical PM of the STB, a detailed understanding of this membrane at the molecular level is not available presently.

Proteomics analysis of the apical PM of the STB is a direct unbiased way to obtain information related to the protein composition of this membrane that may shed light on its biology and pathophysiology. The dynamic range of protein expression in biological compartments (e.g., biological fluids, cells, tissues, organs) can range over several orders of magnitude [5]. This range of expression presents practical problems for proteomics analysis; the severity of the problem depends in part on the methodologies used to analyze the proteome (e.g., mass spectrometry, protein microarrays, 2-dimensional gel electrophoresis) [5,6]. Several studies have addressed this issue and presented methods by which a given sample can be simplified thus facilitating proteomics analysis. These methods include enrichment of specific organelles from cells or tissues [7,8]. The net effect of simplifying the mixture of proteins increases the prospects for detection of less abundant proteins.

Key features of our approach in the placenta have been developing methods for isolating highly-enriched preparations of MV from the STB, and subsequent reduction of non-PM proteins from these MV, which further enriches for integral PM proteins. These fractionation and sub-fractionation methodologies are important for analyzing membrane proteins many of which will be in low copy number compared to the structural and house-keeping proteins present in cells and tissues [9]. Herein, we present the methods used to isolate a highly enriched preparation of the apical PM of the STB that is amenable to proteomics and biochemical analysis.

Materials and methods

Reagents and Supplies

Murine monoclonal antibodies were: anti- α -tubulin (clone DM1A), Accurate Chemical & Scientific Corp., Westbury, NY), anti-LAMP-1 (clone H4A3) and LAMP-2 (clone H4B4) (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-placental alkaline phosphatase (PLAP) (clone 8BS, Sigma-Aldrich, St. Louis, MO), anti-actin (clone AC15, Sigma-Aldrich), anti-CD31 (Dr. P. Newman, Milwaukee, WI), anti-complex V, subunit α (Mito Sciences, Eugene, OR), anti-ezrin (clone 18) and anti-BiP (clone 40) were from BD Transduction Laboratories (San Jose, CA), and anti-dysferlin (Vector Labs., Burlingame, CA). Polyclonal antibodies used were a chicken anti-caveolin 1 α (CAV 1 α) that has been characterized previously [10,11] and rabbit anti-CAV 1 (BD Transduction Labs.). Horseradish peroxidase-labeled donkey anti-mouse, anti-chicken, and anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Poly (acrylic acid-co-maleic acid) sodium salt, protease inhibitor cocktail (cat. #P8340), Ludox CL cationic colloidal silica, and Histodenz. were purchased from Sigma-Aldrich (St. Louis, MO). The BCA protein determination and SuperSignal chemiluminescent kits and colloidal coomassie blue G250 were from Pierce Biotechnology (Rockford, IL). Criterion pre-cast 1-D SDS-PAGE gradient gels and sodium dodecyl sulfate (SDS) were from Bio-Rad Laboratories (Hercules, CA). Fuji Super RX x-ray film was obtained from Fisher

Scientific (Pittsburgh, PA). Glutaraldehyde and formvar were from Electron Microscopy Sciences (Hatfield, PA). Colloidal gold (15 nm) was from Ted Pella, Inc. (Reading, CA). Epon embedding reagents were from Fluka (Sigma-Aldrich). RPMI-1640 medium, fetal calf serum, and penicillin/streptomycin were from Invitrogen. Conventional cationic colloidal silica [12] was from Dr. D.E. Stolz (University of Pittsburgh). All aqueous solutions were prepared with Milli-Q water (Millipore, Bedford, MA). Other reagents and supplies were as we have described previously [10,13].

Cells and Tissue

HL-60 cells were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Human term (39-41 week) placentas were obtained with informed consent according to a protocol approved by the Biomedical Sciences Institutional Review Board at Ohio State University, Columbus, OH. Tissue samples only from uncomplicated cesarean-section deliveries were used. In all cases, tissue processing was initiated as soon as possible following delivery (within 20 min). Placental tissue was cut into segments (~12 cm × 8 cm) and placed into beakers containing ice-cold PBS. The basal plate was dissected away from these segments to expose the placental villous tree. A total of 15 term placentas were used in the development of the methods herein described.

Coating plasma membranes with cationic colloidal silica particles: electron microscopy

Plasma membrane coating with cationic colloidal silica particles (CCS) was modeled in HL-60 cells using the method of Jacobson and colleagues [12]. In brief, cells were centrifuged from the culture medium and washed in PBS. After the PBS wash, pelleted cells were resuspended in 5 ml of coating buffer [20 mM MES, pH 5.5, 100 mM NaCl, 5 mM MgCl₂] and then added drop-wise to 30 ml of coating buffer containing 1% CCS (w:v). Cells were incubated with CCS for 3 minutes and then centrifuged at low speed (60 × g for 5 min) to minimize cell rupture. Cells were then resuspended in coating buffer and washed by centrifugation. The washed cells were resuspended in 5 ml coating buffer and then added drop-wise to 30 ml of coating buffer containing polyacrylic acid (1 mg/ml) and incubated for 3 minutes. The cells were then pelleted and washed in Tris homogenization buffer [125 mM NaCl, 25 mM Tris HCl, pH 7.4, 5 mM MgCl₂]. Placental tissue dissected to expose the villous tree (see above) was washed extensively in cold PBS to remove as much blood as possible. The placental tissue was then washed three additional times in cold coating buffer [20 mM MES, pH 5.5, 100 mM NaCl, 5 mM MgCl₂ and 280 mM sorbitol], which was used in subsequent steps with the placental tissue. The tissue was then incubated in 1% CCS (w:v) in coating buffer for three minutes with constant agitation and then washed two times in coating buffer before incubation in cold polyacrylic acid (1 mg/ml in coating buffer) with constant agitation. This was followed by two washes in Tris homogenization buffer.

In separate preparations, the 1% CCS (w:v) (Jacobson's method) was replaced with 1% Ludox CL colloidal silica (smCCS) (w:v) in coating buffer for labeling the apical PM of the STB. In all other aspects, the procedure for coating the apical PM of the STB with smCCS was identical to that described for CCS.

To assess CCS and smCCS binding to HL-60 cells and the placenta, the washed samples were processed for electron microscopy (EM). They were fixed in 2% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, for 1 hour and subsequently washed in cacodylate buffer. Washed tissue was post-fixed in ferrocyanide-reduced osmium, dehydrated in a graded series of ethanol, and embedded in Epon resin as is routine in our laboratory [14]. Thin sections were cut and then stained prior to examination with a Philips CM12 electron microscope operated at 60 kV.

Size analysis of cationic colloidal silica particles by electron microscopy

The size distribution of the CCS and smCCS particles was determined by EM. Droplets of CCS or smCCS (20 μ l) were applied to formvar-coated EM grids. Excess liquid was wicked away with a small piece of filter paper. The CCS and smCCS samples had been doped with 15 nm colloidal gold particles that served as an internal standard. The grid preparations were air dried before examination with the electron microscope. The sizes of the silica particles and the colloidal gold particles were determined by direct measurements of the particles from standard enlargements of electron micrographs.

Preparation of placental microvilli: homogenization and centrifugation

The washed segments of placental tissue coated with smCCS and cross linked with polyacrylic acid were dissected into smaller pieces prior to homogenization. Routinely 60 – 80 grams of wet tissue was collected. The trimmed tissue, in Tris homogenization buffer [125 mM NaCl, 25 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$] containing protease inhibitor cocktail, was disrupted with a PowerGen 7000 homogenizer (Fisher Scientific, Pittsburgh, PA). The resulting crude tissue homogenate (CTH) was filtered through successively smaller Nytex mesh (1mm, 250 μ m, and 100 μ m), to generate a crude tissue filtrate (CTF). The CTF was centrifuged at $900 \times g$ at 4°C for 10 minutes. The resulting crude placental supernatant (CPS) was not processed further, but the crude placental pellet (CPP) was resuspended in homogenization buffer to a final volume of 20 ml. The resuspended CPP was then mixed 1:1 with 100% Histodenz to yield 50% Histodenz (v:v).

Ultracentrifuge tubes (38 ml Nalgene) were loaded with a 65% Histodenz (v:v) cushion that was overlain with a layer of 60% Histodenz (v:v) to which was added a layer of 55% Histodenz (v:v), each layer was 2 ml. The bulk of the centrifuge tube was then filled with the CPP fraction in 50% Histodenz (v:v). The remainder of the tube was filled with homogenization buffer. The tubes were centrifuged at $65,000 \times g$ for 22 min at 4°C using a SW 28 swinging bucket rotor in a Beckmann L7 ultracentrifuge. Fractions of the CPP were recovered at the 0-50%, 50-55%, and 55-60% Histodenz interfaces (v:v), as well as a pellet under the 65% Histodenz cushion (v:v). The pellet was resuspended in homogenization buffer and then dispersed with a Dounce homogenizer. The resuspended pellet was mixed 1:1 with 100% Histodenz to yield 50% Histodenz (v:v) and the smCCS coated membrane fractions were centrifuged and pelleted as described above, but through a single layer of 65% Histodenz (v:v). The resulting pellet was resuspended in homogenization buffer and fractions were equally distributed into 1.5 ml microfuge tubes and pelleted in a microfuge. The resulting pellets were washed two additional times in homogenization buffer to remove the Histodenz. The final pellets and all of the different fractions mentioned above were flash frozen in liquid nitrogen and then stored in a -80°C freezer until used. The protein concentration of all of the fractions was estimated using the BCA assay with BSA being used to construct standard curves.

In addition to biochemical analysis, the morphology of the smCCS-coated MV fractions was assessed by EM. Material pelleting through the 65% Histodenz (v:v) was washed in homogenization buffer and then fixed and processed for thin section EM as described previously for the HL-60 cells and placenta.

Analysis of isolated microvilli: immunoblots

Immunoblots were used to assess placental fractionation for the preparation of isolated STB MV. The various fractions from homogenization and centrifugation procedures were loaded onto 7 or 12% 1-D PAGE gels to resolve proteins in the fractions prior to their transfer to nitrocellulose membranes. All fractions were loaded at equal protein concentration. Non-specific protein binding sites were blocked with 5% non-fat milk in Tris-buffered saline

containing 0.2% Tween 20 (TBST). These blots were probed with anti-PLAP, which served as a marker for the STB PM. Antibody binding was detected with species-specific secondary antibody labeled with horseradish peroxidase using a chemiluminescence reaction. Control incubations were identical except the primary antibody was omitted.

The degree of enrichment of PLAP in the isolated MV was estimated by immunolabeling. A fixed concentration of the CTH was loaded onto a 12% 1-D PAGE gel; an equal concentration of PPM protein was subjected to a series of dilutions that were then loaded onto the same gel. Following electrophoresis the proteins were transferred to nitrocellulose and probed with anti-PLAP as described above.

The relative amount of other selected proteins in the MV was also estimated by immunoblotting. In these cases, the CTH and PPM fractions were loaded onto 1-D PAGE gels at equal protein concentration. Following electrophoresis, the proteins were transferred to nitrocellulose and probed with antibodies to actin, BiP, CAV-1, CD31, ezrin, lamin B, LAMP-1, and tubulin as described above.

Sequential extraction of microvilli: 1-D gel electrophoresis and immunoblot analysis

The final pellet of smCCS-coated MV was subjected to a sequential series of extraction steps to reduce non-integral proteins associated with the membrane fraction. The initial step of this procedure consisted of resuspending the pellets in low salt buffer (10 mM Tris, pH 7.4) and incubating for 30 min on ice. The sample was then centrifuged in a microfuge for 30 sec. The supernatant or extract was collected and saved; the resulting extracted pellet was then subjected to extraction with the next solution. In this way the samples were extracted with low salt, high salt (2 M NaCl), high pH (0.1 M sodium carbonate, pH 11), and a chaotropic solution (6 M urea). The resulting pellet was resuspended and extracted in boiling 1% SDS in 10 mM Tris buffer, pH 7.4 or directly in 1-D gel sample buffer. The sequential extraction steps were greatly facilitated by the presence of smCCS. The smCCS-coated membrane could be pelleted rapidly in a conventional microfuge thus not requiring ultracentrifugation to pellet the membranes following each extraction step. A flow diagram summarizing the process of preparing the samples is given (Fig. 1).

The protein concentration for each of the supernatant/extracts as well as the SDS solubilized membranes was measured. The protein profile of each fraction was determined by 1-D PAGE. The gels were stained with Sypro and imaged with a Bio-Rad Versadoc 3000 gel documentation system. The supernatants and solubilized membranes derived from the sequential extraction steps were also analyzed by immunoblotting using antibodies selected to represent cytoskeletal elements, markers for cytoplasmic organelles, and markers for the STB PM.

Results

Coating HL-60 cells and the apical plasma membrane of the STB with CCS and smCCS particles

HL-60 cells were used to model the ability of CCS particles to coat the PM. EM shows that the conventional CCS particles coated the PM of HL-60 cells in a uniform manner (Fig. 2A). The CCS method was also tested with human placentas. EM shows that the CCS particles bound to MV of the STB; however, the labeling was concentrated at the tips of the MV with little labeling of the lateral sides of MV or the planar portion of the membrane at the base of MV (Fig. 2B, C). This suggests that the CCS particles could not penetrate beyond the tips of the MV or, alternatively, CCS-binding sites were restricted to the tips. An alternative to the conventional CCS method was sought. Conventional CCS particles were replaced with Ludox CL cationic colloidal silica particles, which are smaller than the particles found in the

Jacobson preparations, and are subsequently referred to as small CCS (smCCS). EM examination of formvar-coated EM grids containing either smCCS or CCS particles was used to determine their size distribution. Also present on the grids were 15 nm colloidal gold particles serving as an internal standard. Measurements of the particles revealed that the smCCS particles were more homogeneous than the CCS particles in their size distributions. Average sizes were 16.7 ± 2.7 nm for smCCS, 41.2 ± 17.5 nm for CCS, and 14.7 ± 1.1 nm for the colloidal gold.

Placental segments were labeled by substituting smCCS for CCS particles. EM examination of these preparations revealed that the STB-PM were uniformly labeled at the tips, lateral sides, and planar portion of the PM at the base of the microvilli, showing that binding sites for cationic particles were present along the entire apical PM of the STB, and not restricted to the tips of MV (Fig. 3A, B).

Electron microscopic examination of isolated MV fractions reveals relatively large “patches” of MV with a distinctive morphology permitting their identification (Fig. 3C, D). Moreover, the MV was uniformly coated with smCCS particles. While the majority of the structures present in these fractions were MV, other membrane profiles lacking smCCS were also present.

Immunoblot analysis of the fractionated placenta

Proteins in the various fractions generated during the isolation of the placental MV were separated by 1-D PAGE, transferred to nitrocellulose membranes, and probed with anti-PLAP to assess for recovery of the STB PM. While PLAP was present in all of the fractions, it was most concentrated in the final MV fraction (Fig. 4A). In control experiments in which the primary antibody was omitted, the secondary antibody bound to a band with slightly higher mobility than PLAP (Fig. 4A). This migration position was suggestive of being the heavy chain of IgG; this was confirmed in separate blots that were probed with antibody to human IgG (data not shown). This cross reactivity with human IgG was fortuitous since it enabled the monitoring of a soluble protein (IgG), known to be in high abundance in the human placenta, and simultaneously observe the depletion of IgG and the enrichment of PLAP during the generation of the MV fractions.

To estimate the enrichment of PLAP in the MV fractions, the CTH fraction (at a fixed protein concentration) was compared to a dilution series of the MV fraction. In this analysis, the enrichment factor for PLAP varied between experiments but ranged from 200- to 400-fold; that is a 200- to 400-fold dilution of the MV had an immunoblot signal similar to the starting CTH fraction (Fig. 4B).

Sequential extraction of microvilli

The MV and CTH fractions were compared, using immunoblotting, for reduction in or enrichment for marker proteins in the MV fraction. The marker proteins tested were for the nuclear envelope (lamin B), lysosomes (LAMP-1), PMs of cell types other than the STB (CD31 and CAV-1), cytoskeletal proteins (actin, ezrin, and tubulin), and the STB PM (PLAP). All of these marker proteins were largely depleted in the MV except for actin, ezrin, and PLAP which were enriched (Fig. 5A). The MV fractions of placenta were highly enriched for cytoskeletal proteins associated with MV (e.g., ezrin and actin). However, analysis of the proteins integral to the apical PM of the MV should be facilitated by further reducing the amount of non-PM proteins in the sample. The MV fractions were subjected to a sequential series of extraction steps (low salt, high salt, pH 11, urea, and finally boiling SDS). Each extract was collected and its protein concentration determined. The percentage of protein recovered in each extract fraction obtained from five separate placental

preparations averaged: $3.1 \pm 0.1\%$ in low salt, $16.6 \pm 5.6\%$ in high salt, $20.3 \pm 5.5\%$ in pH 11, $14.7 \pm 3.1\%$ in urea, and $27.3 \pm 9.8\%$ in SDS. The cationic silica pellet, which remained after the sequential extraction, contained $17.9 \pm 3.9\%$ of the starting protein that was present in the isolated membrane fraction. The extracts were subjected to 1-D PAGE analysis to compare their banding patterns in stained gels. There were clear differences in the pattern of bands and/or the staining intensities of specific bands in each of the extracts indicating that different sets of proteins were removed with different efficiencies in the various extraction conditions (Fig. 5B). In separate experiments, we demonstrated that none of the extraction conditions used alone was as effective at stripping the membrane associated proteins from the MV membranes as the sequential extraction method described above (data not presented).

Analysis of the 1-D PAGE staining patterns, while being very informative, did not address extraction of specific proteins. Immunoblot assays using selected antibodies to marker proteins addressed this question. A major finding from this study was that each of the cytoskeletal proteins monitored displayed different extraction properties. Actin extraction occurred for the most part in the low salt buffer with much smaller amounts being extracted in high salt, high pH, and urea; undetectable amounts were retained in the final pellet (SDS extraction) (Fig. 5C). Ezrin, on the other hand, was not extracted efficiently by low salt, but was extracted by high salt and high pH; undetectable amounts were retained in the final pellet (SDS extraction) (Fig. 5C). Tubulin displayed yet another pattern being extracted primarily with 7 M urea (Fig. 5C). The organelle marker proteins employed also showed different patterns of extraction. Lamin B was extracted in low salt and urea with little extraction in high salt or high pH (Fig. 5C). The mitochondrial marker complex V subunit α was partially extracted in low and high salt and high pH and urea with undetectable amounts were retained in the final pellet (Fig. 5C). The endoplasmic reticulum marker BiP was extracted with high salt, high pH, and urea (low salt conditions were not tested) (Fig. 5C). LAMP-2 was extracted with low salt, high salt, and urea (Fig. 5C). CD31 and CAV1, markers for PMs other than of the apical PM of the STB, were maximally extracted under different conditions. The STB PM-markers PLAP and dysferlin were largely refractive to any of the extraction conditions except for boiling SDS (Fig. 5C).

Discussion

The human genome is composed of about 25,000 genes however, the number of protein species that can arise has been estimated as high as $\sim 10^6$ [15]. In addition to the vast number of possible proteins, the dynamic range of protein expression in cells and biological fluids is also very large; it was estimated that the range in abundance of proteins varies over 7-8 orders of magnitude in cells, while that in serum may be 9 orders of magnitude or possibly higher [5,16]. Adding to this complexity is the fundamental biological concept that protein expression is spatially and temporally restricted. These factors make global proteomics analysis of a complex structure such as the human placenta a daunting prospect.

Numerous approaches have been used to address these problems in proteomics studies. A major theme in these efforts has been to simplify the system under consideration. A benefit of simplification of the entire proteome (i.e., forming a subproteome) is that lower abundance proteins are more likely to be identified [17,18]. There are several approaches that can be used individually, or in combination, to isolate enriched subcellular fractions from cells or tissues. These strategies begin with disruption of cells or tissues, usually by homogenization or nitrogen cavitation. Different components of the homogenate can be separated based on their biological or physical attributes. Centrifugation techniques are usually a critical component of subcellular fractionation. Other methods for subcellular

fractionation that have been used successfully include free-flow electrophoresis [19], flow cytometry [20], and affinity-based methods such as immunoisolation [21].

The major goal of the present study was to obtain highly enriched preparations of the apical PM of the STB that would enable us to obtain high-quality protein profiling data. This is important considering the critical role of that membrane in maternofetal biology. The strategy employed was to (a) isolate highly enriched fractions of MV from the STB and (b) remove non-PM proteins from those MV prior to proteomic analysis. Several investigators have prepared membrane fractions derived from human placenta that were enriched in the marker protein PLAP; the enrichment factors reported from thirteen different studies were 14- to 37- fold with an average of 21-fold [reviewed in 22]. These preparations consist of a heterogeneous population of vesicles, the subcellular origin of which is impossible to determine based upon the ultrastructural morphology. A higher level of enrichment, specific for the apical plasma membrane of the STB, would facilitate proteomics analysis.

We chose to alter the density of the apical PM of the STB so that it could be separated more readily from other non-coated membranes. The pioneering work of Jacobson and colleagues [12], suggested a methodology that could be applied to the placenta for this purpose. Using that approach, CCS particles are applied to the surfaces of cells to which they bind through electrostatic interactions. The mass added by the CCS facilitates isolation of the coated membranes away from uncoated cellular membranes and organelles. In other experiments we tested the Jacobson procedure with HL-60 cells (a leukemic cell line) and found, by EM, that cells were completely coated with CCS particles. Thus this procedure works in our hands. This method was subsequently applied to the human placental tissue. In this case, the CCS particles heavily labeled the tips of the STB MV, but did not label the lateral sides of the MV or the planar portion of the PM at the base of MV. We conclude that the conventional CCS method is not suitable for isolation of the entire apical PM of the STB. To circumvent this problem, yet still utilize the principle of modifying the density of the apical PM of the STB, we sought other types of CCS particles. We found that Ludox CL cationic colloidal silica, herein called smCCS, could be substituted for the conventional CCS particles of Jacobson. EM of placental tissue coated with smCCS showed uniform decoration of all regions of the MV PM of the STB. This indicated that the entire apical PM of the STB has binding sites for CCS particles, but that only the smCCS particles gained access to all of the sites. Direct comparison of the sizes of the smCCS and CCS particles showed that on average the CCS particles were about 2.6 times as large as the smCCS particles; furthermore, the CCS particles were more heterogeneous in their size distribution than were the smCCS particles. Examination of the target cells or tissue by EM was important to assess the level of labeling with both types of particles.

We next determined whether smCCS would sufficiently alter the density of the target PM to permit its enrichment by centrifugation methods. The smCCS-coated membranes could be isolated, and there appeared to be relatively little contamination with uncoated-membranes or cellular organelles in this fraction as determined by EM. The smCCS-coated membranes were clearly derived from the apical side of the STB since the MV appearance was retained in the isolated material. It should be noted that our ultracentrifugation method was also a modification of the Jacobson procedure. In that procedure there is a 70% Nycodenz (v:v) cushion that is overlain with 50% Nycodenz (v:v) containing the biological sample [12]. Our method uses a 65% cushion of Histodenz (v:v) (equivalent to Nycodenz), that is overlain with two layers of Histodenz, 60% and 55%, to which the 50% Histodenz (v:v) containing the sample is then overlain.

The morphological appearance of the isolated MV PM fraction indicated that it was significantly enriched. Biochemical methods were used to establish the level of enrichment

of this fraction. We compared the PLAP signal in each of the fractions created during the homogenization and centrifugation process using an immunoblot assay. The smCCS-coated fraction was enriched in PLAP in comparison to all of the fractions but especially so when compared to the starting material. In addition, it was found that IgG, which is highly abundant in the human placenta, was greatly reduced in the smCCS fraction. Thus the smCCS-coated fraction was purified away from an abundant soluble protein in placenta. A second assay was used to better assess the enrichment of PLAP in the smCCS-coated fraction. The CTH and smCCS-coated fractions were adjusted to an equal concentration of protein; the smCCS-coated fraction was then diluted several times. An immunoblot was used to determine the level of PLAP enrichment. The enrichment factor for PLAP, while variable, ranged from approximately 200- to 400-fold. This represents a 10- to 20-fold increase in enrichment for this STB PM marker when compared to the approximately 21-fold enrichment reported by others [22].

Immunoblot analysis showed that the MV PM fraction was highly enriched in actin and ezrin; both of these cytoskeletal proteins are known components of MV. Thus at this stage of preparation the sample should be considered as highly enriched MV. In order to obtain greater enrichment for PM proteins, cytoskeletal and membrane associated proteins present in the isolated apical STB MV were further removed following treatment with a sequential series of solutions designed to solubilize and strip proteins that are not integral to the PM. Sodium carbonate at pH 11 has been widely used as a method for stripping non-integral membrane associated proteins [23]. However, we found that the pH 11 solution alone was not capable of removing many proteins associated with the isolated STB MV. Further examination of other solutions used for stripping membrane associated proteins, also showed that no single method tested was completely effective. We therefore designed an approach using a sequential series of solutions with increasing harshness. Having samples coated with smCCS facilitated the handling of the membranes following each extraction, since the membranes could be pelleted in 30 sec using a microfuge rather than longer ultracentrifugation steps. Following this sequential extraction, we demonstrated that the integral membrane marker PLAP was further enriched in the stripped membrane fraction relative to the isolated smCCS-coated apical STB MV. Based upon protein recovery in the extracted smCCS-coated membrane (~20% of the unextracted membrane), the PLAP was enriched a further 5-fold. Thus the final enrichment of PLAP obtained in the apical STB membrane fraction was between 1,000 – 2,000 fold. This level of enrichment will permit a proteomics analysis of these purified apical STB PM samples [24, manuscript in preparation].

We have modified the CCS method of Jacobson [12] such that it can successfully isolate the apical PM of the STB. The major modifications were to use smaller more homogeneously-sized silica particles and modification of the centrifugation conditions. The initial enrichment factor for a PM marker unique to the STB, namely PLAP, ranged from 200- to 400-fold. This compares extremely favorably to the 20-fold enrichment for PLAP obtained [25]; they isolated membrane vesicles for proteomics analysis of the apical PM of the STB. Using the conventional CCS method, 20-fold enrichment of markers for the luminal PM of rat lung endothelial cells was also obtained for a proteomics analysis of that membrane [26]. The reasons for our PM preparation being more highly enriched than what was obtained in these two proteomics studies may be multifaceted. In the first instance, altering the density of the PM with silica particles has clear advantages over isolation of uncoated membrane vesicles. In our method we have direct access to the apical PM of the STB in the placentas whose outer basal plate has been removed by dissection. In comparison, study of endothelial luminal PMs from rat lung required perfusion of the CCS particles [26]. In addition, the smaller more homogeneously-sized smCCS particles may be more efficacious in labeling of membranes than the conventional CCS. Additional density layers to the step gradient

(parfait effect) were included in our centrifugation procedure and we repeat the ultracentrifugation procedure, each of these modifications to the Jacobson method may lead to more highly enriched membrane fractions.

The methodological advancements made in this study have led to the preparation of highly enriched MV and subsequently of the apical PM of the placental STB. These methods should be applicable to the isolation of highly enriched PMs from other cell types and may prove particularly useful for the isolation of other microvillous bearing apical PMs.

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Abbreviations

CAV-1	caveolin-1
CCS	cationic colloidal silica
CPP	crude placental pellet
CPS	crude placental supernatant
CTH	crude tissue homogenate
EM	electron microscopy
PLAP	placental alkaline phosphatase
PM	plasma membrane
smCCS	Ludox CL cationic colloidal silica
STB	syncytiotrophoblast
TBST	Tris-buffered saline with Tween 20

Figure 1.

Flow diagram showing the different steps involved in isolation of STB microvillar fractions and subsequent sequential extraction steps used to enrich for plasma membrane proteins of the apical plasma membrane of the STB.

Figure 2.

The conventional CCS method applied to HL-60 cells and the placenta. (A) An EM of portions of three HL-60 cells showing them heavily labeled with CCS particles as indicated by the dot-like structures at the cell surface (arrows). (B) An EM of the STB. Note the line of heterogeneously sized CCS particles along the tips of the MV. A capillary (Cap) is shown. (C) A higher magnification EM of the region of panel B enclosed with the rectangle. Essentially all of the CCS particles are retained at the tips of the MV (arrowhead) with few particles associated with the lateral aspect of the MV. The basal portion of this PM was devoid of CCS particles (arrows). Bars = 1.0 μm .

Figure 3.

The smCCS method applied to the placenta. **(A)** A low magnification electron micrograph showing labeling of the apical STB-PM; the entire apical surface is heavily coated with the smCCS particles (arrows). A capillary (Cap) and a nucleus (N) of the STB are evident. **(B)** A higher magnification image of the region of panel A enclosed by the rectangle. Note that the smCCS particles completely cover the individual MV (arrowheads) and penetrates all the way to the base of the PM (arrows). **(C)** Isolated STB MV coated with smCCS particles. The majority of the membranes have associated smCCS particles. Uncoated-membrane profiles (arrowhead) are distinct from the coated ones (arrows). The STB MV fragments are in sheets with thin section profiles primarily sectioned perpendicular to the MV or *en face* (rectangle). **(D)** A higher magnification image of the region of panel C enclosed with the rectangle. Note that the membranes of the MV are completely coated with smCCS particles. Bars = 1.0 μm .

Figure 4.

Placental fractionation monitored and enrichment of the PM marker PLAP. **(A)** The different fractions generated during preparation of smCCS-coated STB MV were resolved by 1-D-PAGE and then transferred to nitrocellulose membranes (each lane received an equal protein load). The immunoblot was probed with anti-PLAP and then HRP-labeled secondary antibody. Two major bands were detected, one migrating at the molecular weight position appropriate for PLAP (arrowhead). The other band migrated more rapidly than PLAP (double arrows). Note that the PLAP signal becomes progressively more concentrated the Histodenz fractions, 50-55, 55-60, and 65%. Signal from the lower band was essentially absent from the MV fraction (65%). A similar immunoblot was probed with secondary antibody in the absence of anti-PLAP (2° Ab only). The PLAP-specific band was absent in this control; however, the lower band was present and was essentially the same as it was in the presence of anti-PLAP (double arrows). Separate experiments demonstrated that this band was the heavy chain of IgG (not shown). **(B)** The enrichment of PLAP in isolated MV was estimated in a dilution experiment using an immunoblot assay. Aliquots of CTH and MV were adjusted to achieve equal protein concentrations. The CTH was held fixed while the MV were diluted as indicated. In this placenta preparation, the 1:400 dilution has labeling intensity similar to the CTH. Thus the enrichment factor for PLAP was about 400-fold. The lower band (*) indicates IgG detected by the secondary antibody.

Figure 5.

Reduction of some proteins and enrichment for others in preparation of the MV fraction. **(A)** Equal protein concentration of CTH and isolated MV were prepared for immunoblotting and were probed with antibodies to several marker proteins. These marker proteins fell into two groups, those diminished and those enriched in the MV. The cytoskeletal protein tubulin, markers for PMs other than the STB (CD31 for endothelium and CAV-1 for endothelium and other stromal cells), and organelle markers lamin B and LAMP-1 were all diminished in the MV. On the other hand, the cytoskeletal proteins actin and ezrin were enhanced in the MV as was the STB PM marker PLAP. **(B)** Isolated MV were subjected to sequential extraction with low salt (LS), high salt (HS), pH 11, urea, and SDS. Each lane of this 1-D PAGE displays a different banding pattern, both qualitatively and quantitatively. Equal amounts of protein were loaded into each lane. **(C)** An immunoblot analysis of the sequential extraction of proteins associated with placental MV. Antibodies to marker proteins for different cellular compartments were used: actin and ezrin-microvillar cytoskeletal components; lamin B-nuclear envelope; BiP-endoplasmic reticulum; LAMP-2-lysosomes; complex V α -mitochondria; tubulin-microtubules; CD31 and CAV-1-PM of cells other than the STB. Each of these markers displays a unique pattern of extraction thus illustrating the importance of this sequential approach. Following the sequential extraction, none of these markers were detectable in the final pellet, the SDS extract. PLAP and dysferlin were used as markers for the PM of STB. Note that PLAP and dysferlin were largely retained after the four extraction steps and were only removed with SDS. This indicates that the final product, after the extraction steps, is highly enriched for components of the apical STB PM.