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The post-synthetic sorting of endogenous membrane proteins examined by the simultaneous purification of apical and basolateral plasma membrane fractions from Caco-2 cells

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A subcellular fractionation method to isolate simultaneously apical and basolateral plasma membrane fractions from the human adenocarcinoma cell line Caco-2, grown on filter supports, is described. The method employs sucrose-density-gradient centrifugation and differential precipitation. The apical membrane fraction was enriched 14-fold in sucrase–isomaltase and 21-fold in 5′-nucleotidase compared with the homogenate. The basolateral membrane fraction was enriched 20-fold relative to the homogenate in K^+-stimulated *p*-nitrophenylphosphatase. Alkaline phosphatase was enriched 15-fold in the apical membrane fraction and 3-fold in the basolateral membrane fraction. Analytical density-gradient centrifugation showed that this enzyme was a true constituent of both fractions, and experiments measuring alkaline phosphatase release following treatment with phosphatidylinositol-specific phospholipase C showed that in both membrane fractions the enzyme was glycosyl-phosphatidylinositol-linked. There was very little contamination of either membrane fraction by marker enzymes of the Golgi complex, mitochondria or lysosomes. Both membrane fractions were greater than 10-fold purified with respect to the endoplasmic reticulum marker enzyme α-glucosidase. Protein composition analysis of purified plasma membrane fractions together with domain-specific cell surface biotinylation experiments revealed the presence of both common and unique integral membrane proteins in each plasma membrane domain. The post-synthetic transport of endogenous integral plasma membrane proteins was examined using the devised subcellular fractionation procedure in conjunction with pulse–chase labelling experiments and immunoprecipitation. Five common integral membrane proteins immunoprecipitated by an antiserum raised against a detergent extract of the apical plasma membrane fraction were delivered with the same time course to each cell-surface domain.

INTRODUCTION

Experiments on protein sorting in polarized epithelial cells have revealed differences in the intracellular site of sorting of newly synthesized plasma membrane proteins, depending on cell type. There are currently two documented models of membrane protein sorting. Protein sorting can occur in the *trans*-Golgi network (TGN) prior to vesicular transport to the cell surface, or by transcytosis after delivery to the basolateral cell surface (Bartles & Hubbard, 1988; Simons & Wandinger-Ness, 1990). There appears to be a different emphasis on the mechanism which dominates, depending on the epithelial cell type. In Madin–Darby canine kidney (MDCK) cells, plasma membrane proteins have been shown to be sorted into different constitutive secretory vesicles which are vectorially targeted to the correct domain (Lisanti *et al.*, 1989*a*; Le Bivic *et al.*, 1990). However, in hepatocytes, all apical membrane proteins so far studied have been transported first to the basolateral membrane, from where they are sorted and transcytosed to the apical membrane (Bartles *et al.*, 1987). Both routes have been implicated in Caco-2 cells, where sorting occurs both in the TGN and by endocytosis from the basolateral membrane (Matter *et al.*, 1990*a*).

The methods employed to study membrane protein traffic have differed between hepatocytes *in situ* and cultured epithelial cells. Whereas subcellular fractionation after pulse–chase labelling has been applied to study plasma membrane sorting in hepatocytes (Bartles *et al.*, 1987), cell-surface labelling techniques, such as domian-selective biotinylation/^{125}I-streptavidin blotting, have been used in the cultured polarized epithelial cell line MDCK (Lisanti *et al.*, 1989*a*). To date, the routes of membrane protein sorting have not been shown in the same cell type using both technical approaches, yet this is necessary for direct comparison between different cell types.

Since no fully polarized, differentiated, cultured hepatocyte cell line is available, it has not been possible to apply domain-specific labelling techniques to hepatocytes. In contrast, subcellular fractionation methods are easily applied to hepatocytes *in situ*, but are difficult to apply to cultured cells (Howell *et al.*, 1989). In the present paper we report a method for the simultaneous purification of apical and basolateral membranes from the human colonic adenocarcinoma derived cell line Caco-2 (Fogh *et al.*, 1977), grown as a polarized epithelium on permeable filter supports. We have examined the post-synthetic route of delivery to the cell surface of several endogenous apical and basolateral membrane proteins using the subcellular fractionation procedure devised.

MATERIALS AND METHODS

Cell culture

Caco-2 cells were provided as a gift by Dr. M. Mackay and Dr. I. Hassan of Ciba–Geigy Pharmaceuticals, Horsham, W. Sussex, U.K., and were maintained as described by Woodcock *et al.* (1991). Cells were routinely cultured in flasks, and for subcellular fractionation were grown on collagen-treated 0.45 μm-pore-size 24 mm-diameter polycarbonate Transwell

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CMFM, cysteine- and methionine-free DMEM containing 10% dialysed FCS; GPI, glycosyl-phosphatidylinositol; MDCK, Madin–Darby canine kidney; Biotin-LC-NHS, sulphosuccinimidyl 6-(biotinamido)hexanoate; PBS, phosphate-buffered saline (145 mM-NaCl/7.5 mM-Na_2HPO_4,$2H_2O$/2.5 mM-NaH_2PO_4, pH 7.4); PI-PLC, phosphatidylinositol-specific phospholipase C; TGN, *trans*-Golgi network.

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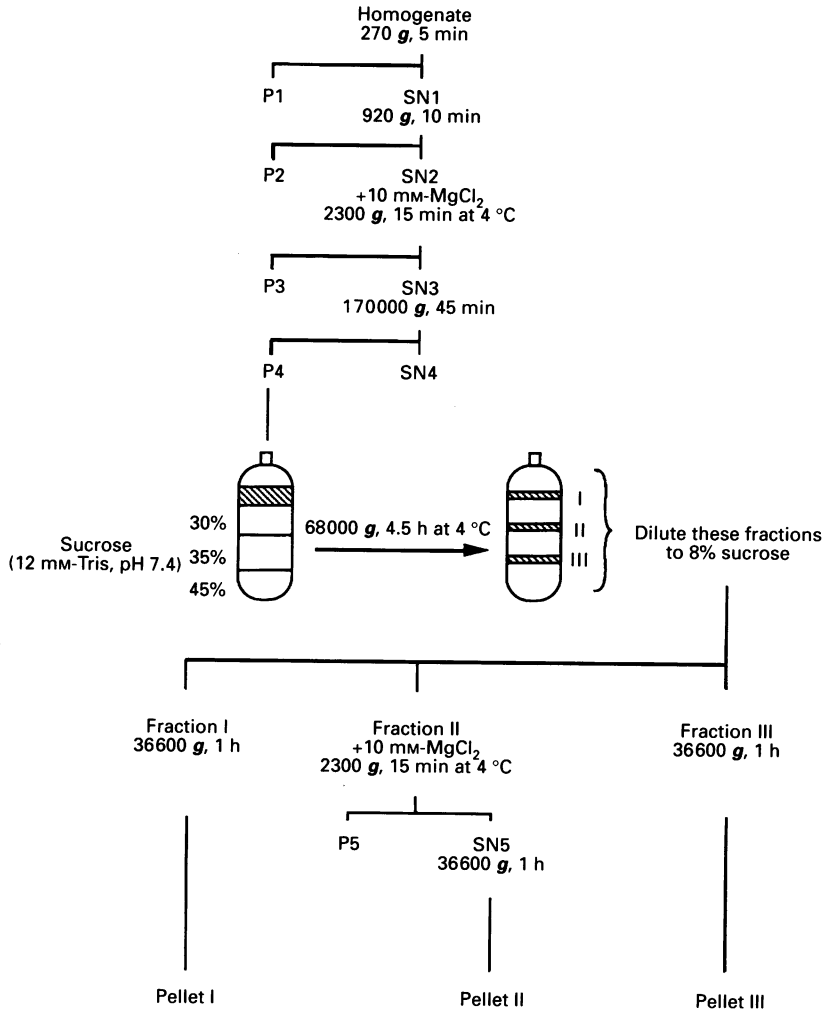

Scheme 1. Flow diagram for the isolation of apical and basolateral membrane fractions from Caco-2 cells

filters (Costar, Cambridge, MA, U.S.A.). In all experiments cells were used 7–9 days after confluence. Caco-2 cells grown as described were found to exhibit a transepithelial electrical resistance of 300–400 $\Omega \cdot cm^2$ (measured with Millicell-ERS; Millipore Corp., Bedford, MA, U.S.A.).

Isolation of fractions enriched in apical (brush border) and basolateral membranes

Apical and basolateral membrane vesicles from filter-grown Caco-2 cells were isolated by a combination of sucrose-density-gradient centrifugation and differential precipitation, adapted from methods previously described for rabbit enterocytes *in situ* (Colas & Maroux, 1980; Stieger & Murer, 1983). The overall fractionation scheme is shown in Scheme 1. Filter-grown Caco-2 cells were rinsed once in ice-cold 0.9% (w/v) NaCl, and once in 250 mM-sucrose/12 mM-Tris/HCl, pH 7.4 (buffer A), and then scraped off with a rubber policeman. The cells were suspended in 5 ml of buffer A. All further operations were carried out at 4 °C. The cells were equilibrated with nitrogen for 10 min at 3795 kPa (550 lb/in²) (Stieger *et al.*, 1988) in a cell disruption bomb (type 4639; Parr Instrument Company, Moline, IL, U.S.A.). The discharge valve was carefully opened, allowing the sample to come out dropwise into a collecting vessel. Microscopic examination of the homogenate showed 95% cell breakage. The homogenate was degassed for 15 min and then centrifuged for 10 min at 270 $g_{av.}$ in an SS34 rotor (Sorvall Instruments Division, Newton, CT, U.S.A.) in a Sorvall RC-5 centrifuge. The resulting supernatant (SN1) was collected and centrifuged at 920 $g_{av.}$ for 10 min in the same rotor. The supernatant (SN2) was collected and $MgCl_2$ was added to 10 mM; it was then stirred for 15 min on ice and centrifuged for 15 min at 2300 $g_{av.}$ in an SS34 rotor. The resulting supernatant (SN3) was pelleted at 170000 $g_{av.}$ for 45 min in a T-1270 rotor (Sorvall) in a Sorvall OTD 65B centrifuge. The crude membrane pellet (P4) was resuspended in 0.5 ml of 250 mM-sucrose/5 mM-Na_2EDTA/12 mM-Tris/HCl, pH 7.4 (buffer B), and layered on the following discontinuous sucrose density gradient (w/w):1.41 ml, 45%; 1.64 ml, 35%; 1.42 ml, 30% in a 13 mm × 51 mm Beckman polyallomer tube. All sucrose solutions were buffered with 10 mM-Tris/HCl, pH 7.4, and their density was checked with a refractometer (Bellingham & Stanley Limited). Centrifugation was for 4.5 h in a Beckman SW 50 swinging bucket rotor in a Beckman L5-50B ultracentrifuge at 68000 $g_{av.}$. Equivalent separation on this gradient could also be achieved by centrifugation in a vertical rotor (Sorvall TV-865) at 219000 $g_{av.}$ for 1.5 h. The bands at the interfaces (Scheme 1) (designated as: I, overlay/30%; II, 30%/35%; III, 35%/45%) were collected with a syringe and needle and diluted to 8% sucrose with buffer A. Fraction II had $MgCl_2$ added to 10 mM, gently agitated on ice for 15 min and then centrifuged for 15 min at 2300 $g_{av.}$ in an SS34 rotor. The supernatant (SN5) and fractions I and III were separately pelleted at 36600 $g_{av.}$ for 1 h in an SS34 rotor. The pelleted fractions I (Golgi-complex-enriched), II

(basolateral-enriched) and III (apical-enriched) were resuspended in a minimal volume of buffer A.

Analytical centrifugation of apical and basolateral membrane fractions

The apical (fraction III) and basolateral (fraction II) membrane fractions collected after sucrose-density centrifugation and differential precipitation were further fractionated by isopycnic centrifugation on vertical 1–22% (w/v) Ficoll gradients as described by Branch *et al.* (1987). Briefly, fractions from three sucrose density gradients were pooled (total protein at least 3 mg), made up to 5 ml in 250 mM-sucrose/1 mM-$MgCl_2$/10 mM-Tes, pH 7.4, and layered on to 30 ml linear gradients of 1–22% Ficoll (Pharmacia) containing 250 mM-sucrose, 1 mM-Na_2EDTA and 10 mM-Tes, pH 7.4, on a 5 ml cushion of 45% Nycodenz (Nycomed) containing 10 mM-Tes and 1 mM-Na_2EDTA, pH 7.4. Centrifugation was for 1 h in a vertical rotor (Beckman VTi 50) at 206000 $g_{av.}$. Gradients were pumped off from the bottom as 0.7 ml samples. The distribution of enzyme markers alkaline phosphatase, 5′-nucleotidase and K^+-stimulated *p*-nitrophenylphosphatase were examined along the gradient.

Antibodies, SDS/PAGE and Western blotting

Rabbit polyclonal antisera were raised against the detergent phase of Triton X-114-extracted integral membrane proteins from apical plasma membrane fractions. Triton X-114 (Sigma) was washed with 50 mM-Tris/HCl, pH 7.4, and used as a detergent/protein ratio of 5:1, as described by Bordier (1981). Proteins of the apical and basolateral membrane fractions were separated on SDS/10%-polyacrylamide slab gels (Laemmli, 1970) and Western-blotted according to Towbin *et al.* (1979).

Biotinylation of the apical and basolateral surfaces of Caco-2 cells

Prior to biotinylation, filter-grown Caco-2 cells were measured for the 'tightness' of the epithelial monolayer. Filter supports showing a transepithelial electrical resistance of less than 300 $\Omega \cdot cm^2$, or transport of > 1% [^{14}C]mannitol (Amersham) across the cell monolayer after 2 h at 37 °C, were not used. Time course and concentration-dependence experiments showed that optimal labelling of Caco-2 cells was achieved with 0.5 mg of sulphosuccinimidyl 6-(biotinamido)hexanoate (biotin-LC-NHS; Pierce)/ml for 30 min at 4 °C (results not shown). Filter chambers were washed for 4 × 10 min in ice-cold phosphate-buffered saline (PBS) containing 0.5 mM-$MgCl_2$ and 0.9 mM-$CaCl_2$ (PBS^+). Biotin-LC-NHS was dissolved in ice-cold PBS^+ to 0.5 mg/ml and used immediately. For labelling of the apical membrane surface, 250 μl of biotin-LC-NHS was added to the apical compartment of the filter chamber and 1 ml of ice-cold PBS^+ was added to the basolateral compartment. Labelling of the basolateral membrane surface was carried out by placing the filter on a 250 μl drop of biotin-LC-NHS on parafilm and adding 1 ml of ice-cold PBS^+ to the apical compartment. After 30 min of gentle horizontal shaking at 4 °C, filter chambers were washed four times in ice-cold PBS^+ containing 50 mM-NH_4Cl. Filters were then excised from the chamber with a scalpel, placed in a 1.5 ml Eppendorf Microfuge tube and extracted with 1 ml of ice-cold lysis buffer [0.15 M-NaCl, 1 mM-EDTA, 1% (v/v) Triton X-114, 10 mM-Tris/HCl, pH 7.4, and 35 μg of phenylmethanesulphonyl fluoride (PMSF)/ml] for 45 min on ice with intermittent mixing. Samples were then clarified by centrifugation at 11000 $g_{av.}$ for 10 min at 4 °C. Phase separation with Triton X-114, as described by Bordier (1981), was carried out on the supernatants to separate hydrophobic 'integral' membrane proteins from hydrophilic 'peripheral' membrane proteins.

SDS/PAGE and electroblotting of biotinylated proteins followed by detection with ^{125}I-streptavidin was carried out as described in Sargiacomo *et al.* (1989). Streptavidin (Sigma) was iodinated with Na^{125}I (Amersham) using the Iodogen method, yielding a specific radioactivity of 5–10 μCi/μg.

Radiolabelling and immunoprecipitation

Confluent monolayers of filter-grown Caco-2 cells were rinsed on both sides of the monolayer in CMFM [cysteine- and methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% dialysed fetal calf serum (FCS)] and incubated for 30 min at 37 °C in fresh CMFM added to both chambers. Each filter was inverted and pulsed for 30 min at 37 °C with 200 μCi of ^{35}S-Translabel (ICN Radiochemicals, Irvine, CA, U.S.A.) in 200 μl of CMFM, added to the basolateral side. The filters were then transferred to a fresh 6-well dish and washed in complete DMEM, and then chased for the indicated times in complete DMEM supplemented with 10 mM-methionine and 10 mM-cysteine. At the end of the chase times the filters were washed in ice-cold PBS, the transepithelial electrical resistance was remeasured to ensure the epithelium had not become leaky, and the cells were scraped into buffer B. Apical and basolateral membranes were isolated as described above. Three filters were used per time point, supplemented with unlabelled carrier cells grown in 150 cm^2 flasks. Isolated apical and basolateral membranes were resuspended in 0.5 ml of 100 mM-NaCl/0.4% SDS/50 mM-Tris/HCl, pH 7.4, and Triton X-100 was added to 2% (v/v). The samples were mixed and centrifuged for 10 min at 11600 $g_{av.}$, and the insoluble pellets were discarded. The fractions were pre-incubated with 1 μg of non-immune rabbit IgG and 20 μl of Protein A–Sepharose (Sigma; 50 mg/ml slurry in PBS) for 2 h, rotating, at room temperature. Protein A–Sepharose was removed by centrifuging at 11600 $g_{av.}$ for 20 min, and the supernatants were transferred to clean Eppendorf Microfuge tubes. Rabbit polyclonal antiserum raised to the apical plasma membrane fraction was added to membrane fractions at a final dilution of 1:500 and incubated, rotating, overnight at 4 °C. Each membrane sample was mixed with 12.5 μl of a 50 mg/ml slurry of Protein A–Sepharose and incubated for a further 2 h, rotating, at room temperature. The samples were centrifuged at 11600 $g_{av.}$ for 20 min and the supernatants discarded. The pellets were washed five times in 100 mM-NaCl/0.1% SDS/0.5% Triton X-100/50 mM-Tris/HCl, pH 7.4, followed by two washes in 50 mM-Tris/HCl, pH 7.4. Fractions were rotated for 10 min between each wash. Samples were prepared for SDS/PAGE by heating to 95 °C in sample buffer (Laemmli, 1970) to release the immunocomplexes from the beads. Proteins were separated on SDS/10%-polycrylamide slab gels (Laemmli, 1970) and visualized by fluorography using EN^{3}HANCE (NEN–Dupont, Boston, MA, U.S.A.) and KODAK X-Omat AR films.

In addition to immunoprecipitation of membrane fractions, immunoprecipitates of integral membrane proteins were also examined after addition of the anti-(apical plasma membrane fraction) serum to intact filter-grown cells.

Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC)

Purified PI-PLC was kindly provided by Dr. M. Low (Columbia University, New York, NY, U.S.A.). Purified apical and basolateral membranes were treated with PI-PLC for 30 min at 37 °C according to Low & Saltiel (1988), and then separated into soluble and particulate fractions by centrifugation (36600 $g_{av.}$, 1 h). This treatment had no effect on the total activity of alkaline phosphatase or 5′-nucleotidase. Data are presented in the text as the means of two experiments.

Table 1. Enrichment and recovery of marker enzymes in purified apical and basolateral membranes

All values are means ± S.D. of five independent experiments. Yields are given as a percentage of the amount determined in the homogenate. Fraction II was assayed after second $MgCl_2$ precipitation step.

	Enrichment (fold, relative to homogenate)			Yield (%)		
Enzyme marker	I	II	III	I	II	III
Alkaline phosphatase	–	3.10±0.07	15.30±0.29	< 0.01	0.55±0.01	16.00±0.31
Sucrase–isomaltase	–	1.95±0.03	14.30±0.20	< 0.01	0.95±0.01	13.40±0.18
Na^+/K^+-ATPase	5.95±0.11	20.0±0.22	1.26±0.04	2.50±0.004	13.00±0.14	1.10±0.04
β-Hexosaminidase	0.11±0.0007	0.05±0.22	0.08±0.003	0.001±0.001	0.03±0.0006	0.15±0.006
α-Glucosidase	1.80±0.04	1.82±0.02	1.24±0.02	0.13±0.003	0.52±0.08	0.61±0.01
Succinate dehydrogenase	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Galactosyltransferase	10.50±0.26	< 0.001	< 0.001	2.10±0.05	< 0.001	< 0.001
5′-Nucleotidase	1.30±0.02	1.30±0.08	21.30±1.70	0.63±0.01	0.58±0.04	11.00±0.88

Table 2. Enzyme content of the fractions obtained during the preparation of apical and basolateral membranes

All values are given as absorbance units (arbitrary)/ml per mg of protein and are the means of five experiments.

	Content (units/ml per mg)						
Enzyme markers	P1	P2	P3	S4	P4	S5	P5
Alkaline phosphatase	0.22	0.29	2.76	0.06	9.76	3.10	1.80
Sucrase–isomaltase	0.15	0.23	3.20	0.08	10.10	2.90	0.60
Na^+/K^+-ATPase	2.90	3.70	5.30	1.07	5.60	19.80	3.50
β-Hexosaminidase	1.30	6.30	17.00	0.08	0.83	0.04	1.09
α-Glucosidase	0.79	1.02	6.70	0.13	11.20	1.70	17.00
Succinate dehydrogenase	4.80	11.40	2.63	< 0.005	0.01	< 0.005	< 0.005
Galactosyltransferase	0.90	1.40	4.60	0.41	10.20	0.41	3.90
5′-Nucleotidase	0.75	1.30	4.40	0.13	17.50	1.20	0.90

Electron microscopy

To the apical and basolateral membrane fractions taken from a single sucrose-density gradient, an equal volume of a solution containing 2% glutaraldehyde, 2% paraformaldehyde in 100 mM-Pipes buffer and 1 mM-$CaCl_2$, pH 7.3, was added. Samples were left overnight at 4 °C and then washed three times in 100 mM-Pipes buffer, and fixed in 1% osmium tetroxide in 100 mM-Pipes buffer for 1 h. Samples were further washed in maleate buffer, stained in 2% uranyl acetate in maleate buffer, dehydrated, and embedded in Spurr resin. Sections were mounted on copper grids and contrast-stained with uranyl acetate and lead citrate.

Assays

The following marker enzymes were used: alkaline phosphatase (Watanabe *et al.*, 1979) and sucrase–isomaltase (Dahlquist, 1968) for apical membranes; K^+-stimulated *p*-nitrophenylphosphatase (Colàs & Maroux, 1980) for basolateral membranes; *N*-acetyl-β-glucosaminidase (Maguire *et al.*, 1983) for lysosomes; neutral α-glucosidase (Peters, 1976) for endoplasmic reticulum; UDP-galactosyltransferase (Bretz *et al.*, 1980) for the Golgi complex; and succinate dehydrogenase (Pennington, 1961) for mitochondria. The distribution of 5′-nucleotidase was also examined, and measured according to Stanley *et al.* (1980). Protein estimations were according to Bradford (1976), using BSA as standard.

RESULTS

The overall method for the purification of membrane fractions enriched in apical and basolateral plasma membranes from the same Caco-2 cells cultured on permeable filters is shown in Scheme 1. The recovery and enrichment of selected marker enzymes in the final membrane fractions are shown in Table 1, and the enzyme content of fractions obtained during the preparative procedure is shown in Table 2. The purification of the apical membranes isolated was assessed by measuring the enrichment of sucrase–isomaltase and alkaline phosphatase (fraction III, Table 1), which are known marker enzymes for the apical cell surface of Caco-2 cells (Hauri *et al.*, 1985). These two enzymes showed similar enrichment, relative to the homogenate, of 14–15-fold, and overall recoveries of 13–16%. The ectoenzyme 5′-nucleotidase, which has not previously been reported as occurring in Caco-2 cells, was also enriched in the apical membrane fraction, by 21-fold with 11% recovery. There was very little contamination of the apical membrane fraction with intracellular marker enzymes for lysosomes, mitochondria or the Golgi complex. The apical plasma membrane fraction was greater than 10-fold purified with respect to the endoplasmic reticulum marker α-glucosidase and the basolateral marker K^+-stimulated *p*-nitrophenylphosphatase (an enzyme activity associated with the Na^+/K^+-ATPase). The enrichment and recovery of the basolateral membrane fraction (fraction II, Table 1) using this same marker enzyme were respectively 20-fold and 13%. Enrichment

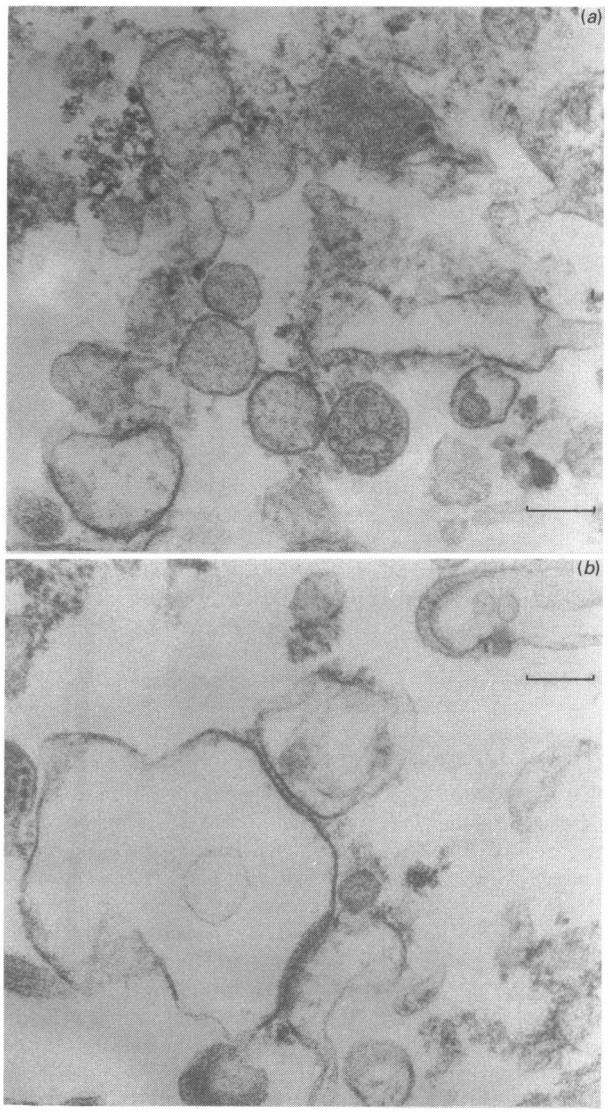

Fig. 1. Electron micrographs of the apical (*a*) and basolateral (*b*) membrane fractions

Bar = 0.5 μm.

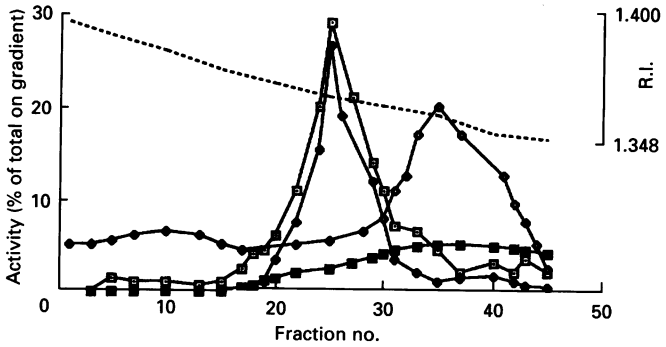

Fig. 2. Distribution of apical and basolateral membranes on a 1–22% vertical Ficoll gradient

Fractions II (basolateral plasma membrane) and III (apical plasma membrane) were loaded on to separate 1–22% Ficoll gradients and centrifuged for 1 h at 206000 $g_{av.}$. The gradient profiles are superimposed for comparison. The distributions of K^+-stimulated *p*-nitrophenylphosphatase (◇) and alkaline phosphatase (■) from fraction II are shown, together with 5′-nucleotidase (◆) and alkaline phosphatase (⊡) from fraction III. The gradient (R.I.) is shown with a broken line.

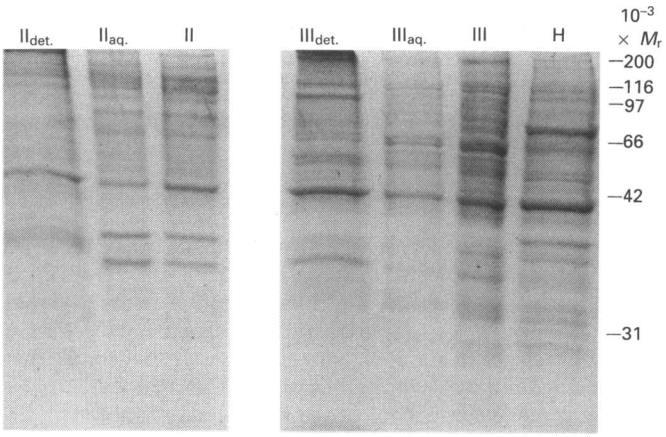

Fig. 3. Protein composition of the apical and basolateral membrane fractions

SDS/PAGE of basolateral (II) and apical (III) plasma membrane fractions before and after Triton X-114 solubilization and temperature-induced phase separation into detergent (det.) and aqueous (aq.) phases. SDS/PAGE of homogenate (H) is also shown. Proteins were stained with Coomassie Blue. The positions of M_r markers are shown.

relative to apical plasma membrane marker enzymes (sucrase–isomaltase and 5′-nucleotidase) and the endoplasmic reticulum marker α-glucosidase was > 10-fold, with little contamination by other intracellular marker enzymes. The activity of K^+-stimulated *p*-nitrophenylphosphatase was not significantly increased (results not shown) when the basolateral membrane fraction was pre-incubated in the presence of 1% SDS prior to assay, indicating that the majority of membrane vesicles in the fraction were right-side out. During the fractionation procedure (Scheme 1) a third membrane fraction was obtained (fraction I, Table 1), in which the Golgi complex marker, galactosyltransferase was enriched 10-fold.

The purity of the apical and basolateral membrane fractions was further examined by electron microscopy. At low magnification (results not shown) no obvious contamination from mitochondria, nuclei or Golgi complex membranes was observed. The membrane fractions differed morphologically from one another, and at high magnification (Fig. 1) their morphological characteristics were apparent. The apical membrane fraction contained spherical membrane vesicles filled with fibrous material and surrounded by a small fuzzy coat. These structures probably represent transverse sections through the microvilli, with the fuzzy coat representing the glycocalyx found on the surface of Caco-2 cells. The basolateral fraction contained larger vesicles with a smooth external surface and an amorphous internal structure. There was evidence of electron-dense attachments between basolateral vesicles, with the characteristic appearance of desmosomes.

Further analysis of the apical and basolateral membrane fractions was carried out by isopycnic centrifugation of the two fractions on a 1–22% Ficoll density gradient (Fig. 2). The apical membrane fraction (fraction III) gave a peak of alkaline phosphatase and 5′-nucleotidase activity at a refractive index of 1.360 on the Ficoll gradient (Fig. 2). The basolateral membrane fraction (fraction II) showed a peak of K^+ stimulated *p*-

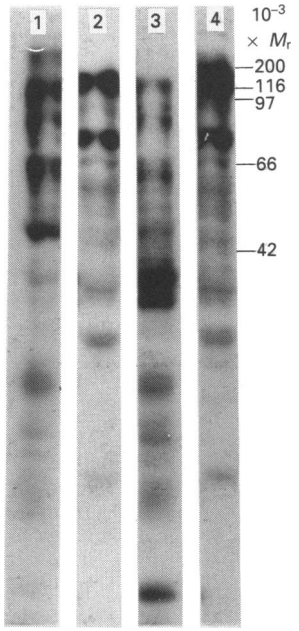

Fig. 4. Cell-surface biotinylation of Caco-2 cells

Labelling of apical (lanes 1 and 2) and basolateral (lanes 3 and 4) membrane proteins on intact Caco-2 cells with biotin-LC-NHS was followed by Triton X-114 solubilization and temperature-induced phase separation into detergent (lanes 1 and 3) and aqueous (lanes 2 and 4) phases. After SDS/PAGE and electroblotting, the biotinylated proteins were detected with ^{125}I-streptavidin. The positions of M_r markers are shown.

nitrophenylphosphatase activity at a refractive index of 1.350 and a low, but significant, alkaline phosphatase activity which peaked at the same density. This observation provides evidence that the apparent 3-fold purification of alkaline phosphatase observed in the basolateral membrane fraction is not simply due to contamination by the apical membrane, but that this enzyme is also a genuine component of the basolateral membrane.

Both 5′-nucleotidase (Bailyes *et al.*, 1990) and alkaline phosphatase (Howard *et al.*, 1987) are linked to the plasma membrane by glycosyl-phosphatidylinositol (GPI) anchors, and active enzyme may be released by PI-PLC treatment, though 5′-nucleotidase is more resistant to this treatment. Since the GPI anchor is recognized as an apical targeting signal (Lisanti *et al.*, 1989*b*, 1990) for integral membrane proteins in polarized epithelial cells, it was important to examine whether both apical and basolateral alkaline phosphatase could be removed by PI-PLC treatment. It was found that apical and basolateral alkaline phosphatase was released to a similar extent by PI-PLC treatment (68 % and 72 % respectively), and to a greater extent than apical 5′-nucleotidase (8 %).

The protein composition of the apical and basolateral membrane fractions isolated by subcellular fractionation was determined by SDS/PAGE followed by Coomassie Blue staining (Fig. 3). Both common and unique integral and peripheral membrane proteins were observed in both fractions. Domain-specific cell-surface biotinylation experiments on intact polarized Caco-2 cell layers also showed labelling of common and unique membrane proteins (Fig. 4). There was a greater difference between the labelling patterns of integral membrane proteins of the two domains than of peripheral membrane proteins (Fig. 4). Polyclonal antisera raised against Triton X-114-extracted proteins from either apical or basolateral plasma membrane fractions showed considerable cross reaction with the two domain-specific membrane fractions in Western blotting experiments (results not shown).

An antiserum raised against the apical plasma membrane fraction was used for immunoprecipitation of plasma membrane fractions obtained from [^{35}S]-methionine/cysteine pulse–chase-labelled cells. Five membrane proteins (M_r 105000, 116000, 160000, 210000 and ~ 300000), which were common to both the apical and basolateral membranes, showed a similar time course of appearance in each domain, with radiolabelled immunoprecipitates being observed 1 h after the start of the chase (Fig. 5). Confirmation that these proteins are genuine components of the plasma membrane was obtained by immunoprecipitation of [^{35}S]-methionine/cysteine pulse–chase-labelled cells after extracellular addition of antiserum. The same five radiolabelled proteins were precipitated (Fig. 5). Since endoplasmic reticulum was the major contaminant of the plasma membrane domain fractions, an experiment was carried out to determine whether the presence of these five proteins in the apical and basolateral plasma membrane fractions was due to endoplasmic reticulum contamination. Addition of anti-(apical plasma membrane) serum to a fraction enriched in endoplasmic reticulum (P5, Table 2) from [^{35}S]-methionine/cysteine pulse–chase-labelled cells did

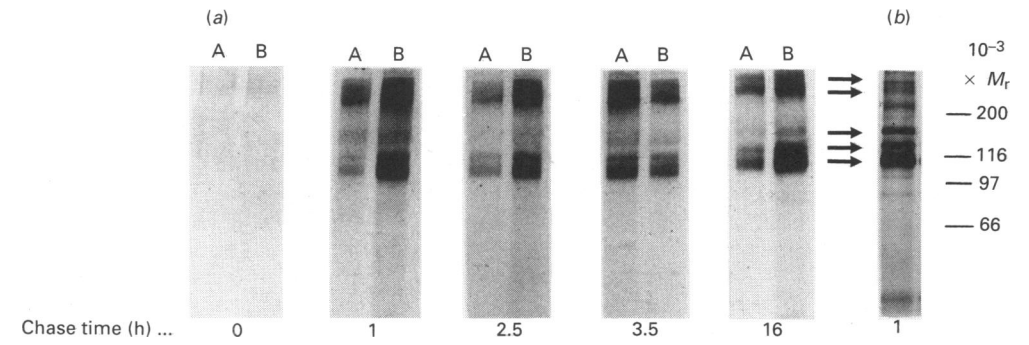

Fig. 5. Immunoprecipitation of (*a*) apical and basolateral plasma membrane fractions and (*b*) intact cells with polyclonal antisera raised against integral membrane proteins of the apical membrane fraction

Caco-2 cells cultured on filters were pulsed for 30 min with [^{35}S]Translabel and chased for the indicated times in DMEM + 10 mM-cysteine and 10 mM-methionine. In (*a*), cells were then fractionated and the apical (A) and basolateral (B) plasma membrane fractions were immunoprecipitated. In (*b*), anti-(apical plasma membrane) serum was added to the intact cells after both a 1 h chase and trypsin treatment to remove cells from the filter. Immunoprecipitates were analysed by SDS/PAGE and fluorography. The arrows show common immunoprecipitated proteins. The positions of M_r markers are shown.

not give an immunoprecipitate containing these five proteins (results not shown).

DISCUSSION

This paper provides the first report of the simultaneous purification of apical and basolateral plasma membrane domains from Caco-2 cells cultured on permeable filter supports. In the protocol described it is the combination of sucrose-density-gradient centrifugation and differential precipitation with bivalent cations that provides a reproducible method. Previous work on gut tissue demonstrated the difficulty of completely separating basolateral membranes from smooth intracellular membranes by sucrose-density-gradient centrifugation alone (Weiser *et al.*, 1978; Ahnen *et al.*, 1983; Moktari *et al.*, 1986). Smooth endoplasmic reticulum was found to co-sediment with the basolateral membrane fraction, but could subsequently be removed by differential precipitation using Mg^{2+} or Ca^{2+} (Schmitz *et al.*, 1973). Bivalent cations have also been shown to be effective in purifying brush border membranes by removing the less positively charged basolateral and smooth membrane components. However, various authors (Booth & Kenny, 1974; Stieger & Murer, 1983) have shown that a significant amount (approx. 30%) of brush border membranes are aggregated along with the basolateral membranes and lysosomes. Differential precipitation with bivalent cations has been used previously to purify apical plasma membranes from cultured Caco-2 cells, with similar enrichment to that obtained in the present experiments (Stieger *et al.*, 1988). There are no previous reports of the isolation of basolateral plasma membrane from cultured Caco-2 cells. Electron-microscopic examination of the apical and basolateral plasma membrane fractions from Caco-2 cells showed their respective similarity to previously isolated apical membrane fractions from rabbit enterocytes (Colas & Maroux, 1980) and basolateral membrane fractions from rat distal colon (Wiener *et al.*, 1989) and rat enterocytes (Scalera *et al.*, 1980).

In the present study it was found that 5′-nucleotidase was a better marker for the apical plasma membranes of Caco-2 cells than either sucrase–isomaltase or alkaline phosphatase. The apical membrane enrichment of 5′-nucleotidase is consistent with its subcellular distribution in other cells (Luzio *et al.*, 1986). In a previous study, sucrase–isomaltase was found in the lysosomes of Caco-2 cells as well as the brush border membrane (Matter *et al.*, 1990*b*), making it unsuitable as a specific apical membrane marker. Similarly, the observation of significant alkaline phosphatase activity in the basolateral plasma membrane fraction also casts doubt on its use as an apical marker. The present data are consistent with previous studies using surface labelling and immunoprecipitation techniques which showed the presence of alkaline phosphatase in the basolateral plasma membrane (Le Bivic *et al.*, 1990). In the current experiments both apical and basolateral alkaline phosphatase were released from membrane fractions by PI-PLC treatment, consistent with the presence of a GPI anchor. The presence of alkaline phosphatase but not 5′-nucleotidase on the basolateral plasma membrane of Caco-2 cells raises questions as to the efficiency of the GPI moiety as an apical targeting signal, and as to whether it causes targeting simply by association with glycolipid clusters targeted to the apical domain (Simons & Wandinger-Ness, 1990).

Domain-selected biotinylation followed by ^{125}I-streptavidin blotting allows the direct visualization of apical and basolateral membrane proteins without the problem of cross-contamination inherent in cell fractionation studies. This is the first study of the apical and basolateral membrane composition of Caco-2 cells using such a method. Although differences in apical and basolateral membrane protein composition in Caco-2 cells by both cell fractionation/SDS/PAGE and domain-selective biotinylation/^{125}I-streptavidin blotting were found, these differences are less dramatic than those seen in MDCK cells (Sargiacomo *et al.*, 1989; Lisanti *et al.*, 1989*a*). This is not due to a lack of polarization of Caco-2 cells, which was demonstrated most clearly by the differential distribution of 5′-nucleotidase and *p*-nitrophenylphosphatase on the apical and basolateral cell surface domains. The more similar membrane protein composition of the apical and basolateral domains of Caco-2 cells compared with MDCK cells may reflect the different physiological functions of the progenitors of these cultured cell lines. It will be of interest to determine whether a larger subset of plasma membrane proteins is transcytosed in Caco-2 cells compared with MDCK cells (Brandli *et al.*, 1990). The membrane traffic pathways of such proteins could provide transcytic routes of targeted drug delivery across the epithelial cell layer lining the gastrointestinal tract.

The availability of a method for the simultaneous isolation of apical and basolateral plasma membrane fractions from cultured Caco-2 cells has allowed us to show simultaneous delivery to the apical and basolateral cell surface of newly synthesized molecules of five proteins common to the two domains. Similar amounts of these radiolabelled proteins were immunoprecipitated from apical and basolateral membrane fractions at all chase times studied, from a 20 min chase (results not shown) to a 16 h chase (Fig. 5). Direct delivery to the basolateral plasma membrane confirms previous reports, using domain-specific cell surface labelling assays, of direct and efficient targeting to this domain (Matter *et al.*, 1990*b*; Le Bivic *et al.*, 1990). We did not observe any proteins which were first delivered to the basolateral domain and subsequently the apical domain, as has been reported for dipeptidyl peptidase IV and aminopeptidase N using domain-specific cell surface assays (Matter *et al.*, 1990*b*). This presumably reflects the fact that only the most immunogenic and immunoprecipitable integral membrane proteins were being analysed. In this context it is of interest that Hauri *et al.* (1985), when isolating monoclonal antibodies to human intestinal microvillus membranes, noted the lack of antibodies to several low-abundance enzymes present in the microvillus membrane. In the present study the observation of a direct delivery route to the apical plasma membrane using techniques similar to those used in studying hepatocytes confirms the previously reported difference in biosynthetic routes in the two cell types (Bartles & Hubbard, 1988).

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