Expression of placental alkaline phosphatase does not correlate with IgG binding, internalization and transcytosis

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The human homologue of FcRn, an IgG Fc receptor expressed in rat villous syncytiotrophoblasts, might be involved in IgG transfer from the maternal to the fetal circulation. However, because the receptor does not bind IgG at the physiological pH of the maternal blood (pH 7.4), FcRn is probably not involved in the initial uptake of IgG. A role in IgG internalization has been suggested for placental alkaline phosphatase (PLAP), which is highly expressed on the apical surface of syncytiotrophoblasts. To determine whether PLAP does indeed have a role in IgG

uptake, we analysed the ability of PLAP to bind, internalize and transcytose IgG in BeWo choriocarcinoma cells endogenously expressing the protein, or in Madin–Darby canine kidney (MDCK) cells transfected with the PLAP cDNA. Although PLAP expression in MDCK cells resulted in increased IgG binding to intact cells, binding was not correlated with the level of PLAP expressed in the different cell lines. Furthermore our findings do not support a role for PLAP in IgG endocytosis or transcytosis.

INTRODUCTION

In humans, passive immunity is transferred prenatally to the developing fetus [1]. Human IgG, but not IgA, IgM or IgE [2], is transported across the placenta with increased efficiency after the 22nd week of gestation [3]. This might be due to the inability of the cytotrophoblast layer underlying the syncytiotrophoblast in the first trimester to transport IgG [4]. However, in the third trimester, the number of cytotrophoblasts is greatly reduced and the feto-maternal barrier then consists of the syncytiotrophoblast and the fetal capillary endothelium [5]. Although Fc receptors have been localized in syncytiotrophoblast as well as in fetal endothelial cells [4,6–25], the precise mechanism by which IgG crosses the term placenta is not fully understood.

As syncytiotrophoblasts are in direct contact with maternal blood, investigations have focused on the uptake of IgG at their apical plasma ('brush border') membrane. Binding of the Fc portion of IgG to the brush border of villous syncytiotrophoblasts [26] led to the isolation of several IgG binding proteins with molecular masses of 60, 45, 37 and 40 kDa [17,27–29]. Moreover, contradictory characteristics of the putative plasma membrane IgG receptor have been described suggesting that no single membrane receptor mediates IgG binding, internalization and transcytosis in syncytiotrophoblasts. First, the affinity constants reported for IgG vary between low affinity (10^6 M⁻¹) and high affinity (10^8 M⁻¹) [28,30–32]. Furthermore a recent report demonstrated the presence of high-affinity (165 nM) as well as low-affinity (58 μ M) IgG-binding sites on isolated villous trophoblasts [33]. Secondly, IgG subclasses exhibit different affinities for binding to placental plasma membrane vesicles (IgG1 = IgG3 > IgG4 > IgG2) [30,34], which correlate well with the respective subclass binding and uptake in isolated trophoblasts [33]. However, Mongan and Ockleford [35] demonstrated only slightly decreased levels of IgG2 in syncytiotrophoblasts *in situ* compared with IgG1. Thirdly, the pH optimum of IgG binding to isolated placental membranes varies from acidic (5.2) to

neutral pH [28,30,31,36,37]. Taken together, multiple IgG receptors seem to be present on the apical surface of syncytiotrophoblasts that might exhibit different subclass specificities. Nevertheless the direct involvement of any of these IgG-binding proteins in IgG endocytosis and subsequent transcytosis to the basolateral membrane has not yet been demonstrated.

Recently, a human homologue (hFcRn) of the neonatal rat intestinal IgG receptor (FcRn) mediating IgG transport from the mother to the offspring has been found in human placental villous syncytiotrophoblasts, non-villous cytotrophoblasts [9,14,19,22], and trophoblast-like cell lines [14]. Although direct evidence for the involvement of hFcRn in transplacental IgG transfer remains to be obtained, its co-localization with IgG in intracellular vesicles (endosomes) in syncytiotrophoblasts [19] supports such a role.

In rat, IgG binding to FcRn occurs at slightly acidic pH (6.0) but is decreased at a pH neutral or higher [38,39]. Therefore FcRn can bind IgG at the acidic pH of the intestinal lumen and, after endocytosis and transcytosis of the ligand across the enterocyte, IgG is released from the receptor at the neutral serosal pH [40]. The apical membrane of placental syncytiotrophoblasts, however, is exposed to the neutral pH of the maternal blood. The hFcRn displays pH-dependent IgG-binding properties similar to those of rat intestinal FcRn [9,22]. Although hFcRn might be present at the apical plasma membrane, binding of IgG to hFcRn might not occur at the apical surface of syncytiotrophoblasts but only after IgG has been internalized into acidic early endosomes. Thus initial IgG internalization could be mediated or supported by an IgG receptor distinct from hFcRn present on the apical plasma membrane of syncytiotrophoblasts. The Fc receptors FcRIIIa and annexin II, both low-affinity IgG-binding proteins, have been suggested to mediate internalization of prebound IgG [41]. A similar role has been proposed for placental alkaline phosphatase (PLAP), a glycosylphosphatidylinositol-anchored protein present on the apical membrane of syncytiotrophoblasts [42,43], owing to the capacity

Abbreviations used: DTAF, 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein; FcRn, neonatal rat intestinal IgG Fc receptor; hFcRn, human neonatal IgG Fc-receptor homologue; MDCK, Madin–Darby canine kidney; PLAP, placental alkaline phosphatase.

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of PLAP to bind the Fc portion of IgG [44]. Indeed, IgG internalization into Hep-2 cells could be inhibited by pre-incubation with anti-PLAP antibodies [45]; also the presence of IgG [46] and PLAP [47] in clathrin-coated vesicle preparations isolated from human placenta was consistent with a role for PLAP as an IgG Fc receptor. Nevertheless a direct correlation between PLAP expression and IgG transport across the placenta has not been observed [48].

In the present study we analysed the possible role of PLAP as an IgG FcR in two epithelial cell models, the trophoblast-derived BeWo choriocarcinoma cells, which endogenously express PLAP [49], and Madin–Darby canine kidney (MDCK) cells, which do not express the protein. Although the expression of PLAP in MDCK cells led to an increased binding of IgG to transfected MDCK cells, bound IgG was not internalized. As expected for the apical localization of PLAP in transfected MDCK cells [50], the increase in IgG binding was restricted to the apical surface. Similarly, IgG that bound at neutral pH to the apical surface of BeWo cells failed to internalize. These findings indicate that, although PLAP enhances the binding of IgG to the apical cell surface, it is not involved in the internalization or transcytosis of IgG.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise noted, all chemicals were purchased from Sigma (Sigma-Aldrich, Vienna, Austria). Dulbecco's modified Eagle's medium was from Life Technologies (Vienna, Austria), and fetal calf serum from Sebak (Seben, Austria). BA 83 nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). The enhanced chemiluminescence kit was from Amersham (Little Chalfont, Bucks., U.K.). Mowiol 4-88 was purchased from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.), and used at a concentration of 10 g/100 ml. FITC was conjugated with human IgG by using FITC on celite as described [51]. The conjugate had a coupling ratio of approx. 7000 molecules of FITC per molecule of IgG. FITC–IgG and FITC–dextran (70 S) were dialysed extensively against Tris-buffered saline, pH 7.34 (TBS) and finally against PBS before use. FITC derivatives in PBS were diluted in phosphate-buffered Leibowitz 15 medium (L-15; Life-Technologies, Vienna, Austria) for binding and transport studies. A protease inhibitor cocktail was prepared by dissolving 10 mg of each protease inhibitor (chymostatin, antipain, leupeptin and pepstatin A) in 1 ml of DMSO. This stock was stored at -20°C and used at a final concentration of 10 $\mu\text{g}/\text{ml}$.

Antibodies

Purified human IgG, human IgG coupled to agarose (IgG–agarose), FITC and 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF), and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma. Rabbit polyclonal anti-(human PLAP) antibody was obtained from Dako A/S (Glostrup, Denmark).

Cell lines and cell culture conditions

BeWo cell clone b24, which maintains the characteristics of the original choriocarcinoma cell line and proliferates rapidly, was kindly provided by Arie van der Ende (Laboratory of Cell Biology, Medical School, University of Utrecht, The Netherlands); MDCK cells strain II were transfected with PLAP cDNA (kindly supplied by D. Brown, SUNY, Long Island, NY, U.S.A.)

[52] by the calcium-phosphate method as previously described [53]. Cells were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% (v/v) fetal calf serum, 100 i.u./ml penicillin and 0.1 mg/ml streptomycin at 37°C under air/ CO_2 (19:1) at 95% humidity. For binding and transport experiments, 5×10^5 cells/ cm^2 (BeWo) or 2.4×10^5 cells/ cm^2 (MDCK) were seeded on 12 mm permeable filters (0.4 μm , low density; Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) to obtain polarized cell monolayers after culture for 4 days (MDCK) or 7 days (BeWo). For immunofluorescence studies, cells were grown overnight on coverslips or on transwell filters for the times indicated above.

Preparation of cell lysates and binding to IgG–agarose

Cells were grown to confluence, rinsed with PBS and collected by scraping and centrifugation. For comparison of expression levels, cell pellets were suspended in 200 μl of lysis buffer [100 mM NaCl/100 mM Tris/HCl (pH 8.0)/0.5% (v/v) Triton X-100, containing protease inhibitors] and left for 10 min on ice, then insoluble material was pelleted [10 min at 12000 g (r_{av} 7.5 cm)]. For IgG binding studies, cells were collected in PBS by scraping and centrifuged [5 min at 1000 g (r_{av} 7.5 cm)]. The cells were resuspended in phosphate buffer (5 mg/ml CHAPS/50 mM NaH_2PO_4 , titrated with 50 mM Na_2HPO_4 to pH 7.4, containing protease inhibitors); after 10 min of incubation at 4°C insoluble material was removed by centrifugation [10 min at 12000 g (r_{av} 7.5 cm)]. The supernatant (100 μg of protein) was incubated with 50 μl (250 μg) of prewashed IgG–agarose in a final volume of 1000 μl of phosphate buffer, pH 7.4, under rotation overnight at 4°C . Unbound proteins were removed by four washes with phosphate buffer, pH 7.4, followed by centrifugation [5 min at 12000 g (r_{av} 7.5 cm)] to collect the agarose. Material still bound to the agarose was dissolved by addition of 16 μl of 4 $\times$ reducing SDS sample buffer, boiled and subjected to SDS/PAGE on 12.5% (w/v) mini-gels. Protein concentration was determined by the method of Bradford [54].

SDS/PAGE and Western blotting

Proteins were dissolved in reducing SDS sample buffer, separated by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) dried milk in PBS/Tween-20 (0.1%, v/v). PLAP was detected by incubation with a polyclonal anti-PLAP antibody (1:500), followed by incubation with a secondary, horseradish peroxidase-conjugated, antibody (1:8000). All blots were developed with the enhanced chemiluminescence kit. Bands of interest were quantified by laser densitometry with an Ultrosan XL (LKB).

Binding and endocytosis studies

Quantification of PLAP expressed at the apical plasma membrane

Confluent cells cultured on permeable filters were placed on ice, washed with PBS⁺ (PBS containing 0.9 mM CaCl_2 and 0.5 mM MgCl_2) and blocked with human IgG (1 mg/ml) in L-15 for 30 min at 4°C . Anti-(human PLAP) antibody (100 μl ; 1:10 in L-15) was bound to the apical plasma membrane at 4°C for 60 min; unbound antibody was removed by washing the cells with PBS⁺, pH 7.4. Cells were then incubated (60 min at 4°C) with FITC-labelled secondary antibody added to the apical chamber (1:50 in L-15). After the cells had been washed with PBS⁺, pH 7.4, filters were removed from the filter holders, cells on filters were lysed in 0.6 ml of PBS, pH 7.4, containing 0.2% (v/v) Triton X-100, then centrifuged at 12000 g (r_{av} 7.5 cm) for

10 min to pellet insoluble material and filter membranes. FITC fluorescence in 250 μ l of supernatant was determined in a Cytofluor 2300 (Millipore Corp., Astroscan Ltd., Isle of Man, U.K.) with a standard filterset [excitation at 485 nm (slit 20 nm); emission at 530 nm (slit 25 nm)]. Background fluorescence was determined under identical conditions but with the first antibody omitted. Results shown are fluorescence (arbitrary units) after the subtraction of background fluorescence.

Binding of FITC-IgG

Confluent cells cultured on permeable filters were preincubated with L-15 containing 0.5% (w/v) BSA for 30 min at 37 °C. Cells were placed on ice and washed with PBS⁺. FITC-IgG (200 μ g/ml), with or without 2 mg/ml IgG, in L-15 was added to the apical (0.5 ml medium) chamber. Cells were incubated at 4 °C for 60 min and unbound FITC-IgG was removed by washing the cells with PBS⁺, pH 7.4. Filters were removed from the filter holders, and cells on filters were lysed in 0.6 ml of PBS, pH 7.4, containing 0.2% (v/v) Triton X-100 then centrifuged at 12000 *g* (r_{av} 7.5 cm) for 10 min to pellet insoluble material and the filter membranes. FITC fluorescence in 250 μ l of supernatant was determined in a Cytofluor as described above. After subtraction of background fluorescence (unlabelled cells) the amount of cell-associated FITC-IgG was calculated with a calibration curve of FITC-IgG in PBS, pH 7.4, containing 0.2% (v/v) Triton X-100.

Transcytosis of FITC-IgG and FITC-dextran

Polarized, confluent cells were incubated for 30 min at 37 °C in L-15 containing 0.5% BSA; 0.5 ml and 1.5 ml of L-15 medium were added to the apical and basolateral chambers respectively. Endocytosis marker (FITC-dextran or FITC-IgG, final concentration 2 mg/ml) was added to the apical medium. Cells were incubated at 37 °C for 60 min. Control incubations were performed under identical conditions at 4 °C to exclude paracellular transport. Cells were then placed on ice, media were collected and cells washed with PBS⁺. FITC fluorescence in 250 μ l of basolateral medium was determined (fluorescence of medium at 37 °C minus background at 4 °C) and the amount of transcytosed marker was calculated with a standard curve of FITC derivatives in L-15 medium. The determination and calculation of cell-associated marker were performed as described above.

Immunofluorescence

Cell surface expression of PLAP

Cells were washed with PBS⁺, cooled to 4 °C and incubated with a polyclonal anti-(human PLAP) antibody (diluted 1:100 in L-15) at 4 °C for 1 h. Unbound antibody was then removed by washing with ice-cold PBS⁺, the cells were fixed for 10 min with methanol (−20 °C), followed by incubation with 10% (v/v) goat serum in PBS (30 min at room temperature) and a DTAF-conjugated secondary antibody [1:250 dilution in 10% (v/v) goat serum for 1 h at room temperature]. To investigate polarized expression, cells were grown on transwell filters and the first antibody was then added to either the apical (100 μ l) or the basolateral (50 μ l) chamber at 4 °C for 1 h. After fixation and incubation with the second antibody, filters were removed from filter holders and mounted with Mowiol.

Binding and internalization of IgG at pH 7.4

Cells were preincubated in serum-free L-15 medium for 30 min, cooled to 4 °C and washed with PBS⁺. Cells were incubated with

IgG in L-15 (final concentrations 50 mg/ml, 5 mg/ml, 500 μ g/ml, 50 μ g/ml and 5 μ g/ml) for 1 h at 4 °C. Unbound IgG was removed by washing with PBS⁺. Cells were either fixed immediately or transferred to 37 °C for 30 min. Cells were washed, fixed with McLean's fixative (2 h at room temperature) [55], quenched with 50 mM NH₄Cl in PBS (10 min at room temperature) and, after internalization, permeabilized with 0.01% Saponin in PBS (2 $\times$ 10 min at room temperature). IgG was detected with an FITC-conjugated goat polyclonal anti-(human IgG) antibody (1:50 in PBS; 60 min at room temperature).

Cells were viewed in an Olympus AH-2 microscope with a $\times$ 63 oil immersion lens. Pictures were taken with Kodak T-Max (1600 ASA) films.

RESULTS

Characterization of transfected MDCK cells expressing PLAP

To determine whether PLAP has a role as an IgG receptor, we analysed the ability of transfected MDCK cells expressing PLAP, and of BeWo cells, which endogenously express PLAP, to bind, internalize and transcytose IgG. MDCK cells, which normally do not express PLAP, were transfected with the PLAP cDNA; stably expressing clones were identified by immunofluorescence with a polyclonal anti-(human PLAP) antibody.

To compare the levels of total PLAP expression in BeWo and transfected MDCK cells, equal amounts of total cell lysate

Figure 1 PLAP expression in BeWo, MDCK and MDCK cells stably transfected with PLAP cDNA (MDCK/PLAP)

(A) Cell lysates (10 μ g of protein per lane) of BeWo (lane B), MDCK (lane M) and MDCK/PLAP (lane M/P) cells were separated by reducing SDS/PAGE [12.5% (w/v) gel], transferred to nitrocellulose and incubated with anti-PLAP antibody. The amount of total PLAP expressed in BeWo cells was much lower than in MDCK/PLAP cells. PLAP was not detected in MDCK cells. (B) Plasma membrane expression of PLAP was investigated by binding of an anti-PLAP antibody to the plasma membrane at 4 °C, followed by fixation and detection with a secondary, DTAF-labelled antibody. The fluorescence intensity of PLAP labelling at the plasma membrane (B) is well correlated with total PLAP expression (A). (C) To analyse whether PLAP expressed in BeWo, MDCK and MDCK/PLAP cells binds IgG, cell lysates were incubated with IgG-agarose at pH 7.4. Bound material was separated by reducing SDS/PAGE [12.5% (w/v) gel], transferred to nitrocellulose and probed with anti-PLAP antibody. PLAP could indeed be identified as an IgG-binding protein in BeWo (lane B) and MDCK/PLAP (lane M/P) cells. Among these cell lines, the amount of PLAP bound to IgG-agarose was comparable to the total expression of PLAP [compare (A) and (C)].

Table 1 Quantification of PLAP expression at the apical plasma membrane of BeWo, MDCK and MDCK/PLAP cells

Filter-grown BeWo, MDCK and MDCK/PLAP cells were pre-equilibrated at 4 °C with 1 mg/ml human IgG in L-15 to block non-specific binding sites. Then an anti-(human PLAP) antibody was bound (4 °C for 60 min) to the apical membrane, followed by incubation with a secondary FITC-labelled antibody (4 °C for 60 min). Unbound antibody was removed, cells on filters were lysed and the fluorescence intensity (arbitrary units) was determined in an ELISA reader. Background fluorescence due to non-specific binding of the secondary antibody was subtracted from the total fluorescence. Results are means $\pm$ S.E.M. for two independent experiments, each performed in triplicate. Transfection of MDCK cells with PLAP cDNA resulted in 5-fold higher levels of PLAP expression at the apical plasma membrane than in BeWo cells.

Cell type	Apical PLAP expression (fluorescence in arbitrary units)
BeWo	325 $\pm$ 45
MDCK	60 $\pm$ 17
MDCK/PLAP	1645 $\pm$ 93

protein were analysed by Western blotting. As shown in Figure 1(A), a 66 kDa band corresponding to PLAP was detected in BeWo and MDCK/PLAP cell lysates, but was absent from lysates from non-transfected MDCK cells. The total PLAP expression level in the transfected MDCK clone was approx. 9-fold that of the endogenous PLAP expression level in BeWo cells as determined by laser densitometry from four independent experiments.

Next we verified by indirect immunofluorescence that PLAP was expressed on the cell surface of transfected MDCK cells and BeWo cells. Anti-PLAP antibody was bound to the plasma membrane at 4 °C. At this temperature endocytosis is inhibited and only plasma-membrane-exposed PLAP will be detected by the antibody. After fixation, cells were incubated with a fluorescence-conjugated secondary antibody. As shown in Figure 1(B), PLAP was detected on the cell surface of BeWo and MDCK/PLAP cells but was not present on the surface of non-transfected MDCK cells. Consistent with published results [49,50,52], PLAP predominantly localized to the apical surface if MDCK/PLAP or BeWo cells were grown on permeable supports to obtain polarized cell monolayers (results not shown).

To compare quantitatively the amounts of PLAP present at the apical cell surface of BeWo, MDCK and MDCK/PLAP cells, anti-(human PLAP) antibody was added to filter-grown cells at 4 °C, followed by incubation with an FITC-labelled secondary antibody at 4 °C. After being washed, cells were lysed and the cell-associated fluorescence was determined (see the Materials and methods section). As shown in Table 1, the amount of PLAP being expressed at the apical surface in PLAP-transfected MDCK cells was approx. 5-fold that in BeWo cells endogenously expressing the protein.

PLAP expression is not correlated with IgG binding

Next we analysed whether PLAP expressed in BeWo or transfected MDCK cells was able to bind IgG. BeWo, MDCK and MDCK/PLAP cell lysates were incubated with IgG-agarose at neutral pH; retained proteins were analysed by SDS/PAGE and Western blotting with an anti-PLAP antibody. As shown in Figure 1(C), PLAP present in the lysates of BeWo and MDCK/PLAP cells was indeed retained on the IgG-agarose. Moreover, the amount of PLAP retained by the IgG-agarose was correlated with the total amount of PLAP present in the cell lysates of

Figure 2 Fluorescence microscopy of IgG binding and internalization in BeWo, MDCK and MDCK cells stably transfected with PLAP cDNA (MDCK/PLAP)

BeWo cells (a–d) were incubated with IgG at concentrations of 50 mg/ml (a, c) and 50 µg/ml (b, d) for 1 h at 4 °C. Cells were either fixed immediately (a, b) or transferred to 37 °C for 30 min (c, d) before fixation. BeWo (e), MDCK (f) or MDCK/PLAP cells (g) were incubated with 500 µg/ml IgG for 1 h at 4 °C and then fixed. IgG was detected with FITC-conjugated anti-(human IgG) antibody. Scale bar, 10 µm. In BeWo cells, IgG binding could be detected at high IgG concentrations (a) but not at low IgG concentrations (b). However, transfer to 37 °C did not result in endocytosis of bound IgG (c, d); most probably IgG was released into the medium. MDCK cells transfected with PLAP (g) exhibited considerably increased plasma membrane IgG staining compared with untransfected cells (f). Again, subsequent endocytosis of prebound IgG was not observed (results not shown).

BeWo and MDCK/PLAP cells (compare Figures 1A and 1C). These results are consistent with the results of a previous study showing that isolated PLAP can bind IgG [44].

We then determined whether IgG could bind to intact cells expressing PLAP. Because PLAP binds IgG with low affinity [44], we used IgG concentrations up to the levels found in maternal blood (50–110 µM). Therefore IgG concentrations ranging from 5 µg/ml to 50 mg/ml were allowed to bind to the cells at 4 °C and pH 7.4. After removal of non-bound IgG by washing, cells were fixed and bound IgG was detected by indirect immunofluorescence. A bright membrane fluorescence was observed for BeWo cells incubated with IgG at a concentration of 50 mg/ml (Figure 2a), which was decreased at 5 mg/ml (results not shown). The membrane-associated fluorescence was weak at 500 µg/ml (Figure 2e) and below the detection level at 50 µg/ml (Figure 2b) and 5 µg/ml (results not shown). Although no

Table 2 Plasma membrane IgG binding is not correlated with PLAP expression

BeWo, MDCK and MDCK/PLAP cells were grown on transwell filters, 200 $\mu\text{g}/\text{ml}$ FITC-IgG was added to the apical medium (pH 7.4) in the absence or presence of 2 mg/ml IgG, and cells were incubated for 60 min at 4 °C. Unbound IgG was removed, cells on filters were lysed and the fluorescence intensity was determined in an ELISA reader. After subtraction of background fluorescence (unlabelled cells) the amount of cell-associated FITC-IgG was calculated with an FITC-IgG calibration curve. Results are means $\pm$ S.E.M. for two independent experiments, each performed in quadruplicate. Transfection of MDCK cells with PLAP cDNA considerably increased IgG binding; nevertheless binding was still lower, as in BeWo cells. Plasma membrane FITC-IgG binding could not be decreased by unlabelled IgG in all cell lines, excluding the presence of a high-affinity IgG receptor.

Cell type	FITC-IgG bound (ng)	
	– IgG	+ IgG
BeWo	94 $\pm$ 3	81 $\pm$ 10
MDCK	11 $\pm$ 2	11 $\pm$ 2
MDCK/PLAP	46 $\pm$ 6	59 $\pm$ 7

binding of IgG (500 $\mu\text{g}/\text{ml}$) to control MDCK cells could be detected (Figure 2f), MDCK/PLAP cells displayed a significant binding of IgG at neutral pH (Figure 2g).

Next, we quantified the binding of IgG to the apical domain of BeWo, MDCK and MDCK/PLAP cells grown on permeable supports to obtain polarized cell monolayers and compared it with the amount of PLAP present at the apical cell surface of these cell lines (see Table 1). FITC-IgG (200 $\mu\text{g}/\text{ml}$) was added to the apical medium at pH 7.4 and allowed to bind at 4 °C. After removing unbound FITC-IgG, cells were lysed and the cell-associated fluorescence was measured to calculate the amount of FITC-IgG bound to the apical surface (Table 2). The expression of PLAP led to an increased binding of IgG to MDCK/PLAP compared with non-transfected cells. Interestingly, however, even though BeWo cells expressed one-fifth the amount of PLAP that MDCK/PLAP cells did at their cell surfaces, the former bound approximately twice as much IgG. Thus IgG binding to intact cells is not correlated with the level of PLAP expressed at the apical cell surface.

Furthermore the presence of a 10-fold excess of unlabelled IgG (2 mg/ml) only insignificantly affected the binding of FITC-IgG to BeWo, MDCK/PLAP or control MDCK cells (Table 2), indicating that the binding of IgG at neutral pH to the different

cells reflected to a large extent a non-specific interaction with the plasma membrane.

In conclusion, there was no correlation between PLAP surface expression levels and binding of IgG to intact cells at pH 7.4. In addition, IgG binding to intact cells at neutral pH could not be competed with excess IgG, indicating that IgG binding to BeWo, MDCK/PLAP and control MDCK cells occurred largely in a non-specific fashion.

PLAP expression is not correlated with IgG endocytosis or transcytosis

Because PLAP has been suggested to have a role in IgG internalization [20,45], we next determined whether BeWo or MDCK/PLAP cells were able to internalize IgG. For this purpose, IgG (5 $\mu\text{g}/\text{ml}$ to 50 mg/ml) was allowed to bind to cells at 4 °C and pH 7.4 (see Figure 2). When cells shown in Figures 2(a), 2(b), 2(e) and 2(g) were warmed to 37 °C for 30 min before fixation to induce internalization of prebound IgG, no intracellular fluorescence staining was observed (Figures 2c and 2d, and results not shown), indicating that although IgG was able to bind to BeWo and MDCK/PLAP cells, it could not be internalized.

One possible explanation for the lack of endocytosis of prebound IgG is that the IgG is bound to the cells at such a low affinity that it dissociates when the cells are transferred to 37 °C. Therefore we next analysed whether IgG continuously present at high concentrations in the apical media at 37 °C was internalized and transcytosed by polarized BeWo or MDCK cell monolayers. FITC-IgG (2 mg/ml) was continuously internalized from the apical surface of BeWo, MDCK and MDCK-PLAP cell monolayers for 60 min at 37 °C, and the amount of IgG that had been internalized or transcytosed was determined (Table 3). In addition, transcytosis of the fluid-phase marker FITC-dextran (2 mg/ml) was also investigated under the same conditions (Table 3). As shown in Table 3, control and PLAP-transfected MDCK cells internalized similar amounts of IgG. Furthermore similar amounts of IgG and the fluid-phase marker FITC-dextran were transcytosed by MDCK and MDCK/PLAP cells, demonstrating that, at least in MDCK cells, PLAP is unable to mediate IgG endocytosis or transcytosis. Despite the lower PLAP expression levels, BeWo cells internalized and transcytosed more IgG than MDCK/PLAP cells. This could be attributed to higher levels of fluid-phase endocytosis and transcytosis in BeWo than in MDCK cells. However, in comparison with FITC-dextran, IgG was preferentially transcytosed in BeWo cells,

Table 3 IgG endocytosis or transcytosis is not correlated with PLAP expression

BeWo, MDCK and MDCK/PLAP cells were grown on transwell filters. Endocytosis of FITC-IgG was determined by adding the marker (2 mg/ml) to the apical chamber and incubating the cells for 1 h at 37 °C. The amount of cell-associated IgG was then determined as described in Table 2. To make a comparative analysis of the transcytosis of IgG and a fluid-phase marker, cells were incubated with 2 mg/ml FITC-IgG or FITC-dextran present in the apical medium for 1 h at 37 °C. The amount of marker being transcytosed into the opposite chamber was assayed as described in the Materials and methods section. Results are means $\pm$ S. E. M. for two independent experiments, each performed in quadruplicate. Continuous endocytosis of FITC-IgG resulted in uptake rates that were indistinguishable in MDCK and MDCK/PLAP cells, but were approx. 5-fold higher in BeWo cells. No significant differences in IgG and dextran transcytosis were observed when MDCK and MDCK/PLAP cells were compared. In contrast, BeWo cells exhibited 20-fold higher rates of IgG transcytosis. Moreover, IgG was preferentially transported in BeWo, but not MDCK or MDCK/PLAP, cells. Consequently PLAP expression does not affect the amount of IgG being endocytosed or transcytosed.

Cell type	FITC-IgG endocytosed (cell-associated) (ng)	FITC-IgG transcytosed (ng)	FITC-dextran transcytosed (ng)
BeWo	649 $\pm$ 16	929 $\pm$ 54	267 $\pm$ 65
MDCK	116 $\pm$ 13	50 $\pm$ 9	59 $\pm$ 15
MDCK/PLAP	122 $\pm$ 31	45 $\pm$ 6	78 $\pm$ 7

suggesting the presence of a specific IgG receptor responsible for IgG transcytosis.

Taken together, our results show that although the expression of PLAP in Fc receptor-negative MDCK cells increased IgG binding to the plasma membrane, PLAP has no role in IgG internalization or transcytosis. Furthermore, even though BeWo cells express lower levels of PLAP, they internalized and transcytosed more IgG than transfected MDCK/PLAP cells. However, the higher levels of IgG transcytosis by BeWo cells were not due simply to a higher rate of fluid-phase endocytosis in these cells, because IgG was preferentially transcytosed compared with the fluid-phase marker dextran, indicating the presence and involvement of a specific Fc receptor in these cells.

DISCUSSION

Several observations have been taken to support a role for PLAP in IgG internalization in the placental syncytiotrophoblast. PLAP is expressed on the apical domain of the syncytiotrophoblast facing the maternal blood from which IgG is taken up. Clathrin-coated vesicles isolated from placenta were found to contain IgG [46] and to be enriched in PLAP [47]. In addition, PLAP has been shown to bind IgG via its Fc region [44], and IgG internalization by Hep-2 cells endogenously expressing PLAP could be inhibited with anti-PLAP antibodies [45]. Although these observations are consistent with a role for PLAP in IgG internalization, they do not directly address the question of whether PLAP does indeed function as an endocytic IgG receptor. To investigate this question, we studied IgG internalization and transcytosis in transfected MDCK cells expressing PLAP and compared it with IgG transport in trophoblast-derived BeWo choriocarcinoma cells endogenously expressing the protein. All experiments were performed at neutral pH (7.4), reflecting the pH of the maternal blood bathing the syncytiotrophoblast.

PLAP present in cell lysates was found to bind to IgG-agarose; the amount bound was closely correlated with the different levels of PLAP present in the lysates from BeWo and MDCK/PLAP cells. This is in agreement with results demonstrating binding of PLAP by IgG in Hep-2 cells [45] and binding of human plasma IgG to PLAP purified from human placenta [44]. However, although the expression of PLAP in MDCK cells led to an increase in plasma membrane IgG binding compared with non-transfected cells, MDCK/PLAP cells bound less IgG than BeWo cells, even though the former expressed approx. 5-fold more PLAP at their surface. This observation indicates that although in MDCK cells PLAP expression leads to an increase in IgG binding, PLAP is not primarily responsible for the IgG binding observed in BeWo cells. Because BeWo cells are trophoblast-derived cells and various IgG-binding proteins had been localized in the syncytiotrophoblast plasma membrane [17,27–29], several IgG-interacting proteins are expected to be also present on the surface of BeWo cells. Indeed, additional proteins not corresponding in size to PLAP were retained if lysates from BeWo cells that had been biotinylated to label plasma membrane proteins were incubated with IgG-agarose (results not shown). We were unable to estimate the contribution of PLAP to total IgG-binding proteins present in BeWo plasma membranes because the anti-PLAP antibodies used did not inhibit the binding of purified PLAP to IgG-agarose (results not shown). However, the conclusiveness of such an experiment is doubtful given the reported variability (0–70%) of inhibition of IgG/PLAP interaction by different anti-PLAP antibodies [44]. Detectable binding of IgG to MDCK/PLAP or BeWo cells required high concentrations of labelled IgG (more than 200 µg/ml) consistent with a low-affinity association, as has been demonstrated for the

IgG–PLAP interaction [44]. However, binding was only marginally affected by the presence of a 10-fold excess of unlabelled IgG, indicating that at neutral pH the binding of IgG to the plasma membrane was largely non-specific. Thus, at neutral pH, binding of IgG to PLAP or to other plasma membrane proteins must either occur in a non-specific manner or be of very low affinity. Indeed, IgG–PLAP interaction was demonstrated to be of low affinity [44].

Our results also argue against a role for PLAP in IgG endocytosis or transcytosis. We were unable to detect the internalization of prebound IgG in MDCK/PLAP or BeWo cells by immunofluorescence. This is in agreement with earlier reports showing either plasma membrane binding of IgG at 4 °C to Hep-2 cells and isolated trophoblasts, or internalization of IgG at 37 °C when continuously present in the medium [33,45,57]. To our knowledge, the internalization of prebound IgG has not been demonstrated in PLAP-expressing Hep-2 cells and isolated trophoblasts. The lack of detectable IgG internalization was not due to the dissociation of the IgG from the cells after warming to 37 °C because in the continuous presence of IgG at 37 °C control MDCK and MDCK/PLAP cells also internalized comparable amounts of IgG, again arguing against a role for PLAP in IgG endocytosis. These results are in contrast with the reported inhibition of IgG internalization by anti-PLAP antibodies when analysed by immunofluorescence in Hep-2 cells [45]. In contrast, transfection of MDCK cells with the closely related PLAP isoform of testis resulted in membrane IgG binding but not in endocytosis [58].

Furthermore apical-to-basolateral transcytosis of IgG by MDCK and MDCK/PLAP cells was similar and occurred at a comparable rate and level to that of the fluid-phase marker dextran. Regardless of the lower PLAP expression levels in BeWo cells, they internalized 5-fold more IgG from the apical domain; IgG transcytosis was also increased compared with MDCK/PLAP cells. Consequently, varying levels of PLAP expressed at the apical plasma membrane were not correlated with IgG endocytosis or transcytosis at neutral pH. The greater amounts of IgG internalized by BeWo than by MDCK or MDCK/PLAP cells in the continuous presence of IgG could reflect a higher fluid-phase endocytic activity of BeWo cells, and/or the involvement of a protein other than PLAP in endocytosis (see below).

Our results are therefore at variance with the work of Makiya and Stigbrand [45], who proposed that the inhibition of IgG internalization into Hep-2 cells by an anti-PLAP antibody was suggestive of PLAP-mediated IgG uptake. The lack of a role for PLAP in IgG internalization and transcytosis is, however, in agreement with recent studies that found no correlation between fetal IgG levels, PLAP phenotypes and PLAP activity [48,59].

However, prebound anti-PLAP antibodies were internalized in BeWo and MDCK/PLAP cells presumably by inducing endocytosis of PLAP via caveolae by cross-linking (results not shown). This observation is in agreement with other studies in which some endocytosis of PLAP was found in BeWo (15% under steady-state conditions) [49] and PLAP-transfected MDCK cells [52]. The low fraction of PLAP localized to clathrin-coated vesicles of BeWo cells (3%) [49], however, is in contrast with the results demonstrating an enrichment of PLAP in clathrin-coated vesicles isolated from human placenta [47]. Because PLAP has also been detected in caveolae of A431 cells [60], the question of whether PLAP is endocytosed via clathrin-coated vesicles, caveolae, or both, remains open.

Despite the fact that BeWo cells did not internalize prebound IgG, they internalized IgG that was continuously present in the apical medium. BeWo cells express hFcRn on their apical surface,

but the receptor binds IgG only poorly at neutral pH (I. Stefaner, M. Schwab, A. Stefanescu, W. Hunziker and R. Fuchs, unpublished work). One possibility is that at high IgG concentrations the receptor binds the ligand with low affinity at pH 7.4 and consequently has a role in endocytosis. It might also be possible that IgG-binding proteins such as PLAP, annexin II or FcRn further increase the IgG concentration in the proximity of the membrane, thereby supporting IgG endocytosis in syncytiotrophoblasts and BeWo cells. Although our results exclude a direct involvement of PLAP in IgG endocytosis and transcytosis, more information is required for definition of the exact role of annexin II and FcRn in IgG transport. Current results suggest an involvement of these proteins in IgG internalization but not in translocation to the basolateral surface [19,41]. The colocalization of annexin II and IgG in endosomes [21,41] which was taken to indicate annexin-mediated IgG binding and uptake, could be due to the reported association of annexin II with the cytoplasmic side of endosomes and its role in endosome fusion [61,62].

Alternatively, fluid-phase endocytosis could account for the bulk of initial IgG uptake by BeWo and, by analogy, syncytiotrophoblast cells. This possibility would be consistent with the observation that the amount of apically internalized IgG, as well as that of the fluid-phase marker dextran, is correlated with their concentration in the apical medium [63], and I. Stefaner, M. Schwab, A. Stefanescu, W. Hunziker and R. Fuchs, unpublished work). Indeed, IgG is present at high enough concentrations (50–110 μ M) in the maternal blood to be available for fluid-phase internalization. Uptake of IgG by fluid-phase endocytosis would be consistent with the observation that not only IgG, but also IgA and IgM, are present in endosomes of placental villous syncytiotrophoblasts [19]. Because underlying fetal endothelial cells contained only IgG, syncytiotrophoblasts could take up serum proteins non-specifically according to their concentrations in the blood. Transcytosis of IgG to the fetal side would require binding to FcRn in acidic endosomes. Such a role for hFcRn might account for the preferential transcytosis of IgG compared with that of dextran in BeWo cells. Our results are therefore in line with transport studies in isolated trophoblasts [57]. There, IgG was preferentially transported in the apical-to-basolateral direction when compared with the fluid-phase marker horseradish peroxidase, whereas both markers were transported equally well in the opposite direction. Whether hFcRn is indeed responsible for IgG transcytosis in BeWo and syncytiotrophoblast cells remains to be elucidated. The subclass specificity (IgG1 = IgG2 > IgG3 > IgG4) of human FcRn suggested by sequence comparison with rat FcRn has not yet been verified [64]. However, this proposed specificity does not correspond to the subclass specificity of IgG transport observed in the perfused placenta [65] and in isolated trophoblasts [33].

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