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The major soyabean allergen P34 resists proteolysis *in vitro* and is transported through intestinal epithelial cells by a caveolae-mediated mechanism

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Abstract

Soya is considered to be one of the eight most significant food allergens. Among the allergenic soya proteins determined to date, P34 has been identified as one of the immunodominant soya antigens. Sensitisation to a specific food antigen like P34 generally follows the transit of intact antigens across the intestinal barrier and usually occurs in infants, who are most susceptible to food allergies. In the present study, we used the intestinal epithelial cell line IPEC-J2, which was originally derived from the jejunum of a neonatal piglet, to recapitulate the infant intestinal epithelium and study the binding and uptake of P34 protein. P34 was partially resistant to degradation in an *in vitro* proteolysis assay. IPEC-J2 cells were able to endocytose intact P34, as shown by immunofluorescence and immunoelectronmicroscopy methods. P34 associated with lipid raft microdomains of IPEC-J2 cells, and disruption of caveolae/lipid raft microdomains using methyl-β-cyclodextrin abolished P34 endocytosis, indicating that the observed endocytosis was mediated by caveolae. Using IPEC-J2 cells grown on Transwell filters, we further demonstrated that P34 is transported through the epithelial monolayer by transcytosis. Piglets frequently show hypersensitivity to soya antigens, and in this study, we show that healthy adult pigs with dietary exposure to soya protein mount an antibody response to soyabean protein P34, suggesting that this protein has entered the body, probably through gastrointestinal uptake. In summary, our data suggest that soya P34 resists proteolysis in the gastrointestinal tract and is transported through the intestinal epithelial barrier, thereby allowing sensitisation of immune cells in the sub-epithelial compartment.

Key words: Soyabean protein P34: Allergens: Endocytosis: Transcytosis: Proteolysis *in vitro*

Food allergies are an increasing public health problem, especially in young children. About 4–8 % of all children suffer from food allergies, compared to only 1–2 % of adults[1,2]. Dissecting the mechanism of allergy induction is the basis for developing novel therapeutic or preventive approaches. Therefore, understanding how allergenic food proteins reach immune cells in the intestinal lamina propria below the epithelial barrier is a top research priority.

Dietary antigens enter the body via the gastrointestinal tract. Sensitisation with an unknown allergen then requires its uptake by the intestinal mucosa[3], processing by immune cells such as sub-epithelial dendritic cells, and presentation to lymphocytes[4,5].

The soyabean protein P34 (Gly m BD 30K, Gly m 1) is the main soyabean allergen for soyabean-sensitive patients[6]. This protein fulfils all criteria of allergenic plant proteins, including intermediate molecular weight (32 kDa)[7,8], glycosylation[8,9] and low isoelectric pH (4·5)[10]. P34 is of further interest because of its sequence homology with other important allergens like Der p 1 (European house dust mite cysteine protease), Ara h 1 (main allergen of peanuts) and cows' milk protein 2-S1-casein[2].

We have previously developed a novel method to isolate and purify P34 from soyabeans for *in vitro* studies[8]. To determine how soya P34 interacts with intestinal epithelial cells, we used the IPEC-J2 cell line generated from the

Abbreviations: FIFC, fluorescein isothiocyanate; MβCD, methyl-β-cyclodextrin; TEER, transepithelial electrical resistance.

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neonatal piglet jejunum[11] for our study. Since these cells are neither immortalised nor transformed and are derived from neonatal cells[12], IPEC-J2 cells probably represent the infant enterocyte layer more accurately than commonly used cell lines such as CaCo-2, which are derived from adult carcinoma cells. IPEC-J2 cells form a confluent monolayer, express the *tight junction* proteins claudin-3 and -4 as well as occludin at the apical membrane and have a mucous layer of MUC3[11,13].

The pig may be an interesting animal model for soya allergy in human infants, since piglets frequently develop soya hypersensitivity after weaning, with a similar duodenal lymphocyte influx as seen in human infants with food intolerance[14]. Furthermore, the physiology, the development after birth and the structure of the gastrointestinal tract are highly comparable between pigs and humans[15].

In the present study, we have used purified P34 to study binding to the surface of IPEC-J2 cells, uptake and cellular transport in order to understand the pathophysiological basis for the initial step of the induction of allergy – namely the delivery of the complete protein to immune cells in the intestinal lamina propria.

Methods

Purification of P34

The purification of soyabean protein P34 was performed as described earlier[8]. First, a protein preparation of 77 % purity was obtained by carbonate extraction from the oil body fraction obtained from ground soyabeans[7,8]. Afterwards, a protein solution of 99 % purity was obtained using a further chromatographic separation on a Butyl Sepharose 4 FF column (two-step or one-step gradient elution with $(NH_4)_2SO_4$). The purity of both protein preparations was determined using a densitometry technique. For this purpose, protein preparations were separated with SDS-PAGE and gels were stained with Coomassie brilliant blue[8]. The transcytosis and lipid raft isolation experiments, which involved specific detection of P34 with a monoclonal antibody, were performed with the 77 % pure preparation; all other experiments were performed with the 99 % pure preparation of P34. Repeat experiments for fluorescence activated cell scanner (FACS) assays were performed with either the 99 % or the 77 % pure P34 preparation; similar results were obtained with both preparations.

Cells

IPEC-J2 cells (a generous gift from Dr P. Schierack, Institute of Microbiology and Epizootics, Freie Universität Berlin, Germany) from the porcine intestine[11] were cultured at 39°C (porcine body temperature) and 5 % CO_2. The cells were seeded in cell culture flasks at a concentration of 13 300/cm^2 in Dulbecco's modified Eagle's medium–Ham's-F12 (1:1) medium supplemented with 5 % fetal calf serum, 1 % insulin–transferrin–selenium, 16 mM-HEPES (all PAN Biotech) and 5 µg/l epithelial growth factor (BD Biosciences).

P34 proteolysis in vitro

To investigate the *in vitro* proteolysis of P34, a protocol of Boisen[16] with an adaptation according to Miller *et al.*[17] was used. Dried soyabeans were soaked overnight in distilled water or left untreated, ground and transferred into 25 ml phosphate buffer (0·1 M, pH 6). To recapitulate gastric proteolysis, 10 ml of 0·2 M-HCl solution were added and the pH was adjusted to 2·0. Next, 1 ml pepsin solution (28·5 mg porcine pepsin in 0·1 M-HCl, 0·7 Federation Internationale Pharmaceutique Unit (FIPU)/mg; Merck) was added to each sample to achieve a final pepsin concentration of 0·8 mg/ml. Samples were then incubated in a shaking water-bath for 2 h at 39°C. After that, the samples were placed on ice, 10 ml phosphate buffer (0·2 M, pH 6·8) and 5 ml 0·6 M-NaOH were added, and the pH value of all solutions was adjusted to 6·8. To model intestinal proteolysis, 1 ml of freshly prepared pancreatin solution (including 50 mg pancreatin from porcine pancreas, activity equivalent to 4 × United States Pharmacopoeia (USP) specifications; Sigma) was added to the solutions of homogenised soyabeans after pepsin treatment. In one aliquot of each preparation, porcine bile extract (Sigma) was added at a pancreatin–bile extract ratio of 1:6·25 (w/w) according to the *in vitro* digestion procedure of Miller *et al.*[17]. After two further hours of incubation in the shaking water-bath, all samples were placed on ice; solutions were transferred into tubes and centrifuged (10 000 ***g***, 4°C, and 5 min). Proteins in the supernatant were precipitated using TCA solution and analysed using SDS-PAGE (in the presence of β-mercaptoethanol) and immunoblot. P34 was detected with the monoclonal mouse antibody F5 (anti-P34, a generous gift from Professor T. Ogawa, Kansai University of Welfare Sciences, Kashihara, Osaka, Japan) or a rabbit polyclonal serum anti-P34 (generated by injecting highly purified P34 into rabbits by Seqlab Sequence Laboratories). Bands were visualised using the BM Chemiluminescence Western Blotting Kit (mouse/rabbit, Roche) and an Alpha-Ease® FC Imaging System.

Immunodetection of P34 in IPEC-J2 by electron microscopy

For electron microscopy of IPEC-J2 cells, uncoated Transwells with a pore size of 1 µm were used (Greiner Bio-one). Confluent monolayers were incubated with P34 for 2 h following a standardised immunostaining protocol. Cells on Transwell inserts were washed with phosphate buffer three times and fixed (room temperature, 10 min). After three further washing steps, the cells were permeabilised (5 min), washed again and then blocked for 10 min (room temperature) with 1 % bovine serum albumin. The primary antibody (mAb F5) was added for 2 h or overnight. The Transwell inserts were washed afterwards. After a second blocking step, cells on the inserts were incubated with the secondary antibody (biotinylated anti-mouse IgG; Biozol) for 2 h. After a washing step, the cells were incubated with ABC-solution for 1 h (Vectastain® Elite® ABC Kit; Vector Laboratories, Inc.). The secondary antibody was visualised with diaminobenzidin in the presence of 0·3 vol.% H_2O_2). Pre-fixation for electron microscopy was

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performed with 1–3 vol.% glutaraldehyde. Fixed Transwell membranes with cells were punched out, washed in phosphate buffer and then fixed for 30 min in osmium tetroxide (1 g/100 ml, Science Services) followed by a washing step with buffer and an ascending ethanol series (60–99 vol.%) to dehydrate samples. Next, the cells on membranes were embedded between two foils in durcupan (Fluka). Areas of interest were chosen using light microscopy, were cut out and glued on a blank capsule of durcupan. Semi-thin sections (0·5–2 μm), which were stained with toluidine, and ultra-thin sections (50–80 nm) were made using an Ultracut S (Reichert-Jung). The electron microscopic analysis was carried out on a LEO 906 E microscope (Zeiss) equipped with a digital BioScan camera (Gatan).

Lipid raft isolation using a sucrose-density-gradient centrifugation

Plasma membrane lipid rafts contain high levels of cholesterol and sphingolipids. Cell extraction with non-ionic detergents such as Brij-58 leaves the detergent-resistant membrane fraction containing lipid rafts and caveolae intact. Due to their high buoyancy correlated with the special lipid composition of these detergent-resistant membranes, they float up on specially designed sucrose gradients and form rings in the upper tube area, which can be collected[18,19]. In this gradient, all soluble molecules remain at the bottom of the tubes.

For the experiment, confluent monolayers of IPEC-J2 cells were incubated with 1 mg/ml P34 for 2 h, harvested using trypsin–EDTA and lysed on ice in lysis buffer (50 mM-HEPES; AppliChem, pH 7·4, 100 mM-NaCl, 3 % Brij-58, 1 mM-PMSF, 5 mM-EDTA, 1 mM-Na_3VO_4, 50 mM-NaF, 10 mM-$Na_4P_2O_7$). The lysate (1 ml) was homogenised in an equal amount of 80 % sucrose solution (sucrose in MNE buffer: 25 mM-2-(N-morpholino)ethanesulfonic acid) (MES), pH 6·5, 5 mM-EDTA, 150 mM-NaCl; sucrose and MES from Fluka; phenylmethylsulfonyl fluoride (PMSF), EDTA, Na_3VO_4, NaF, $Na_4P_2O_7$ from Sigma) and transferred to centrifugation tubes. Finally, this mixture was covered with ice-cold 30 % sucrose solution (2 ml) and 5 % sucrose solution (1 ml). Centrifugation was performed in an SW55Ti rotor of a Beckman TL-100 ultracentrifuge (Beckman Coulter) for 20 h and 100 000 ***g*** at 4°C. After centrifugation, fractions were collected, separated using SDS-PAGE and blotted on nitrocellulose membranes. On these membranes, protein P34 (with mAb F5), flotillin (with a goat pAb, Abcam) and caveolin-1 (with a rabbit pAb, Abcam) were detected as previously described. In addition, clathrin was detected as a raft-negative control protein, as also previously described[20] (using a mouse IgG1, BD Biosciences).

Analysis of P34 endocytosis by flow cytometry

The endocytosis of proteins and their cell surface binding is detectable by flow cytometry if the protein of interest is labelled with a fluorescent tag. For the experiments in this study, IPEC-J2 cells were plated at 110 000 per well on twenty-four-well plates. They reached confluence after 24 h and were kept on serum-free medium afterwards. On day 9 of culture, cells were treated for 2 h with fluorescein isothiocyanate (FITC)-labelled P34 (99 %, 150 μg/ml) at 39 and 4°C. In some experiments, cells were pre-treated with 10 mM-methyl-β-cyclodextrin (MβCD; Sigma) for 30 min to disrupt lipid rafts, with the continuing presence of MβCD during the uptake experiment. At the end of the experiment, the cells were washed with PBS, harvested with trypsin–EDTA and analysed on an FACS Calibur flow cytometer (Becton Dickinson). The mean fluorescence of at least 15 000 cells was determined using CellQuest software (Becton Dickinson). Dead cells were excluded by propidium iodide staining (5 μg/ml; Sigma).

Transcytosis of P34

In a Transwell system, cells are exposed to medium on their apical and basolateral surface. Transcytosis of P34 can be measured by its detection in the basolateral compartment following its application to the apical surface of a tight monolayer of IPEC-J2 cells. For the experiment, IPEC-J2 (50 000) were seeded on collagen (Type 1)-coated Transwell inserts, twelve-well format, pore size 0·4 μm (Corning Life Sciences). After reaching confluence (usually 1 d later), the cells were treated with serum-free medium for 8 d. IPEC-J2 cells usually reach a maximal transepithelial electrical resistance (TEER) after 8 d[21]. On day 9 (8 d in serum-free medium), different concentrations of P34 were added to the apical compartment for 3 h. Since the detection of P34 in the basolateral compartment with the monoclonal antibody F5 was highly specific, the 77 % enriched P34 solution was used. After the incubation, the medium collected from the basolateral compartment was precipitated with TCA and loaded on an SDS-PAGE gel. After electrophoresis, proteins were transferred to a blotting membrane, and P34 was detected with antibody F5 as previously described. TEER-measurement was performed after the experiment with a Millicell-ERS Volt-Ohm Meter of Millipore (Schwalbach) to assess epithelial integrity.

Detection of P34 antibodies in the sera of un-suckled newborn piglets and adult pigs

The ninety-six-well plates (Maxisorp; Nunc) were coated with purified P34 (99 % enriched solution) overnight at 4°C (10 μg P34/ml, 100 μl/well). Next day the wells were washed with PBS and blocked with goat serum for 1 h at room temperature (dilution 1:100 in PBS, 100 μl/well; Dianova). After the next washing step with PBST (phosphate-buffered saline plus 0·05 vol.% Tween20), pig sera (*n* 3) were added for 2 h at 37°C in three different dilutions (each 100 μl/well). The secondary peroxidase-labelled antibody (goat anti-swine IgG, Jackson Immunoresearch, dilution 1:10 000) was incubated after a further washing step for 1 h at room temperature. Finally, antibody binding was visualised using *O*-phenylene diamine following the manufacturer's instructions (Sigma*Fast*™ OPD Sigma-Aldrich). Plates were read in a CM Sunrise Microplate Reader (Tecan) at 450 nm. Sera of un-suckled newborn Landrace piglets were generously

provided by FBN Dummerstorf. Sera of conventionally fed adult Land Race pigs were generously provided by IMTM GmbH. The diet for the adult pigs (K849/08 deuka SFT grainy, Deutsche Tiernahrung GmbH&C *ad libitum*) contained soyabean meal (obtained after solvent extraction of oil from soyabean flakes) and soya oil.

The amount of soyabean meal in the diet was about 15 g/kg feed, which contained about 46–47·5 % soya protein (approximately 7 g/kg feed; manufacturer's specifications). According to Kalinski *et al.*[22], the content of P34 in soyabean protein is about 2–3 %. Therefore, the estimated final concentration of P34 in the diet was calculated as 1·38–2·14 mg/kg feed.

Statistical analysis

Data are expressed as single and mean values with their standard errors. Significance between values was assessed by paired Student's t test. Values of $P<0·05$ were considered statistically significant.

Results

P34 partially resists in vitro *proteolysis*

To investigate whether P34 may escape degradation by digestive enzymes in the gastrointestinal tract, we subjected ground, unprocessed soyabeans to an *in vitro* proteolysis protocol and then analysed the resulting protein solutions for the presence of non-degraded P34. Intact, non-degraded P34 was detected in all preparations as a 32 kDa band, independent of soyabean pre-treatment (soaked or dry) and bile salt supplementation (Fig. 1). With the monoclonal antibody F5, only intact P34 (Fig. 1, left panel) was detected; whereas with the polyclonal antibody serum (anti-P34), several additional protein bands could be visualised (Fig. 1, right panel). A large fragment of P34 with a size of about 20 kDa, probably corresponding to a P34 fragment, and a 40–55 kDa band, which possibly represented the pro-P34 polypeptide (approximately 47 kDa) or a P34 dimer (approximately 53 kDa), were detected. The band patterns were largely unaffected by the *in vitro* proteolysis treatment, and neither soaking of the soyabeans nor the incubation with bile extracts had a striking influence on the detected protein bands. However, the larger 40–55 kDa protein band was less prominent in the preparations if bile extracts were added. Importantly, a percentage of the P34 remained intact after *in vitro* enzymatic treatment. Thus, our data suggest that intact P34 may reach the intestinal epithelial cells.

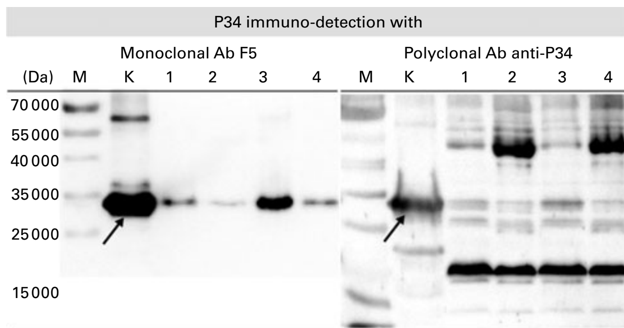

Fig. 1. *In vitro* proteolysis procedure with soyabeans. Ground soaked or dry soyabeans were incubated in solution with pepsin plus pancreatin or with both plus additional bile extract for each 2 h. After centrifugation, supernatants of each mixture were precipitated and separated using SDS-PAGE. P34 was immuno-detected with either P34-binding mAb F5 or a polyclonal antibody serum (anti-P34). In the figure, a blot of a representative experiment is shown: lane M: marker, lane K: control, lane 1: represents P34 in the protein/enzyme solution of dry soyabeans treated without bile extract, lane 2: dry soyabeans treated with bile extract, lane 3: soaked soyabeans treated without and lane 4: soaked soyabeans treated with additional bile extract. The arrows mark the band of P34 at about 32 kDa. Results are representative of two experiments.

P34 binds to the surface of and is endocytosed by IPEC-J2 epithelial cells

To determine whether P34 is internalised by intestinal epithelial cells, IPEC-J2 cells were incubated with P34-FITC and then analysed by flow cytometry, fluorescence microscopy and electron microscopy. As shown in Fig. 2(a), large amounts of FITC-labelled P34 were internalised by IPEC-J2 cells incubated at 39°C. In contrast, the cells incubated at 4°C had much lower mean fluorescence intensity, consistent with a low level of surface-bound protein. Protein uptake by IPEC-J2 cells was not specific to P34, since incubation with BSA-FITC or DQ-ovalbumin™ gave similar results (data not shown).

Endocytosis of P34-FITC by IPEC-J2 cells was confirmed by the microscopic analysis of cytospin preparations (Fig. 2(b)). Two different distribution patterns of P34 within IPEC-J2 cells were visible: either a diffuse FITC signal (cell at the top) or a granular stain with very light FITC spots was detected (cell at the bottom).

P34 was also visualised inside epithelial cells using electron microscopy and F5 antibody labelling. Fig. 2(c)–(e) present examples of intracellular P34, or fragments of P34, distributed either in small aggregates (Fig. 2(c)) or in large vesicles (diameter of about 200–1000 nm, Fig. 2(d) and (e)), similar to the distribution patterns observed using fluorescence microscopy (Fig. 2(b)). The results obtained by flow cytometry and microscopy indicate endocytosis of P34 in IPEC-J2 cells.

P34 is associated with lipid raft microdomains of the plasma membranes and endocytosed via caveolae/lipid rafts

To determine the mechanism of P34 endocytosis, 'detergent-resistant' microdomain isolation was performed to study a possible association of P34 with lipid rafts and caveolae as an alternative mechanism to the classical clathrin-mediated endocytosis. Lysates of IPEC-J2 cells that had been cultured in the presence of P34 were treated with detergent and separated by a sucrose-density-gradient centrifugation. The detergent treatment disrupts all cell membrane domains other than lipid rafts and caveolae. After the centrifugation, two rings were seen (R1 and R2, Fig. 3(a)). The rings, the intermediate phase (I) as well as eight additional fractions

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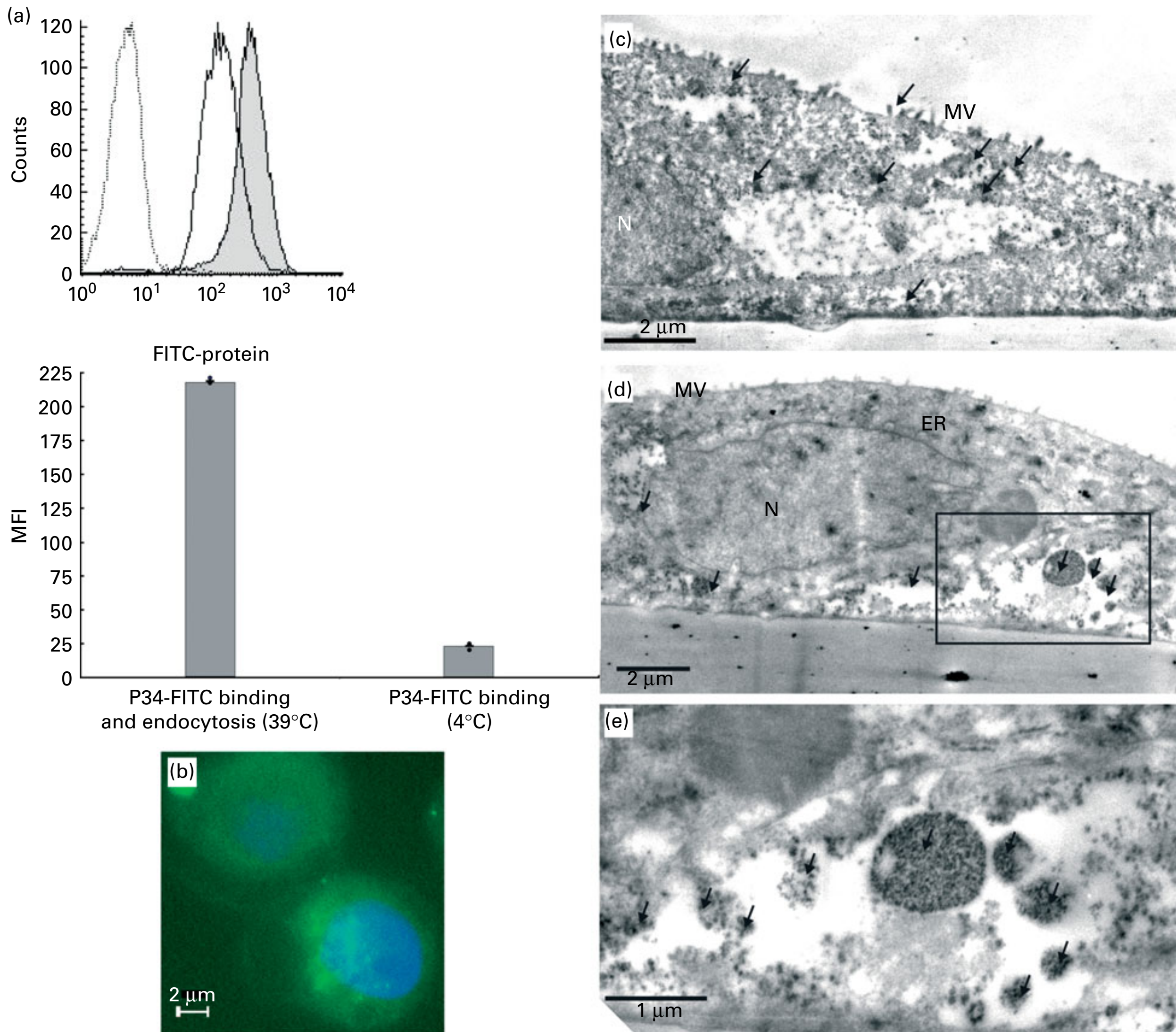

Fig. 2. Endocytosis and surface binding of P34 at IPEC-J2 cells. (a) IPEC-J2 cells were incubated with 150 µg/ml fluorescein isothiocyanate (FITC)-labelled P34 at 39°C (▭) and 4°C (▭) for 2 h. Afterwards, cells were trypsinised and analysed using flow cytometry. The histogram shows cells incubated with no P34-FITC (⬚) and cells incubated with P34-FITC at 4 or 39°C. Below, mean fluorescence intensity (MFI) values from one representative experiment (out of three experiments) are shown. Mean values were represented by vertical bars, single data points (*n* 3 in every experiment) are represented as dots. Background MFI was 10·7. (b) Cytospin preparations of IPEC-J2 cells with internalised P34-FITC: the cell nuclei are stained with 4′,6-diamidino-2-phenylindolole (DAPI) (blue); inside the cell P34-FITC signals can be seen (green). Bar = 2 µm. (c–e) Electron microscopy of intracellular P34: IPEC-J2 cells, grown on Transwell filters (pore size = 1 µm), were incubated with P34 and immunostained using immunohistochemistry for P34 (mAb F5 and a peroxidase conjugate using diaminobenzidin as substrate, black immunoreactivity, arrows). (c, d) Bar = 2 µm and (e) bar = 1 µm. (e) Magnification of the marked area in (d). MV, microvilli; ER, endoplasmatic reticulum; N, nucleus.

(F1–F8) were collected (Fig. 3(a) and (b)). Collected fractions were separated using SDS-PAGE and blotted. The marker proteins clathrin (here as 'raft negative protein'), caveolin-1 (caveolae), flotillin (planar lipid rafts) as well as P34 were immuno-detected on the blotting membranes (Fig. 3(b)).

P34 was predominantly detected in fractions F1 and F2 in this representative experiment. Caveolin-1 and flotillin were detected in fractions F1 and R2. Caveolin-1, flotillin and P34 were visualised together in fraction F1 (Fig. 3(b)). Clathrin was found in the lower fractions F5–F8 together with all soluble cellular components including the majority of intracellular and membrane proteins. These fractions contained only small amounts of P34. This implies that the major proportion of protein P34 was bound to isolated lipid complexes and floated up.

The findings indicate that P34 associates with caveolae/lipid raft sub-domains and that caveolae contribute to P34 uptake in IPEC-J2 cells.

To verify the results of the preceding experiments, the plasma membrane of IPEC-J2 cells was treated with MβCD before and during P34-FITC incubation to disrupt lipid raft domains. Thereafter, intracellular FITC-signals were measured using flow cytometry. As shown in Fig. 3(c), treatment of IPEC-J2 cells with MβCD significantly inhibited uptake (39°C) and binding (4°C) of P34. P34 endocytosis in IPEC-J2 cells was significantly inhibited ($P < 0·0001$) to 44 %, and binding of P34 at the

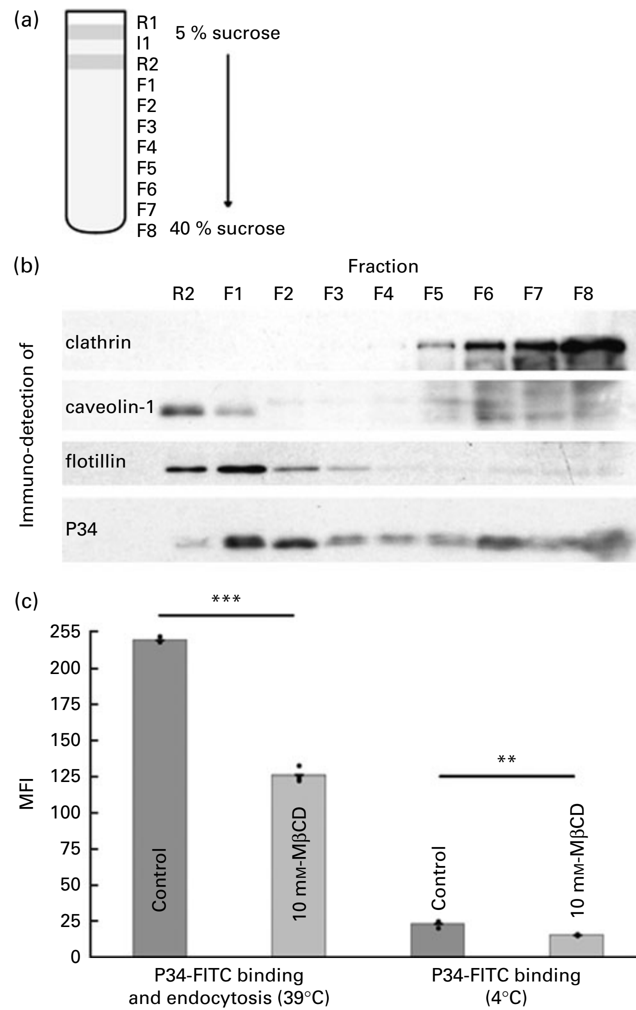

Fig. 3. P34 association to lipid raft fractions and P34 endocytosis inhibition by methyl-β-cyclodextrin (MβCD). (a, b) Lipid raft isolation and P34 association: high-buoyancy detergent-resistant membrane fractions (lipid rafts and caveolae) of P34 incubated IPEC-J2 cells were isolated and separated using a sucrose density gradient ultra centrifugation. The ring patterns and fractions which develop during centrifugation can be seen in (a). (b) Immunoblots for every fraction illustrated in (a) on which clathrin, caveolin-1, flotillin and P34 were immuno-detected. (c) MβCD inhibits P34 endocytosis: mean fluorescence intensity (MFI) measured using flow cytometry of IPEC-J2 cells treated with P34-fluorescein isothiocyanate (FITC) and MβCD before and during protein incubation at 4 and 39°C is shown. The three single values are shown as dots; the mean values are shown as vertical bars. Background MFI was 10·7. Results are given of one representative experiment (out of three). Mean values were significantly different: $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$, Student's *t* test.

IPEC-J2 cell surface was significantly reduced ($P<0{\cdot}01$) to about 33 % of the initial value after the MβCD treatment. These results suggest that the integrity of lipid raft microdomains/caveolae was necessary for P34 uptake by enterocytes.

P34 is transported through IPEC-J2 cells

To analyse whether P34 is transcytosed through IPEC-J2 cells, different amounts of protein P34 were added to the apical surface of a confluent monolayer of IPEC-J2 cells grown in a Transwell system. After 3 h, proteins in the basolateral compartments were recovered, precipitated, separated using SDS-PAGE and blotted, and P34 was detected by Immunoblot (Fig. 4). Integrity of the epithelial monolayer was confirmed by analysis of TEER values, which were 2493 (SEM 463) $\Omega \times cm^2$ after P34 treatment, and 2458 (SEM 773) $\Omega \times cm^2$ in control cultures. These values were comparable to those measured by other researchers[13,23].

Using mAb F5, P34 was detected as an intact protein in the basolateral compartment (Fig. 4), suggesting that P34 was transported through IPEC-J2 cells.

P34 antibodies are present in porcine sera

P34-binding antibodies were measured in the sera of conventionally reared adult pigs fed a diet containing soyabeans and in the sera of neonatal, un-suckled piglets by ELISA. Fig. 5 shows the absorbance values of one representative experiment. All tested sera and serum dilutions obtained from adult pigs fed a diet with soya contained antibodies to P34 (Fig. 5, right side). In contrast, no P34-binding antibodies were detected in the sera of newborn, un-suckled piglets (Fig. 5, left side), indicating that serum antibodies to P34 were associated with dietary exposure to soya protein.

Discussion

The monomeric glycoprotein P34 is the main soyabean allergen in soyabean-sensitive patients. To elucidate the mechanisms of oral allergy induction by P34, we here used *in vitro* proteolysis and analysed P34 uptake in intestinal epithelial cells in order to determine whether intact P34 can access the sub-epithelial lamina propria and thus allergy-inducing immune cells in the intestine.

Our data suggest that P34 may partially resist digestion in the gastrointestinal tract, since intact P34 was detected after proteolysis *in vitro*. Although not a defining characteristic of dietary allergens[24], proteins resisting a proteolytic digestion and the acidic pH-value of the stomach have a higher probability of stimulating immune responses[25,26]. Allergenic P34 that remains intact after exposure to gastric acid and digestive enzymes is available for transport through the intestinal barrier and thus allergy induction.

Possibly, the glycosylation level of the P34 contributed to its increased resistance to proteolytic degradation[27]. Protein solubility is another factor that determines the efficiency of proteolytic degradation. P34 is a protein of low solubility[2]

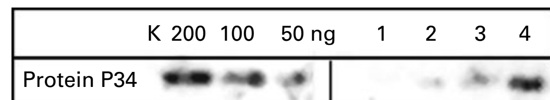

Fig. 4. Transcytosis of P34 through a monolayer of IPEC-J2 cells. IPEC-J2 cells were grown in a Transwell system until they had reached confluence. Then, different concentrations of P34 (50, 250, 500 and 1000 µg/ml; 77 % purity) were added to the apical compartment of the Transwell for 3 h. After that, protein in the supernatants of the basolateral compartments were precipitated, separated using SDS-PAGE, transferred on blotting membranes and P34 was immuno-detected. The Immunoblot shows the transcytosed P34 detected with the monoclonal antibody F5 (lanes 1–4). As control (K), P34 was loaded at the following concentrations: 200, 100, 50 ng/lane.

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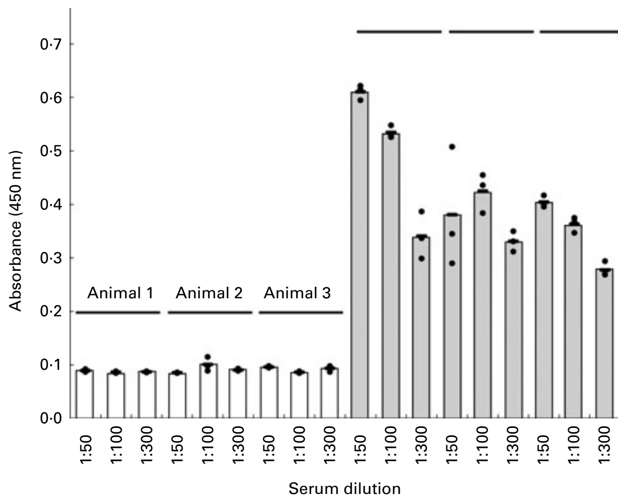

Fig. 5. P34 binding antibodies in the sera of pigs. The sera of two different pig groups were tested in different dilutions for the presence of P34-binding antibodies in an ELISA system. The results (absorbance values) of a representative experiment are shown here with triplicates of each sera and dilution. Sera on the left side of the graph (□) were taken from three different newborn, un-suckled piglets. The sera of the right side of the graph (▨) were obtained from three conventionally kept adult pigs fed a diet containing soya. Each sample is given as a single point and the mean values are given as bars. This experiment was repeated with similar results.

that usually associates with the oil bodies of disrupted soyabean cells (soyabean oil bodies)[7]. In the digestive tract, biliary acids act as emulsifiers to improve the solubility of dietary components and enhance protein proteolysis[28]. Here, the addition of bile extracts to the soya preparations did not result in complete digestion of P34. However, the absence of bile extracts improved the recovery of a soyabean protein of higher molecular weight (between 40 and 55 kDa), which may correspond to either the native unprocessed P34 protein with the pre- and pro- region still attached (approximately 47 kDa)[7] or a dimer of P34 (approximately 53 kDa) which may have been present, although SDS-PAGE gels were run in the presence of β-mercaptoethanol[29].

Interestingly, in earlier *in vitro* proteolysis studies, P34 fragments were detectable only for 8 min, indicating that P34 was not resistant to treatment with pepsin solution[24,30]. However, these studies used a purified P34 solution as a substrate, whereas we used ground, untreated soyabeans for our *in vitro* proteolysis experiments to closely model the natural dietary source of P34. Since the dietary matrix has an impact on protein digestibility[31], these differences in the experimental protocol may account for the discrepancy in the results.

In addition to the intact P34, fragments of P34 were detected after *in vitro* proteolysis using a polyclonal antibody. The most abundant fragments recovered were about 20 kDa and about 25–30 kDa. As also previously shown, the monoclonal antibody F5 detected only intact P34[32]. This antibody recognises the ^{121}GYETLI^{126} epitope, which contains a tyrosine residue as a predicted pepsin restriction site and which is therefore possibly lost during *in vitro* proteolysis[33]. Notably, our *in vitro* proteolysis protocol may have yielded additional smaller fragments of P34 (<20 kDa) that were not recovered by the TCA precipitation protocol and that thus remained undetected. However, the focus of the present study was on the intact protein rather than protein fragments. Thus, no attempt was made to identify possible P34 protein fragments in any of the experiments.

Having shown that a proportion of P34 resists *in vitro* proteolysis, we next studied binding, uptake and transport of P34 by small-intestinal epithelial cells (IPEC-J2). Our data suggest that P34 binds to the cell surface of IPEC-J2 cells, is taken up by a caveolae-dependent mechanism, and is released into the basolateral compartment. Using flow cytometry, we showed that P34 binds to the enterocyte surface at 4°C, which may be facilitated by the high glycosylation level of P34[8,9]. Exploratory binding assays in which purified P34 and selected monosaccharides of the P34 glycosylation were co-incubated on the surface of an enterocyte monolayer suggested that fucose may be involved in enterocyte surface binding (E Sewekow, unpublished results). After incubation at 39°C, P34 protein was visualised inside the epithelial cells in vesicle-like structures and aggregates by microscopy. Using density gradient-based centrifugation to isolate lipid rafts, we revealed, for the first time, an association of P34 with lipid raft microdomains detected by the presence of both caveolin-1 and flotillin in the same fraction. Caveolin-1 stabilises the plasma membrane association of caveolae[34], and flotillin is known as a component of morphologically defined caveolae[35]. The presence of caveolin-1 and flotillin in the upper fractions of the density gradient indicates an effective separation of the lipid raft membrane sub-domains. Importantly, P34 was associated with smaller caveolae aggregates consistent with un-fused single caveolae that contribute to cellular transcytosis (fractions F1 and F2 in Fig. 3), rather than with larger high-buoyancy aggregates formed by multiple fusion of caveolae (fraction R2). The presence of P34 in lower fractions after the density gradient-based centrifugation was probably due to its partial liberation from lipid raft domains during sample preparation.

The association of P34 with lipid rafts/caveolae suggests an involvement of those membrane sub-domains in P34 endocytosis and transport. Caveolae-mediated endocytosis can be distinguished from clathrin-dependent endocytosis and pinocytosis because of its sensitivity towards reagents like nystatin, filipin and MβCD, which disrupt caveolae by depleting cholesterol from the cell membranes[36–39]. Here, we show that IPEC-J2 cells treated with MβCD bound and endocytosed significantly less FITC-labelled P34, confirming our hypothesis that caveolae are involved in epithelial uptake of P34.

Previous studies have shown transcytosis of intact and also of biologically active proteins through epithelial cell layers[32,40–42]. While the major proportion of any endocytosed protein is degraded within the cell, a small proportion of intact protein may be released into the basolateral compartment[43]. In IPEC-J2 cells, transcytosis of *Escherichia coli* F4 fimbriae by a clathrin-mediated mechanism has recently been demonstrated[44]. Our results indicate that P34 is transcytosed through intact monolayers of IPEC-J2 cells. Although we cannot completely exclude that some paracellular protein leakage occurred in addition to protein transcytosis, the high TEER measured was consistent with a tight monolayer.

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Thus, our present study confirms earlier observations of P34 transport through human epithelial colorectal adenocarcinoma cells (Caco-2)[32].

The detection of P34-binding IgG antibodies in the sera of pigs fed a diet containing soya supports the hypothesis that transepithelial transport of intact P34 also occurs *in vivo*, since a systemic immune response requires the presence of antigen at systemic sites. In mice fed high concentrations of purified P34, intact protein and peptide fragments were detected in plasma samples[45]. Because of a low concentration of P34 in conventional pig feed ($1{\cdot}38{-}2{\cdot}14 \times 10^{-4}$ wt%), we did not attempt to detect P34 protein in porcine serum samples. Notably, clinical soya hypersensitivity occurs spontaneously in swine, but exclusively in piglets after weaning[46]. Thus, P34 transport may preferentially occur at a certain stage of epithelial development after birth, i.e. in a certain age group, consistent with the preferential induction of food allergies during early childhood. The fact that pigs naturally develop symptoms of soya hypersensitivity as well as antibodies to P34 and our results showing transepithelial transport of intact P34 in porcine enterocytes implicate the pig as a potentially useful model animal for studies of soya allergy.

In summary, we have in this study characterised one potential pathway for the transport of intact soyabean allergen P34 from the diet to systemic immune cells. Our observations that P34 resists *in vitro* proteolysis, is transcytosed via a caveolae-dependent mechanism, and initiates a systemic immune response *in vivo* may also apply to the transport of other important allergens of close sequence homology with P34, the peanut allergen Ara h1 and the cows' milk allergen 2-S1-casein[2].

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