Mechanisms of epithelial translocation of the α_2-gliadin-33mer in coeliac sprue

M Schumann,[1] J F Richter,[2] I Wedell,[1] V Moos,[1] M Zimmermann-Kordmann,[3] T Schneider,[1] S Daum,[1] M Zeitz,[1] M Fromm,[2] J D Schulzke[1]

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[1] Department of Gastroenterology, Charité, Campus Benjamin Franklin, Berlin, Germany; [2] Institute of Clinical Physiology, Charité, Campus Benjamin Franklin, Berlin, Germany; [3] Institute for Biochemistry und Molecular Biology, Charité, Campus Benjamin Franklin, Berlin, Germany

Correspondence to: Professor Dr Joerg D Schulzke, Klinik für Gastroenterologie, Charité, Campus Benjamin Franklin, 12200 Berlin, Germany; joerg.schulzke@charite.de

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ABSTRACT

Background and aims: The α_2-gliadin-33mer has been shown to be important in the pathogenesis of coeliac disease. We aimed to study mechanisms of its epithelial translocation and processing in respect to transcytotic and paracellular pathways.

Methods: Transepithelial passage of a fluorescence-labelled α_2-gliadin-33mer was studied in Caco-2 cells by using reverse-phase high-performance liquid chromatography, mass spectrometry, confocal laser scanning microscopy (LSM) and fluorescence activated cell sorting (FACS). Endocytosis mechanisms were characterised with rab-GFP constructs transiently transfected into Caco-2 cells and in human duodenal biopsy specimens.

Results: The α_2-gliadin-33mer dose-dependently crossed the epithelial barrier in the apical-to-basal direction. Degradation analysis revealed translocation of the 33mer polypeptide in the uncleaved as well as in the degraded form. Transcellular passage was identified by confocal LSM, inhibitor experiments and FACS. Rab5 but not rab4 or rab7 vesicles were shown to be part of the transcytotic pathway. After pre-incubation with interferon-γ, translocation of the 33mer was increased by 40%. In mucosal biopsies of the duodenum, epithelial 33mer uptake was significantly higher in untreated coeliac disease patients than in healthy controls or coeliac disease patients on a gluten-free diet.

Conclusion: Epithelial translocation of the α_2-gliadin-33mer occurs by transcytosis after partial degradation through a rab5 endocytosis compartment and is regulated by interferon-γ. Uptake of the 33mer is higher in untreated coeliac disease than in controls and coeliac disease patients on a gluten-free diet.

Coeliac disease is an autoimmune disease caused by the ingestion of gliadins in disease-susceptible individuals, where a chronic inflammatory process in the small intestine results in villous atrophy and leads to diarrhoea and symptoms related to malabsorption of nutrients. Susceptibility is partially explained by the HLA haplotype HLA-DQ_2 and -DQ_8 as factors predisposing to coeliac disease.[1] Gliadins are a group of proteins that are ingredients of wheat, barley and rye. After passing the intestinal epithelial border gliadins trigger a T_H1-dependent inflammatory reaction leading to the destruction of villus structure in the duodenal mucosa. Recently, evidence has been presented that a 33 amino acid fragment of α_2-gliadin fulfils three major criteria for being an important trigger in the inflammatory process:[2] (1) it is resistant to further (intraluminal) intestinal digestion; (2) it is a substrate of the tissue transglutaminase (tTG), the major autoantigen of coeliac disease; and (3) after deamidation by tTG, the 33mer sequence contains three previously described coeliac disease-specific T cell epitopes. Functional assays revealed that the 33mer directly activates gut-derived HLA DQ_2-restricted T-cell clones of coeliac disease patients.

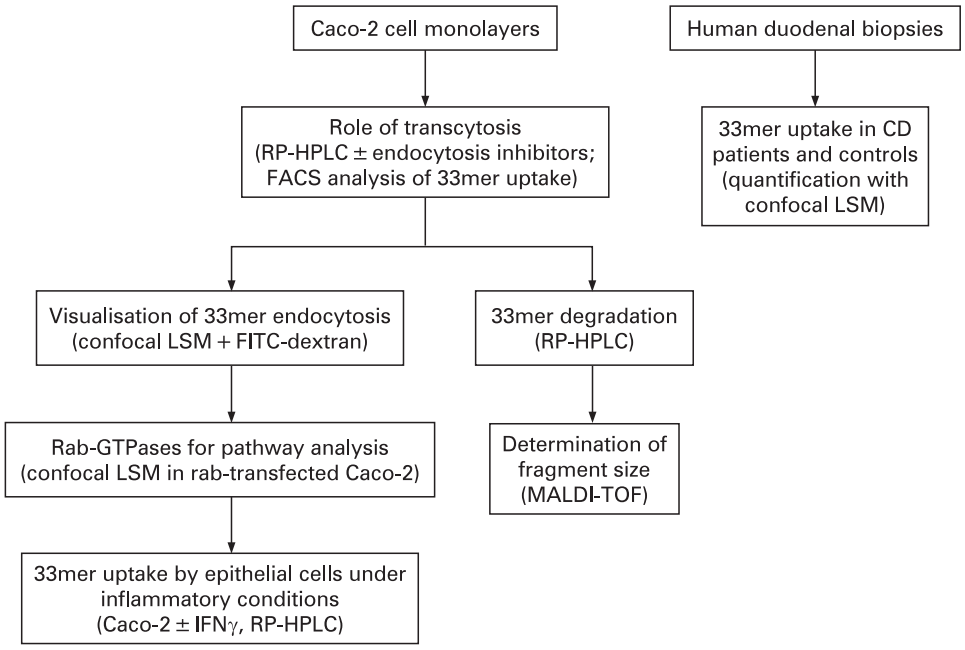
However, up to now it is not clear how the 33mer crosses the epithelial barrier of the intestine. Furthermore, it is not known whether or not the epithelium plays a role in processing the 33mer. Research on the role of the adaptive immune system and its genetic background revealed that changes within this system are important for the pathogenesis of coeliac disease but are not the only cause for development of the disease.[3] Recent research focussed on processes in enterocytes being the first steps towards a breakdown of the epithelial barrier in the disease.[4] Therefore, it seems justified to look for additional mechanisms to explain the pathogenesis of coeliac disease. A recent study on sprue patients revealed a higher transepithelial translocation of the intact 33mer in active coeliac disease compared to healthy control subjects as well as an incomplete degradation of the 33mer during intestinal transport.[5] These data seem to suggest that intraepithelial degradation of the 33mer is an important step, indicating that the transcellular route could be important for the translocation of the 33mer through the epithelial barrier. Therefore, we performed experiments to analyse potential mechanisms of transepithelial translocation in a cell culture-based epithelial model as well as in coeliac disease specimens. Using a fluorescently labelled α_2-gliadin-33mer we present evidence that polypeptide transcytosis by epithelial cells significantly contributes to the translocation of either the intact or partially degraded 33mer peptide and that uptake is stimulated by interferon-γ (IFN-γ).

METHODS

Materials

Minimal essential medium (MEM) plus Glutamax for Caco-2 cells, fetal bovine serum (FBS), antibiotics, trypsin/ethylenediamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) were from Gibco/Invitrogen (Karlsruhe, Germany). Complete protease inhibitor tablets and Fugene 6 transfection reagent were from Roche Diagnostics (Mannheim, Germany). Mount Fluor ProTaqs mounting medium was from Biocyc (Luckenwalde, Germany). IFN-γ was from PeproTech Inc. (Rocky Hill, NJ, USA). All other chemicals were from Sigma–Aldrich (Schnelldorf, Germany).

Figure 1 The experimental plan.



Cell culture and transwell system

The human colon carcinoma cell line Caco-2, which is known to resemble enterocytes in their transport function,[6 7] was from ATCC/LGC Promochem (Wesel, Germany) and was grown on PCF transwell filters (0.4 μm pore size; Millipore, Schwalbach, Germany) in media containing 10% fetal calf serum plus antibiotics. After a 2 week differentiation period PCF transwell filters were transferred from 6 cm dishes to 24 wells. For the 33mer translocation assay Cy^3-33mer (1 μmol/l) was added apically to serum-free media. Samples from apical and basal compartments were collected for reverse-phase high-performance liquid chromatography (RP-HPLC) after different 33mer incubation periods. The integrity of the Caco-2 cell monolayers was checked in parallel by measuring transepithelial resistance (R^t) in the same set of cells. R^t was determined in the culture dishes as described previously.[8]

Patients and healthy controls

We recruited three patient groups: (1) healthy controls without any clinical symptoms typical of coeliac disease at the time of the biopsy, a negative tissue transglutaminase IgA serology and a normal duodenal mucosal architecture under a normal (gluten-containing) diet; (2) coeliac disease patients who were under a gluten-containing diet who had a partial villous atrophy on duodenal biopsy, a positive tissue transglutaminase IgA serology (except one IgA deficient coeliac disease patient) and a positive HLA-DQ2 test; and (3) coeliac disease patients who were under a gluten-free diet. Written consent was obtained from all patients in our study.

Synthesis of the Cy^3-labelled α_2-gliadin-33mer

Synthesis of the 33 amino acid α_2-gliadin peptide (amino acids 56–89 of α_2-gliadin, LQLQPFPQPQLPYPQPQLPYPQPQPF; MW 3908) was performed using a solid phase approach (Fmoc/Bu^t strategy) at Biosyntan (Berlin, Germany). Subsequent N-terminal labelling of the 33mer polypeptide with the Cy^3 dye (Amersham Biosciences, Munich, Germany) was done according to the manufacturer's protocol. The labelled 33mer peptide was purified by using RP-HPLC. The identity of the peptide was verified by using matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF) yielding a molecular mass of the unlabelled and the Cy^3-labelled peptide of 3909 and 4525 Da, respectively.

Analysis by RP-HPLC

Reverse-phase HPLC was performed to analyse translocated Cy^3-labelled α_2-gliadin-33mer. Separation according to hydrophobicity was achieved with a RP18 HPLC column and a Shimadzu HPLC system with a detector for fluorescent light according to the Methods section in the Supplemental material.

Mass spectrometry

The basal compartment of 33mer-treated Caco-2 cells grown on PCF filters was analysed by preparative RP-HPLC and MALDI-TOF according to the Methods section in the Supplemental material. MALDI spectra of the 33mer and its fragments were evaluated taking the sequence of the native peptide and the different possible adducts (H^+, Na^+ or K^+) into account.

Flow cytometric analysis

Confluent Caco-2 cells on 12 wells were incubated with Cy^3-33mer (5 μmol/l) and bovine serum albumin (0.1%) for 40 min at 37°C and 4°C, respectively. Trypsinisation was stopped with FBS-containing medium. Cells were centrifuged (150 *g*, 5 min, 4°C) and resuspended in PBS plus EDTA (10 mmol/l). Fluorescence-activated cell sorting (FACS) was done using a FACS–Calibur Flow Cytometer (BD Biosciences, Heidelberg, Germany), collected, and analysed with CellQuest software (BD Biosciences). Gates were set on a sideward scatter/forward scatter (SSC/FSC) dot blot. At least 50 000 cells were analysed for Cy^3 fluorescence.

Confocal laser scanning microscopy

Co-localisation studies with fluorescein isothiocynate (FITC)–dextran (MW 4400 Da) and Cy^3-33mer added to the apical compartment of filter-grown Caco-2 cells were carried out as described in the Methods section of the Supplemental material.

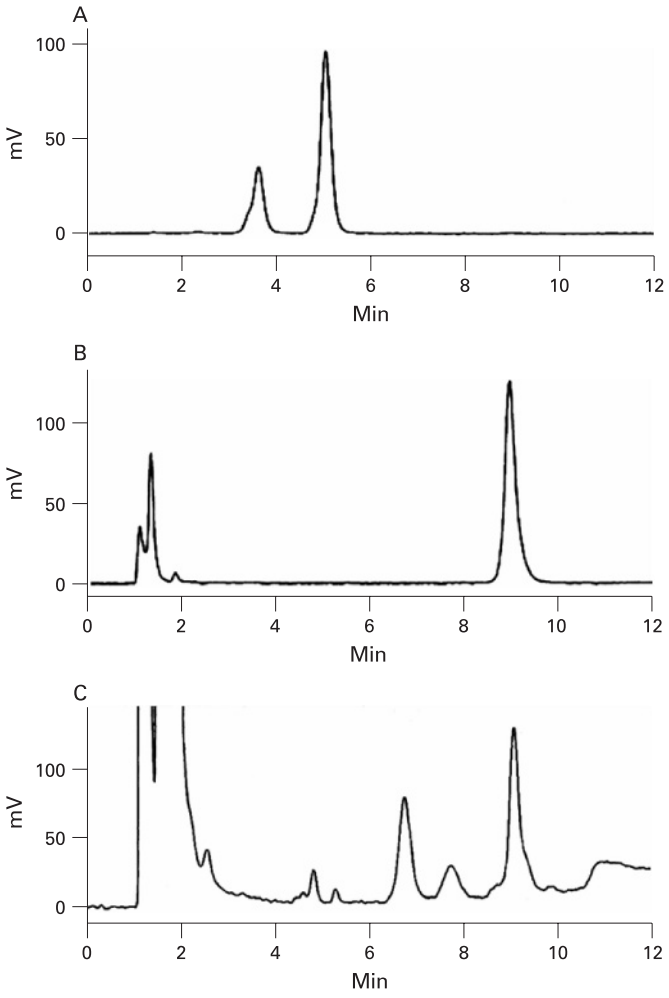


Figure 2 Reverse-phase high-performance liquid chromatography (RP-HPLC) of the Cy^3-33mer and the Cy^3-NHS dye. (A) RP-HPLC of the Cy^3-NHS dye: retention time, R_t = 3.6 and 5.1 min. (B) RP-HPLC of the Cy^3-33mer, R_t = 9.0 min. (C) The RP-HPLC patterns of the 33mer found in the basal compartments of Caco-2 cells grown on PCF filters 6 h after Cy^3-33mer (1 μmol/l) had been added apically.

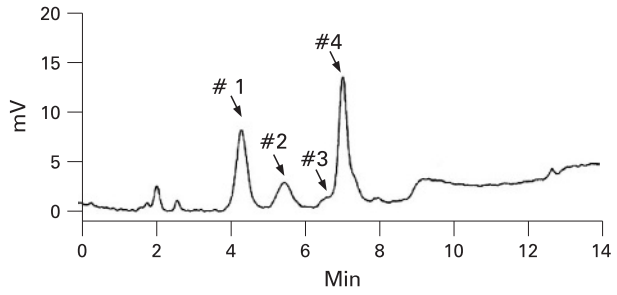
For rab-GTPase co-localisation studies, Caco-2 cells were transfected with rabEGFPC1-DNA. Three days later Cy^3-33mer was added to the apical cell compartment and confocal laser scanning microscopy (LSM) was performed as described in the Methods section of the Supplemental material.

Analysis of human biopsy specimens

Human duodenal biopsy specimens were analysed in miniaturised Ussing chambers as previously described.[9] After luminal addition of Cy^3-33mer, tissues were stained for E-cadherin and quantified for epithelial Cy^3 with the confocal LSM as described in the Methods section of the Supplemental material.

Statistical analysis

Results are expressed as means, with the standard error of the mean (SEM). Statistical analysis was performed using the Student t test and, for the studies on human samples, with the Mann–Whitney U test. $p<0.05$ was considered significant.



HPLC peak no	HPLC R_t (min)	Molecular mass (daltons)	N (amino acids)	Peptide sequence
#1	6	877	2	Cy^3-LQ
#3	8	2219	13	Cy^3-LQLQPFPQPQLPY
#3	8	3318	23	Cy^3-LQLQPFPQPQLPYPQPQLPYPQP
#3	8	3692	26	Cy^3-LQLQPFPQPQLPYPQPQLPYPQPQLP
#4	9	4525	17	Cy^3-LQLQPFPQPQLPYPQPQ
#4	9	4525	19	Cy^3-LQLQPFPQPQLPYPQPQLP
#4	9	4525	33	Cy^3-LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF

Figure 3 Mass spectrometry analysis of the 33mer fragments. After 6 h of Cy^3-33mer incubation the 33mer was partially degraded to several fragments. Fragment size was analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF) and peptide sequence of the 33mer fragments was deduced from the molecular mass of the fragment deciphered by MALDI-TOF.

RESULTS

The aim of this study was to characterise the mechanism for transepithelial passage of the α2-gliadin-33mer. An experimental plan is shown in fig 1.

RP-HPLC of the Cy^3-NHS dye and Cy^3-33mer

To establish HPLC-based separation of the Cy^3-labelled α_2-gliadin-33mer we analysed various concentrations of the polypeptide with RP-HPLC and compared it to the Cy^3-NHS dye alone (fig 2). The R_t of the intact Cy^3-33mer was 9.0 min; RP-HPLC analysis of the Cy^3-NHS dye revealed two peaks at 3.6 and 5.1 min, respectively. MALDI-TOF analysis of the 9.0 min peak identified a compound with a molecular mass of 4525 Da, accounting for the Cy^3-conjugated 33mer (fig 3). The detection limit of the Cy^3-33mer was 0.1 pmol. A linear increase of the 9.0 min peak, quantified as the integrated area under the curve (AUC), was seen with increasing concentrations of Cy^3-33mer. Neither cell media nor bathing solution affected these results.

Intestinal epithelial cells take up Cy^3-33mer

To evaluate whether or not intestinal epithelial cells (IECs) take up the gliadin peptide, Caco-2 cells were incubated in the presence or absence of Cy^3-33mer. Cy^3-positive cells were counted by FACS (fig 4). The distribution of the Caco-2 cells according to the SSC/FSC dot blot appeared normal in granulation and size of the isolated Caco-2 cells. After 40 min of Cy^3-33mer incubation 2.9% of the Caco-2 cell population was Cy^3-positive. The distribution of Cy^3-positive cells in the SSC/FSC dot blot was very similar to the total cell population, excluding a specific subpopulation of IECs to take up the 33mer. The uptake of Cy^3-33mer was temperature sensitive, since incubation at 4°C resulted in a Cy^3-positive cell population similar in size to that of control cells.

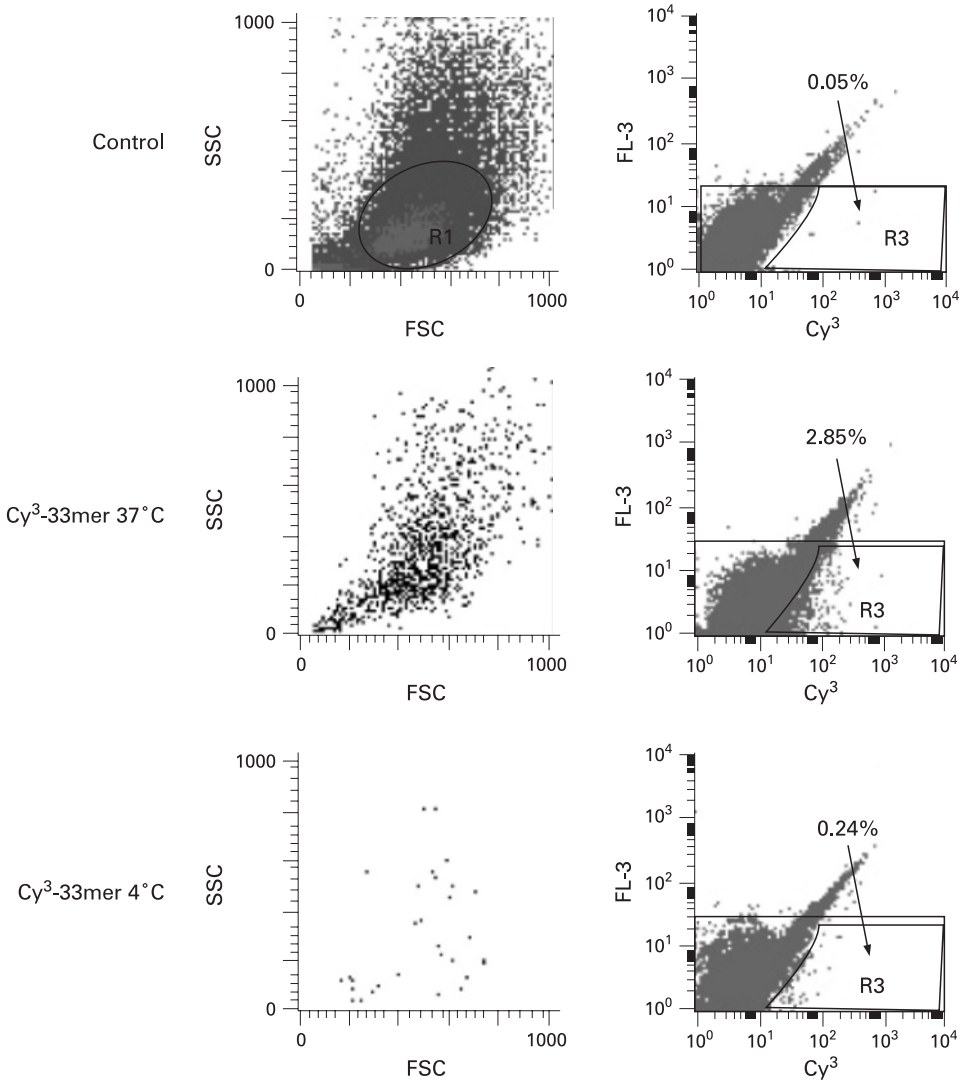


Figure 4 Fluorescence activated cell sorting (FACS) of Caco-2 cells after short-time incubation with the 33mer. Caco-2 cells were incubated with or without Cy^3-33mer, isolated and analysed by FACS. 33mer uptake by Caco-2 cells was active, since the Cy^3-positive cell population was much higher when the gliadin peptide was incubated at 37°C than at 4°C. This was repeated twice in independent experiments giving the same result. SSC, sideward scatter; FSC, forward scatter; FL-3, fluorescent channel for green emission (not used); R3, region 3 represents the population of Cy^3-positive cells.

Kinetics of the translocation of the 33mer and of 33mer fragments

We analysed the translocation of the α_2-gliadin-33mer through the epithelial cell layer of confluent Caco-2 cells growing on PCF transwell filter systems. At different time points after apical addition of the Cy^3-α_2-gliadin-33mer, the basal compartment was analysed by RP-HPLC. A linear increase in the amount of intact 33mer was detected over time (fig 5). Furthermore, the RP-HPLC yielded additional peaks to those from the intact 33mer or the Cy^3 dye (at $R_t = 6.4$, 7.8 and 8.4 min; fig 2C and D). These peaks were found in the basal compartment as early as 6 h after apical addition of Cy^3-33mer.

Quantification of overall 33mer degradation in Caco-2 cells revealed that 64% of the total 33mer added to the apical compartment was degraded by 6 h. In the absence of cells, RP-HPLC analysis showed that the 33mer was stable. To analyse whether the fragmentation of the 33mer was an effect related to intestinal epithelial cells, unpolarised HEK-293 cells were incubated with the Cy^3-33mer. Remarkably, the RP-HPLC showed a completely different HPLC pattern with R_t values of 8.8, 9.3, 10 and 10.5 min (data not shown). In order to further characterise and identify potential 33mer metabolites, eluates of the RP-HPLC were collected at 1-min intervals and mass spectrometry was performed (fig 3). MALDI-TOF revealed that the peak at $R_t = 9$ min contained not only the intact Cy^3-33mer but two more fragments of the 33mer. Also, the "shoulder" of this peak ($R_t = 8.4$ min) included four other fragments of the 33mer.

Transcytosis as a mechanism for 33mer translocation

In order to evaluate transcytosis as a potential mechanism for 33mer translocation from apical to basal, endocytosis was inhibited in Caco-2 cells by pre-incubating the cells with inhibitors of endocytosis, namely methyl-β-cyclo-dextrin and nystatin.[10 11] While the inhibition of endocytosis had no effect on the transepithelial resistance R^t (data not shown), the apical-to-basal translocation of the intact Cy^3-33mer was reduced to 25% (SEM 5%) of control for methyl-β-cyclo-dextrin and to 23% (SEM 6%) of control for nystatin after 6 h of 33mer translocation (fig 6).

Involvement of the small Rab GTPases in transcytosis

The underlying mechanisms of the apical-to-basal transcytosis as an uptake mechanism for polypeptides in intestinal epithelial cells are so far unknown. First, we compared the uptake of the Cy^3-33mer to FITC-labelled dextran (MW 4400 Da), a pinocytosis marker,[12] by co-incubating both substances with confluent

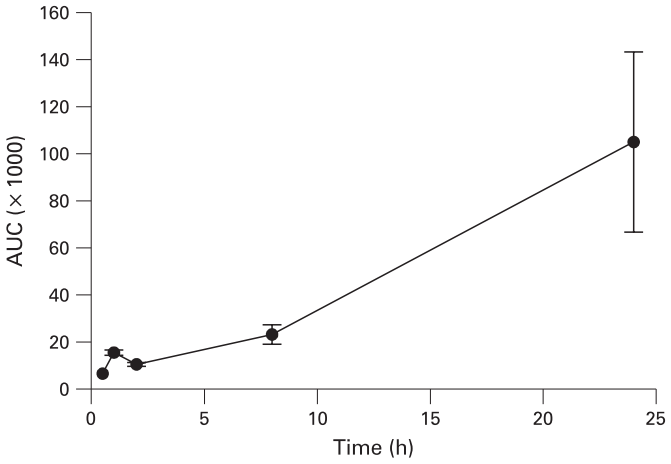


Figure 5 Translocation of the α-gliadin-33mer. Cy^3-33mer was added apically to Caco-2 cells grown on PCF filters and samples from the basal compartment were collected. Reverse-phase high-performance liquid chromatography was carried out as described under Methods. The ordinate indicates the translocated Cy^3-33mer quantified as the integrated area under the curve. Transepithelial resistance was measured in Caco-2 cells as described under Methods and was 362 Ω cm^2. AUC, area under the curve.

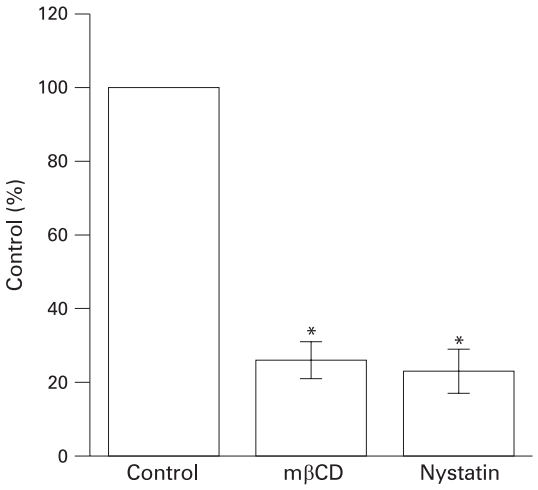


Figure 6 Inhibition of transcytosis of the Cy^3-33mer. Caco-2 cells were grown on PCF transwell filters. For inhibition of endocytosis cells were pre-incubated with methyl-β-cyclo-dextrin (2 mmol/l) or nystatin (10 μg/ml). The Cy^3-33mer (1 μmol/l) was added to the apical compartment of the transwell system. Fluid samples from the basal compartments were collected 6 h later and analysed by RP-HPLC (n = 4, *$p<0.05$).

Caco-2 cells for 30 min at 37°C. Co-localisation of FITC–dextran and Cy^3-33mer was observed (fig 7A).

Since there is evidence for a role of rab-GTPases in non-intestinal epithelial cells,[13] we transiently transfected Caco-2 cells with rab-GFP fusion plasmids and checked for rab-GTPases playing a role in the 33mer uptake by IECs (fig 7B–D). Co-localisation of Cy^3-33mer and rab5-GFP was detected as early as 30 min after 33mer addition. Interestingly, no co-localisation was seen in rab4-GFP and rab7-GFP-transfected cells when the Cy^3-33mer was added (fig 7C,D).

Interferon-γ increases epithelial 33mer translocation

After pre-treatment of Caco-2 cells with IFN-γ (1000 U/ml, 48 h) the epithelial 33mer translocation was increased by about 40% (fig 8).

Epithelial 33mer uptake in human duodenal biopsy specimens

Duodenal biopsies of patients with coeliac disease on either a gluten-containing or a gluten-free diet and healthy controls were obtained during endoscopy and mounted into miniaturised Ussing chambers. Cy^3-labelled 33mer (20 μmol/l, 60 min) was added to the mucosal compartment. After completion of the Ussing experiment the samples were analysed with confocal microscopy to (1) determine the mucosal distribution of the 33mer peptide, and (2) compare the epithelial 33mer uptake of coeliac disease patients with that of healthy controls. In order to discriminate epithelial and non-epithelial 33mer, the samples were counterstained with E-cadherin. Quantification of the epithelial Cy^3 signal was performed with the Zeiss LSM software as explained in the Methods section. The 33mer was found in epithelial cells as well as in the adjacent subepithelium (fig 9A). The intraepithelial 33mer signal resembled endocytotic vesicles (fig 9A, insert). Most important, the mucosal 33mer uptake in coeliac disease patients on a gluten-containing diet was significantly higher than that of both healthy controls and coeliac disease patients on a gluten-free diet (fig 9A–C). Furthermore, no 33mer was detected in crypt cells (fig 9D).

DISCUSSION

Potential pathways for the passage of luminal macromolecules through the epithelial barrier include the paracellular as well as the transcellular route. In coeliac disease, it is known that transepithelial translocation of gliadin peptides is perturbed in patients suffering from active disease.[5] Matysiak-Budnik and co-workers have shown an incomplete degradation of the α_2-gliadin-33mer in patients with active coeliac disease, suggesting but not confirming a role for transcytosis in the uptake of the 33mer.[5] The present study was designed to clarify the mechanisms of the intestinal transepithelial passage of one important gliadin peptide, the α_2-gliadin-33mer.

Caco-2 cells were used since they represent not only a well-characterised cell model for polarised epithelial transport but also show similarities to small intestinal enterocytes.[7 14] First, we have shown that the intact 33mer can pass the epithelial barrier composed of Caco-2 although the transepithelial resistance (R^t) measured was high enough to ensure paracellular tightness.

Second, RP-HPLC analysis detected a degradation of the 33mer, which was as high as 64% after 6 h of 33mer. This degradation was not seen in cell-free 33mer samples and was also different with non-epithelial HEK cells. MALDI-TOF analysis showed that the additional HPLC peaks occurring after incubation with Caco-2 cells represent distinct 33mer fragments. Therefore, we conclude that a considerable portion of the 33mer is digested by epithelial cells, most likely during transcytosis.

As a third important aspect, three independent sets of experiments uncovered endocytosis as an uptake mechanism of the α_2-gliadin-33mer by intestinal epithelial cells. (1) FACS revealed an active uptake of the 33mer, since Cy^3-positive cells were diminished in the 4°C setting. (2) Biochemical inhibition of endocytosis in Caco-2 cells resulted in a 75% decrease in apical-to-basal translocation of the intact 33mer compared to control cells. (3) Confocal LSM of intestinal epithelial cells revealed Cy^3-positive intracellular vesicles that co-localised with the pinocytosis marker FITC–dextran.[12] Taken together, these data establish apical-to-basal transcytosis as a mechanism for

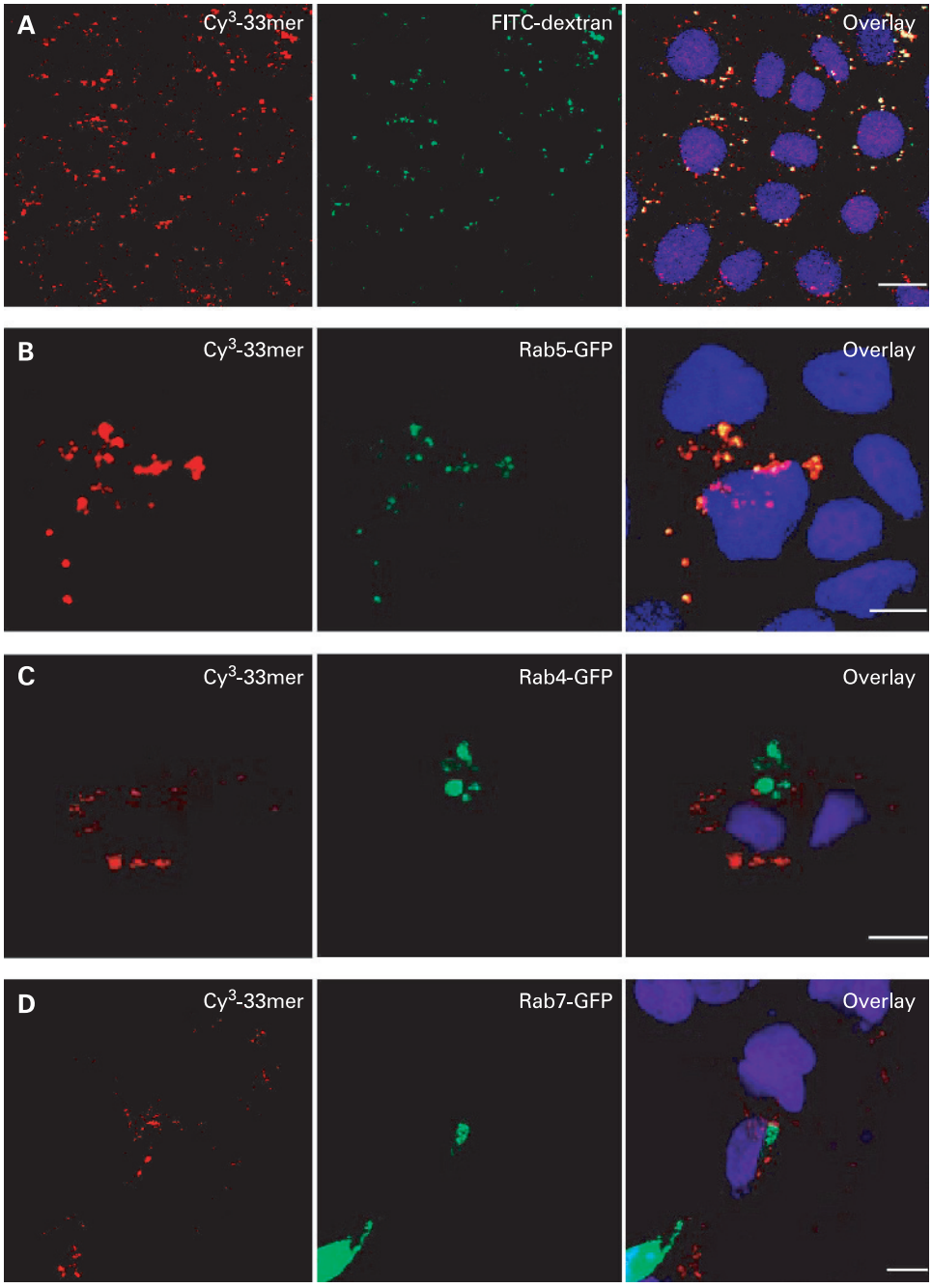


Figure 7 Confocal laser scanning microscopy of Caco-2 cells incubated with Cy³-33mer. (A) Caco-2 cells were incubated with the Cy³-33mer and fluorescein isothiocyanate–dextran (MW 4400 Da) for 30 min at 37°C as described under Methods. These experiments were repeated several times in independent experiments giving the same result. Size bars are 20 μm. (B–D) Caco-2 cells were transfected with rab5- (B), rab4- (C) and rab7-pEGFPC1 constructs (D) as described under Methods. Cy³-33mer was added to transfected cells for 1 h at 37°C. Size bars are 10 μm. Experiments were repeated twice giving the same result.

transepithelial passage of gliadin peptides. This translocation mechanism was verified in the human setting by confocal microscopy of human duodenal biopsy specimens. Using E-cadherin as a marker for epithelial cells and a 33mer quantification strategy based on confocal LSM analysis we showed that the epithelial uptake was about 10-fold higher in coeliac disease patients than in healthy controls. Interestingly, this effect was reversed in coeliac disease patients on a gluten-free diet, implying that the underlying mechanism is secondary to the coeliac disease-associated inflammation that is "turned off" once the inflammation has been treated appropriately.

Lastly, we show that the 33mer translocation increases with IFN-γ, the dominating cytokine in coeliac disease.[15 16] This finding is in accordance with a study by Terpend and co-workers[17] who proposed an increase of epithelial polypeptide translocation with IFN-γ.

Transcytosis as an intestinal uptake mechanism of macromolecules has already been suggested by a number of studies.[18 19] However, so far, direct experimental evidence for endocytosis playing a pivotal role in gliadin uptake in coeliac disease has been rather limited. Zimmer and co-workers[20 21] performed two studies based on electron microscopy of coeliac disease specimens, where gliadin was found in intracellular compartments of enterocytes. Friis and co-workers[22] documented the uptake of pepsin/trypsin-digested gliadin by human enterocytes in a small group of coeliac disease patients. The underlying mechanism of apical-to-basal transcytosis in epithelial cells, however, remained largely unknown. By using electron microscopy, gliadin seemed to be localised to late endosomes or lysosomes of jejunal enterocytes in coeliac disease patients.[20 21] In the present study we used confocal laser scanning microscopy to analyse the intracellular route in more detail and found

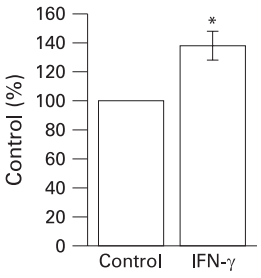


Figure 8 Effect of interferon-γ (IFN-γ) on epithelial translocation of the 33mer. Caco-2 cells were pre-incubated with or without IFN-γ (1000 U/ml) for 48 h. 33mer translocation was analysed with reverse-phase high-performance liquid chromatography as described under methods (n = 6, *p<0.05).

co-localisation of the 33mer with rab5-GFP after 60 min of 33mer incubation. Rabs are small GTPases that have distinct functions in the regulation of intracellular vesicular trafficking.[23] Consistent with the idea of rab5 having a role in the early steps of transcytosis, it is localised to the apical as well as the basal early endosome in polarised cells, where it regulates the fusion of clathrin-coated pits with early endosomes.[24 25] In contrast ab4 and rab7, which localise to recycling endosomes and late endosomes,[23] did not show any co-localisation with the 33mer, although the cells were analysed up to 4 h after apical addition of the gliadin peptide.

Nevertheless, one should allude to the paracellular pathway as a second transepithelial passage route. The main paracellular barrier is formed by the tight junctions (TJs).[26] In general, it is assumed that under physiological conditions macromolecules in the kilodalton range cannot pass the TJ barrier, as already shown using ionic lanthanum as an electron dense marker.[27] The TJ permeability for macromolecules increases in a number of situations, including mucosal inflammation.[8 19 28] Previous data from our own group as well as others have revealed a reduced transepithelial electrical resistance in active coeliac disease. This points to a contribution of the paracellular barrier defect in the small intestine, since endocytosis/transcytosis is an electrically silent phenomenon. Further direct support came from freeze–fracture electron microscopy studies, in which a reduced number of horizontally oriented TJ strands was observed in the duodenal mucosa of coeliac disease patients and from the identification of zonulin as a modifier of TJs.[29–33]

On the basis of our present findings and those in the literature, mucosal uptake of antigens like the α_2-gliadin-33mer most likely occurs in untreated coeliac disease via both the transcellular and the paracellular pathways. However, it is possible that, initially, transcytosis is the major route of antigen entry into the subepithelial compartment. On the other hand, it also appears possible that minimal TJ alterations represent initial paracellular leaks in this respect.[33]

Clearly, as shown in this study as well as by others, the antigen uptake into the inflamed mucosa is markedly increased.[34] In analogy to the application of endopeptidases as luminal therapeutic tools that degrade toxic gliadin sequences, drugs that modify the epithelial uptake of gliadins and their fragments might be a future therapeutic implication for coeliac disease as well. Finally, endocytotic uptake of peptides is a *conditio sine qua non* along the hypothesis of intestinal epithelial cells functioning as non-professional antigen-presenting cells

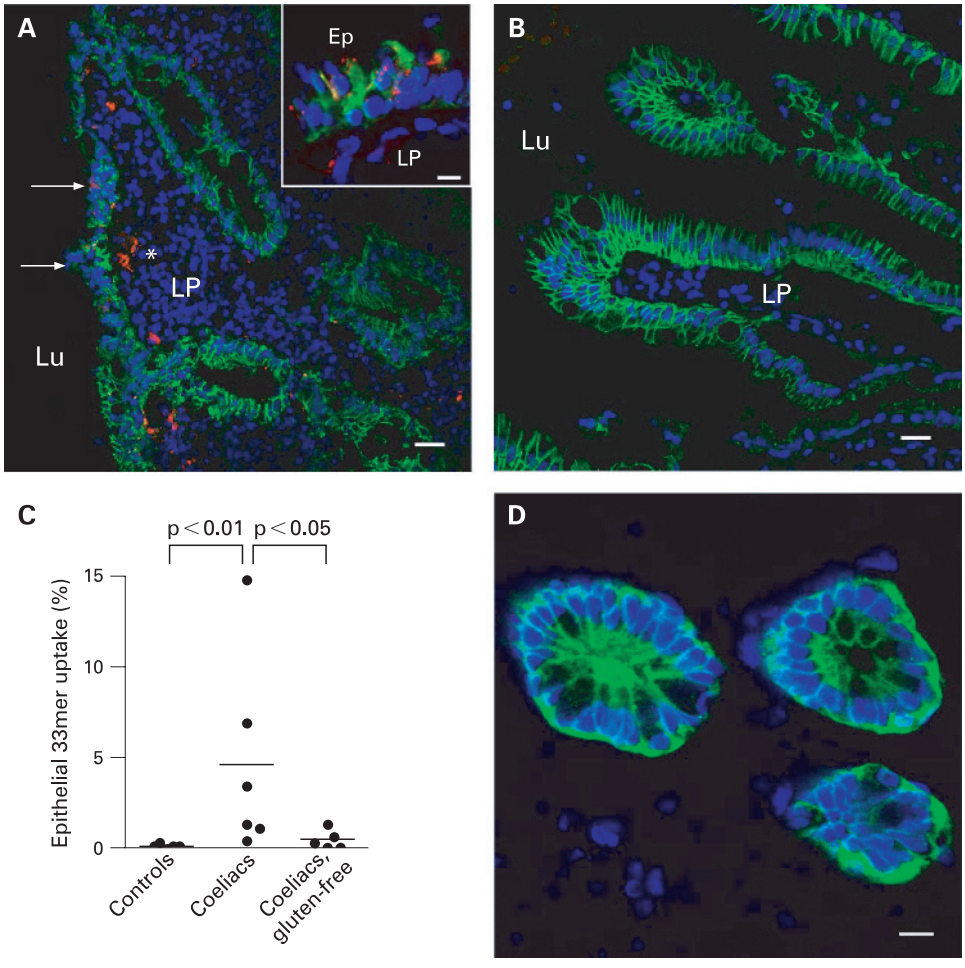


Figure 9 Distribution of the 33mer in the duodenum of coeliac disease patients. Duodenal biopsy specimens of healthy controls (n = 5), patients with coeliac disease on a gluten-containing diet (n = 6) and coeliac disease patients on a gluten-free diet (n = 5) were analysed within the miniaturised Ussing chamber as described under Methods. Cy^3-33mer (20 μmol/l, red fluorescence) was added to the mucosal compartment of the Ussing chamber. After 1 h, the biopsy specimens were cryoconserved, stained with E-cadherin (green fluorescence) and 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) (nuclei in blue) and analysed with confocal laser scanning microscopy (LSM). (A) In duodenal mucosa of coeliac disease patients, the Cy^3-33mer was detected intraepithelially (arrows) as well as subepithelially (asterisk). The inset shows E cadherin-positive epithelial cells that have taken up the 33mer. (B) No Cy^3 signal was detected in the mucosa of healthy controls. (C) LSM-assisted quantification of the Cy^3-33mer in epithelial cells revealed a significantly higher epithelial 33mer take-up in coeliac disease patients compared to healthy controls. In coeliac disease patients adhering to a gluten-free diet this effect was reversed. Epithelial cells are defined as cells positive for E-cadherin staining. (D) The lack of 33mer detection in coeliac intestinal crypts. Lu, lumen; LP, lamina propria; Ep, epithelial cells. Size bars are 10 μm.

during intestinal inflammation including coeliac disease.[35–37] Future prospects of following studies on the importance of the epithelial antigen uptake will include the comparative uptake of different gliadin fragments, like the peptide p31–43/p31–49 that directly affects via epithelial cells a response of the innate immune system.[4]

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M Schumann, J F Richter, I Wedell, V Moos, M Zimmermann-Kordmann, T Schneider, S Daum, M Zeitz, M Fromm and J D Schulzke

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