

# The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*

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## Summary

**Background** There have been only a few studies of how allergens cross the airway epithelium to cause allergic sensitization. House dust mite fecal pellets (HDMFP) contain several proteolytic enzymes. Group 1 allergens are cysteine peptidases, whilst those of groups 3, 6 and 9 have catalytic sites indicative of enzymes that mechanistically behave as serine peptidases. We have previously shown that the group 1 allergen Der p 1 leads to cleavage of tight junctions (TJs), allowing allergen delivery to antigen presenting cells.

**Objective** In this study we determined whether HDMFP serine peptidases similarly compromise the airway epithelium by attacking TJs, desmosomes and adherens junctions.

**Methods** Experiments were performed in monolayers of MDCK, Calu-3 or 16HBE14o-epithelial cells. Cell junction morphology was examined by 2-photon molecular excitation microscopy and digital image analysis. Barrier function was measured as mannitol permeability. Cleavage of cell adhesion proteins was studied by immunoblotting and mass spectrometry.

**Results** HDMFP serine peptidases led to a progressive cleavage of TJs and increased epithelial permeability. Desmosomal puncta became more concentrated. Cleavage of TJs involved proteolysis of the TJ proteins, occludin and ZO-1. This was associated with activation of intracellular proteolysis of ZO-1. In contrast to occludin, E-cadherin of adherens junctions was cleaved less extensively. Although Calu-3 and 16HBE14o-cells expressed tethered ligand receptors for serine peptidases, these were not responsible for transducing the changes in TJs.

**Conclusion** HDMFP serine peptidases cause cleavage of TJs. This study identifies a second general class of HDM peptidase capable of increasing epithelial permeability and thereby creating conditions that would favour transepithelial delivery of allergens.

**Keywords:** asthma, house dust mite allergens, tight junctions, serine peptidases, adherens junctions, allergic sensitization, occludin, claudins

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## Introduction

House dust mites (HDM) are an important cause of allergic asthma [1–3] and many, if not all, denominated allergens from this source are found at high concentration in their faecal pellets. When inhaled, house dust mite faecal pellets (HDMFP) impact on the hydrated lining of the airways and discharge their contents onto the surface of an epithelium that normally prevents the entry of foreign proteins [4]. This raises the possibility that responses of epithelial cells to the concerted actions of the contents of HDMFP could be crucial to the development of sensitization and allergic responses [5]. In this regard, it has been hypothesized that HDM allergens might facilitate their own delivery to dendritic antigen presenting cells and polarize T cell responses into an allergic phenotype [5].

The most extensively studied HDMFP allergen is Der p 1, a protein that shares sequence identity with the catalytic site of the plant enzyme, papain [5]. Mechanistically, papain is a cysteine peptidase as determined by the amino acids that comprise the charge relay system of its catalytic site. Consequently, Der p 1 is also viewed as behaving as a cysteine peptidase, and shows functional attributes expected of this. However, at least three other HDM allergens (Der p 3, Der p 6 and Der p 9) are proteolytic enzymes. The amino acid sequences of these enzymes suggest that they belong to the family of enzymes known as serine peptidases [5]. In having a different charge relay system and catalytic subsite structure, serine peptidases have substrate requirements and inhibitor susceptibilities that differ from those of cysteine peptidases.

Serine peptidase activity accounts for 79% of the proteolytic activity of house dust [6], making the effects of these enzymes potentially more significant than those of Der p 1, depending upon the substrates that they target. Several proteolytic effects of allergens could be relevant to sensitization and the initiation of allergic responses [5–9]. In addition to increasing the leakiness of airway epithelium [7–9], Der p 1 cleaves the low affinity IgE receptor of B cells (FcεRII, CD23) [10,11] (albeit without affecting the development of allergy), it attacks the α subunit of the human T cell interleukin-2 receptor (CD25) [12], it degrades α<sub>1</sub>-proteinase inhibitor [13], and polarizes murine T cell responses into a Th2 phenotype [14]. Der p 9, the only serine peptidase HDM allergen whose biology has been studied specifically to date, has been shown to increase the release of GM-CSF, IL-6 and IL-8 from a transformed epithelial cell line by proteolysis of an unknown target(s) [15].

Our earlier investigations demonstrated that peptidases from HDMFP increase epithelial permeability by an unknown mechanism, ultimately causing some cells to become apoptotic [9,16]. In the case of Der p 1 we recently

identified [9] that occludin and claudin family adhesion proteins of tight junctions (TJs) are important targets of this HDM allergen. As TJs regulate paracellular permeability [17,18], their specific disruption accounts for the previous observed increase in epithelial permeability [9,16].

In the present study we investigated the effects of HDMFP serine peptidases on structure-function of epithelial TJs. Putative cleavage sites, differing from those of Der p 1 were identified in TJ adhesion proteins, suggesting that HDMFP have multiple opportunities to 'open' TJs, compromising the ability of airway epithelial cells to function as an effective barrier. These results suggest a novel therapeutic rationale for the prevention and/or treatment of allergic diseases by modulating the process of allergen delivery.

## Methods

### *Preparation of serine peptidases from HDM faecal pellets*

Serine peptidase activity from faecal pellet-enriched *D. pteronyssinus* culture medium (Commonwealth Serum Laboratory, Parkville, Australia) was prepared as described [16]. These previous studies confirmed that this fraction is enriched in the serine peptidase allergens (Der p 3, Der p 6 and Der p 9) [16]. Protein content of the extracts was measured using the Coomassie Blue technique with bovine serum albumin as standard [19]. Enzymatic activity was measured at 37 °C by spectrophotometry at 405 nm using N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (625 μM) as substrate [9]. Preparations were analysed for the presence of endotoxin by Limulus amebocyte lysis assay (ICN Pharmaceuticals Ltd, Basingstoke, Hampshire, UK) and were found to be below the assay limit of detection (0.06 ng/mL).

### *Cell culture and permeability measurement*

Madin-Darby canine kidney (MDCK), Calu-3 and 16HBE14o-cells were cultured as described previously [16,20]. Calu-3 and 16HBE14o- are immortalized cell lines derived from human bronchial epithelium. Both cell lines express TJs, adherens junctions and desmosomes, enabling them to develop functional barrier properties that make them excellent paradigms of bronchial epithelium [9,16,20]. Comparisons were made with the MDCK epithelial cell line which has intercellular junctions that have been extensively characterized.

For immunocytochemistry, cells were grown on glass coverslips coated with an ultra-thin layer of Matrigel (Universal Biologicals, London, UK) [20]. Permeability of D-[<sup>14</sup>C]-mannitol (NEN Du Pont, Brussels, Belgium) was measured according to published methods [20] in confluent monolayers grown on a Matrigel undercoat in 0.1 μm pore

diameter Transwells (Corning-Costar Ltd, High Wycombe, Buckinghamshire). Allergen preparations were diluted in minimum essential medium with Earle's salts (EMEM) for addition to cells. Controls comprised EMEM alone.

#### *Immunostaining and 2-photon molecular excitation microscopy*

The morphology of TJs and desmosomes was studied by fluorescent antibody labelling in methanol-fixed cells [20]. Fluorescein dichlorotriazine (DTAF) or tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies (Chemicon International Ltd, Harrow, UK) were used for labelling. Occludin was immunostained using monoclonal antibody (mAb) MOC37 [21], ZO-1 with mAb R40.76 [22], and desmoplakin with mAb 11-5F [23]. Proteinase activated receptors (PARs) were visualized using rabbit polyclonal antisera raised against specific peptide sequences for these proteins. F-Actin was stained using rhodamine phalloidin (Molecular Probes Europe, Leiden, The Netherlands). For F-actin staining, cells were fixed for 10 min in 3.7% v/v formaldehyde solution in phosphate buffered saline (PBS), washed extensively, and permeabilized for 10 min with 0.1% v/v Triton X-100. After washing with PBS, cells were stained with rhodamine phalloidin for 45 min.

Two-photon molecular excitation microscopy (2PMEM) was performed on a modified LSM410 microscope (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) illuminated by a Ti:sapphire modelocked laser (Coherent Laser Group, Santa Clara, California, USA). Two-photon excitation of fluorochromes was attained at 810 nm using laser pulses of 80–100 fs duration [9,24]. Quantitative analysis of changes in TJs and desmosomes was conducted using programs written by us for the IDL software data visualization environment (Research Systems Inc, Boulder, Colorado, USA). Three-dimensional isosurface reconstructions were prepared using IRIS Explorer (Numerical Algorithms Group, Oxford, UK) running on a Silicon Graphics Indigo 2 computer loaded with SG Extreme Graphics hardware (Silicon Graphics, Reading, Berkshire, UK). Details of these procedures have been described elsewhere [9].

Confocal microscopy with single-photon excitation was performed using a Zeiss LSM 510 microscope with excitation/emission conditions set for FITC. Digital image stacks were acquired and processed using a Silicon Graphics Indy workstation.

#### *Immunoblotting*

Whole cell extracts were prepared for electrophoresis and immunoblotting as described elsewhere [9]. Detection was

by enhanced chemiluminescence technique (ECL) (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Primary antibodies for blotting in MDCK cells were MOC37 for occludin, R40.76 for ZO-1 and DECMA-1 for E-cadherin. Details of these antibodies have been described elsewhere [9,20–22]. In human cells, occludin and ZO-1 were blotted using polyclonal antibodies obtained from Zymed Inc., San Francisco, CA, USA.

#### *HPLC/electrospray ionization mass spectrometry*

The potential for HDMFP serine peptidases to cleave TJ adhesion proteins was investigated using synthetic oligopeptides as described elsewhere [9].

#### *RT-PCR detection of PARs*

Total RNA was prepared from 16HBE14o- and Calu-3 cells (approximately  $10^6$  in each case). Additionally, we studied the cell lines A549, NCI-H292 and MDCK cultured as described [20], and primary cultures of human lung fibroblasts [25]. RNA was extracted using 4 M guanidinium thiocyanate denaturing buffer, 2 M sodium acetate, pH 5.2, phenol and chloroform-isoamyl alcohol mixture. cDNA was synthesized from 5 µg RNA using 4 µL reverse transcriptase buffer (5x), 1 µL of 0.27 µg/µL p(dT)<sub>12–18</sub> primer (Amersham Pharmacia Biotech), 2 µL 100 mM dithiothreitol, 2 µL dNTP (5 mM each) (Amersham Pharmacia Biotech), 1 µL of 40 U/µL RNasin (Promega, Southampton, Hampshire, UK) and 400 U of MMLV reverse transcriptase (GIBCO-BRL). PCR amplification was performed using RedTaq DNA polymerase (Sigma, Poole, Dorset, UK), dNTP and the equivalent of 0.2 µg original total RNA. Forward and reverse primers for human PARs were as follows:

PAR-1 sense 5'-TGTGAAGTATCATGTTTATG-3', antisense 5'-TTCGTAAGATAAGAGATATGT-3', PCR product 708 bp; PAR-2 sense 5'-AGAAGCCTTATTGGTAAGGTT-3', antisense 5'-AACATCATGACAGGTCGTGAT-3', product 582 bp; PAR-3 sense 5'-CTGATACCTGCCATCTACCTCC-3', antisense 5'-AGAAAAGTGTGCCCCACACC-3', product 382 bp; PAR-4 sense 5'-ATTACTCGGACCCGAGCC-3', antisense 5'-TGTAAGGCCACCCCTTCTC-3', product 392 bp. Amplification of β-actin with the sense and antisense primer pair 5'-GGCTCTCTTCCAGCCTTCCTTCCT-3' and 5'-CACAGAGTACTTGCCTCAGGAGG-3' (product 240 bp) acted as an internal control. After initial denaturation at 94 °C for 3 min, PCR was performed for 35 cycles with final extension at 72 °C for 10 min. Thermocycling conditions for PAR-1 and -2 involved denaturation at 97 °C for 15 s, annealing at 57 °C for 60 s and extension at 72 °C for 90 s.

For PAR-3 and PAR-4 denaturation was at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 90 s. Electrophoresis was carried out on 1.2% agarose gels and stained with ethidium bromide for visualization. PCR products were isolated using a QIAquick gel extraction kit (Qiagen Ltd, Crawley, West Sussex, UK) and their identities confirmed using an Applied Biosystems ABI 377 automated DNA sequencer.

#### Treatment of cells with PAR activating peptides

Putative hexapeptide activators of human PARs were synthesized with amidated C-termini as follows (single letter amino acid notation): SFLLRN-NH<sub>2</sub> for PAR-1, SLIGKV-NH<sub>2</sub> for PAR-2, TFRGAP-NH<sub>2</sub> for PAR-3 and GYPGQV-NH<sub>2</sub> for PAR-4 [26,27]. It should be noted that in the case of the PAR-3 sequence, and in contrast to the other receptor ligands, no evidence exists to support its ability to act as an activator of signal transduction. Peptides were diluted in EMEM for application to monolayers of 16HBE14o-human bronchial epithelial cells grown on Matrigel-coated glass coverslips.

#### Statistics

Statistical significance, calculated from analysis of variance modules of the Statistica program suite (Statsoft, Tulsa, OK, USA), was taken as  $P < 0.05$  and is indicated in the figures by asterisks.

#### Results

##### Effects of HDMFP serine peptidases on intercellular junctions

Under control conditions, both MDCK and Calu-3 cell lines formed confluent monolayers containing TJs (Figs 1 and 2). TJs of MDCK cells were visualized by immunostaining of the cytoplasmic TJ protein ZO-1 (Fig. 1a), whereas those of Calu-3 bronchial epithelial cells were studied by immunostaining of the transmembrane protein occludin (Fig. 2a). In both cell lines, TJs appeared as virtually continuous rings, apically arranged to the punctate staining pattern created by desmosomes (Figs 1b and 2b). This is disclosed more clearly in 3-dimensional isosurface reconstructions of control cells (Figs 1c and 2c).

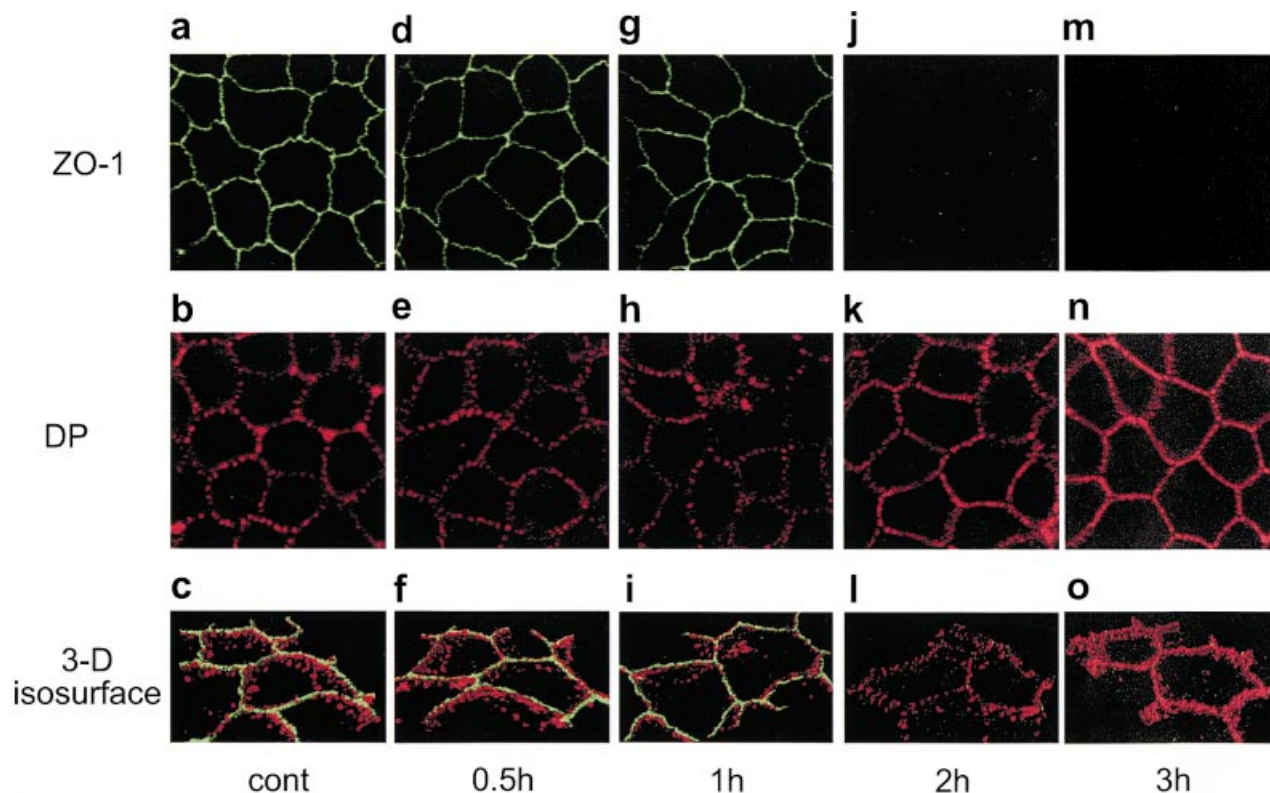


**Fig. 1.** Effects of HDMFP serine peptidases on TJs and desmosomes of MDCK cells. TJs were visualized by immunostaining of ZO-1 and desmosomes by desmoplakin. Images were acquired by 2PMEM and are shown in extended focus (3–4 µm thick) for each cell junction and as 3-dimensional isosurface reconstructions of both. Depicted are control cells (cont, panels a, b, c) and those at various time points indicated following exposure to HDMFP serine peptidase. Serine peptidase activity was 63.3 nmol N-benzoyl-Phe-Val-Arg-*p*-nitroanilide degraded/min. This is equivalent to approximately 1200 HDMFP.

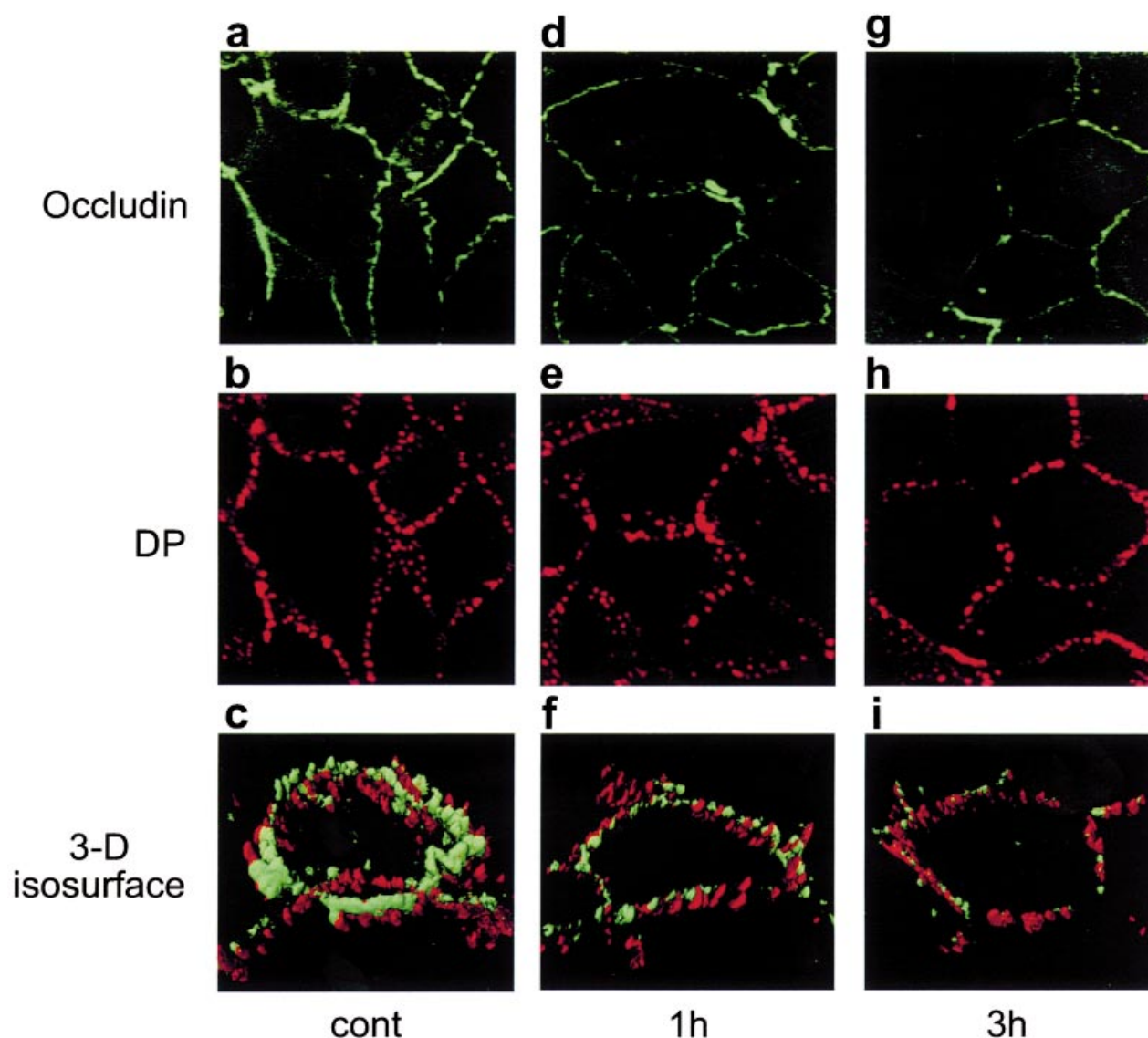


**Fig. 2.** Effects of HDMFP serine peptidases on TJs and desmosomes of Calu-3 human bronchial epithelial cells. Other details as shown in the legend to Fig. 1, except that TJs were visualized by immunostaining of occludin.

Treatment of MDCK cells with the HDMFP serine peptidase mixture evoked a time-dependent decrease in ZO-1 localization at the TJs of MDCK cells (Fig. 1). Changes were also evident in the desmosomal staining pattern of treated MDCK cells (Fig. 1). Qualitatively similar changes were produced in the TJs and desmosomes of Calu-3 cells (Fig. 2).

The acquisition of images by 2PMEM, which minimizes photobleaching outside any given focal plane, allowed quantification of the changes produced by peptidase treatment at intercellular junctions. Figure 3 (a) shows an analysis of the decrease in the TJ localization of ZO-1 in MDCK cells. A significant reduction was achieved within 30 min of exposure to the peptidases, but by 2 h junctional

staining was reduced to <20% of control levels. In contrast, the amount of cytoplasmic staining of immunoreactive ZO-1 remained constant throughout this period (Fig. 3, a). The initial changes in MDCK cell TJs following HDMFP serine peptidase treatment appeared to be due to a decrease in junctional protein and was not associated with an obvious breakage in the rings of ZO-1 immunostaining. This impression was confirmed by quantitative analysis of TJ breakage based on measures of protein content and fragmentation of the ZO-1 immunostaining pattern (Fig. 3b). In untreated confluent cell monolayers the number and length of the breaks in the ZO-1 staining of TJ rings is normally low, and this situation remained essentially unchanged for the first 60 min following exposure to the

peptidases (Fig. 3b). However, after 2–3 h, ZO-1 staining passed a threshold beyond which contiguity of the rings could not be maintained. Both the number and length of breaks in the TJ rings was significantly increased in these cases (Fig. 3, b).

Figure 3 (c, d) illustrates the corresponding data for Calu-3 bronchial epithelial cells. Whilst the overall effect of the HDMFP serine peptidases was similar to those described above, some details differ. These differences may occur either because the cell lines are not the same, or because the plaque and transmembrane proteins behave differently. Figure 3 (c) shows that the initial response in Calu-3 cells was a small, but significant, increase in TJ occludin fluorescence intensity 30 min after treatment. However, this was followed by a time-dependent reduction in the intensity of junctional occludin staining, which was not as marked as that seen in MDCK cells. Figure 3 (c) also shows that, unlike MDCK cells, there was a significant increase in the cytoplasmic fluorescence following peptidase

treatment. Compared to ZO-1 in MDCK cells, immunostaining of occludin in Calu-3 cells revealed a larger number of breaks in the continuity of TJ protein staining under control conditions, despite the formation of low permeability monolayers. This could indicate that other TJ adhesion proteins play significant roles in the permeability properties of Calu-3 TJs. Following peptidase treatment there was no change in the average length of breaks in peripheral staining per cell, although by 3 h there was an increase in the mean number of these breaks (Fig. 3, d).

Digital image analysis revealed the nature and extent of changes that took place in desmosomes following exposure of epithelial monolayers to the HDMFP serine peptidases. Figure 4 (a) shows measurements of the intensity of desmosomal puncta and the cytoplasmic immunofluorescence due to immunoreactive desmoplakin staining in MDCK cells. The initial effect of the peptidases was to decrease the intensity of desmosomal puncta, but by 3 h this response was reversed to a significant increase

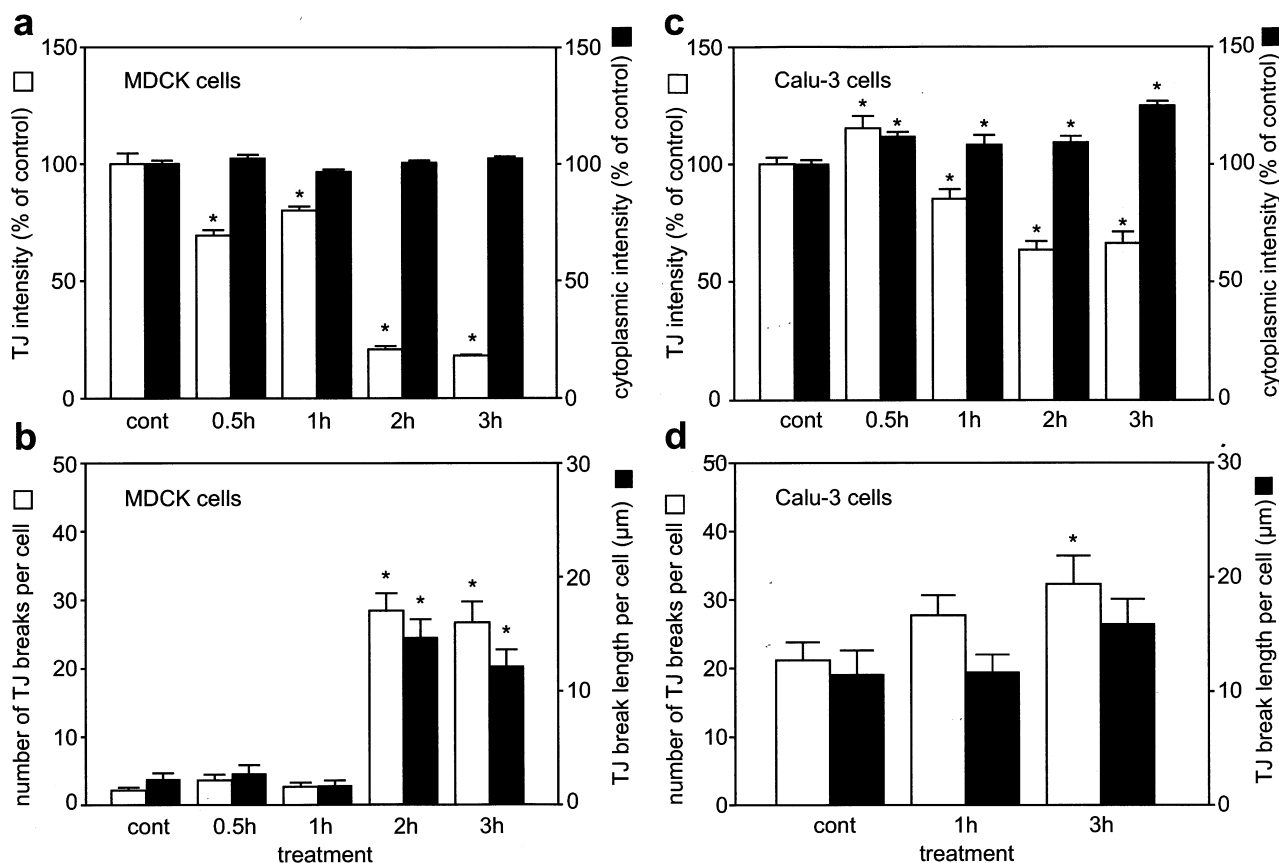


**Fig. 3.** Quantitative analysis of changes in lateral cell adhesions in MDCK cells (a, b) and Calu-3 cells (c, d) under control (cont) conditions and following HDMFP serine peptidase exposure. (a) Time-dependent changes in the amount of immunoreactive ZO-1 within TJs and in the cytoplasm of MDCK cells. (b) Number of breaks in TJ strands per cell and break length per cell. (c) Time-dependent changes in the amount of immunoreactive occludin within TJs and in the cytoplasm of Calu-3 cells. (d) Number of breaks in TJ strands per cell and break length per cell. Data are shown as the mean  $\pm$  SE mean in 10–20 cells. Asterisks indicate significant differences ( $P < 0.05$ ) from control values.

(Fig. 4a). Cytoplasmic staining initially decreased following treatment, but was followed by a reversal to a significantly increased cytoplasmic staining (Fig. 4, a). The average number of desmosomal puncta per cell was unchanged by treatment, although there was a tendency to decreased size (Fig. 4b). In Calu-3 cells, the effects of the peptidases on the intensity of desmosomal puncta and fluorescence of the cytoplasmic compartment differed from MDCK cells because it did not initially decrease (Fig. 4c). However, the extent of the increase in both measurements varied over the time course of the experiment (Fig. 4c). There were no significant changes in the average number and size of desmosomal puncta per cell in Calu-3 monolayers (Fig. 4, d).

#### Effects of HDMFP serine peptidases on epithelial permeability

Under control conditions, monolayers consisting of MDCK,

Calu-3 or 16HBE14o- cells formed tight barriers with low permeability to mannitol (Fig. 5 a, b, c). The permeability of the monolayers was significantly increased following exposure to HDMFP serine peptidases (Fig. 5 a, b, c).

#### HDMFP serine peptidases cause cleavage of occludin, ZO-1 and E-cadherin

The morphological evidence summarized in Figs 1 and 2 indicated that an early effect of the serine peptidases was to disrupt TJs. This suggested that occludin, an adhesion protein of TJs and previously identified by us as a major target of Der p 1, may be a substrate for these enzymes. To test this, we immunoblotted occludin in protein extracts of control and peptidase allergen treated cells.

Figure 6 (a) shows that in untreated monolayers of MDCK cells occludin was present mainly in a ~65 kDa form, with additional, diffusely staining bands of higher

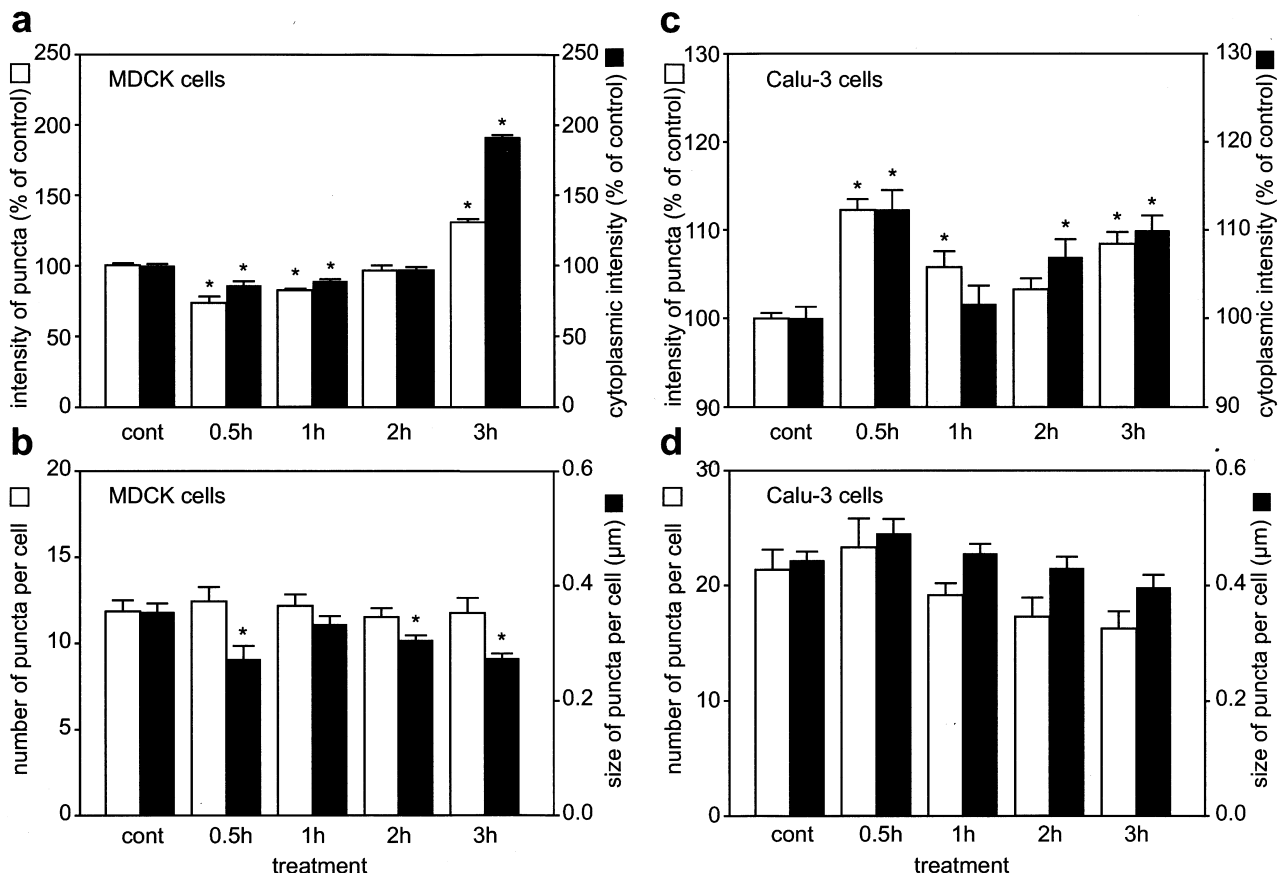


**Fig. 4.** Quantitative analysis of changes in lateral cell adhesions in MDCK cells (a, b) and Calu-3 cells (c, d) under control (cont) conditions and following serine peptidase exposure. (a) Time-dependent changes in the intensity of fluorescent antibody labelling of desmosomal puncta and in immunoreactive desmoplakin in the cytoplasm of MDCK cells. (b) Number and size of desmosomal puncta in MDCK cells following HDMFP serine peptidase treatment. (c) Time-dependent changes in the intensity of fluorescent antibody labelling of desmosomal puncta and in immunoreactive desmoplakin in the cytoplasm of Calu-3 cells. (d) Number and size of desmosomal puncta in Calu-3 cells following HDMFP serine peptidase treatment. Data are shown as the mean  $\pm$  SE mean in 10–20 cells. Asterisks indicate significant differences ( $P < 0.05$ ) from control values.

apparent molecular weight. Following exposure of confluent cell monolayers to the peptidases, the 65 kDa occludin band was reduced in intensity and new bands with apparent molecular weights of 47 kDa and 35 kDa appeared. The cytoplasmic TJ protein ZO-1 generated a ~197 kDa product following treatment of the cells with HDMFP serine peptidases (Fig. 6, b). E-cadherin, the adhesion protein of adherens junctions in these cells, also exhibited evidence of proteolysis since there was a time-dependent reduction in the intensity of the 120 kDa band

(Fig. 6, c). However, the effect of treatment on E-cadherin was much less marked than for the TJ proteins.

Figure 6 (d, e) illustrates the corresponding changes in occludin and ZO-1 in Calu-3 cells following their treatment with HDMFP serine peptidases. As shown in Fig. 6 (d), the immunoblotting profile of occludin in untreated Calu-3 cells was more complex than that seen in MDCK cells. However, major bands were visible at  $\geq 65$  kDa consistent with unmodified and possibly isoform or post-translational variants of this protein. Fragmentation of these forms of occludin occurred following treatment of the cells with the peptidases and bands at apparent molecular weights of 52, 43 and 34 kDa increased in intensity. All of the bands detected in this experiment represent proteins that retain a whole or partial intracellular domain of occludin because the polyclonal antibody used for immunoblotting in Calu-3 cells reacts with an intracellular C-terminal epitope of the protein. Similar to the proteolysis of ZO-1 in MDCK cells, immunoblotting revealed clear evidence of ZO-1 cleavage which involved the formation of a ~192 kDa product (Fig. 6, e).

To ascertain whether occludin is degraded by the serine peptidases, we constructed oligopeptide substrates from regions of the extracellular domains of occludin in which we have previously found Der p 1 cleavage sites. Figure 7 (a) shows degradation by the serine peptidases of the 12mer peptide AWDRGYGTSLG which corresponds to residues 88–99 of the first extracellular loop of occludin. Major cleavages in this peptide were detected between the AW, WD, RG and YG residues, with other residues being attacked less effectively, suggestive of a facile tryptic cleavage in concert with several chymotryptic attacks (Fig. 7, b). These findings suggest that putative cleavage sites exist for the HDM serine peptidases in the TJ adhesion protein occludin. The topography of the putative cleavage sites in this peptide sequence is summarized in Fig. 7 (b).

Regions of the extracellular domains of claudin-1 were also studied for their potential susceptibility to cleavage by HDM serine peptidases.  $^{64}\text{KVFDSSLNLS}^{74}$  and  $^{138}\text{WYGNRIVQ}^{144}$  from the first and second extracellular loops of murine claudin-1 both acted as substrates for the HDM serine peptidases.  $^{38}\text{WYGNRIVQ}^{144}$  was cleaved between WY (chymotryptic), RI (tryptic), YG and NR (Fig. 8, a).  $^{64}\text{KVFDSSLNLS}^{74}$  was cleaved between residues KV (tryptic), LL and LN (chymotryptic) (Fig. 8, b). The topography of the putative cleavage sites in these peptide sequences is summarized in Fig. 8 (c).

#### *HDMFP serine peptidases do not transduce changes in intercellular adhesion through proteinase activated receptors (PARs) 1–4*

To ascertain whether the effects of the serine peptidases on

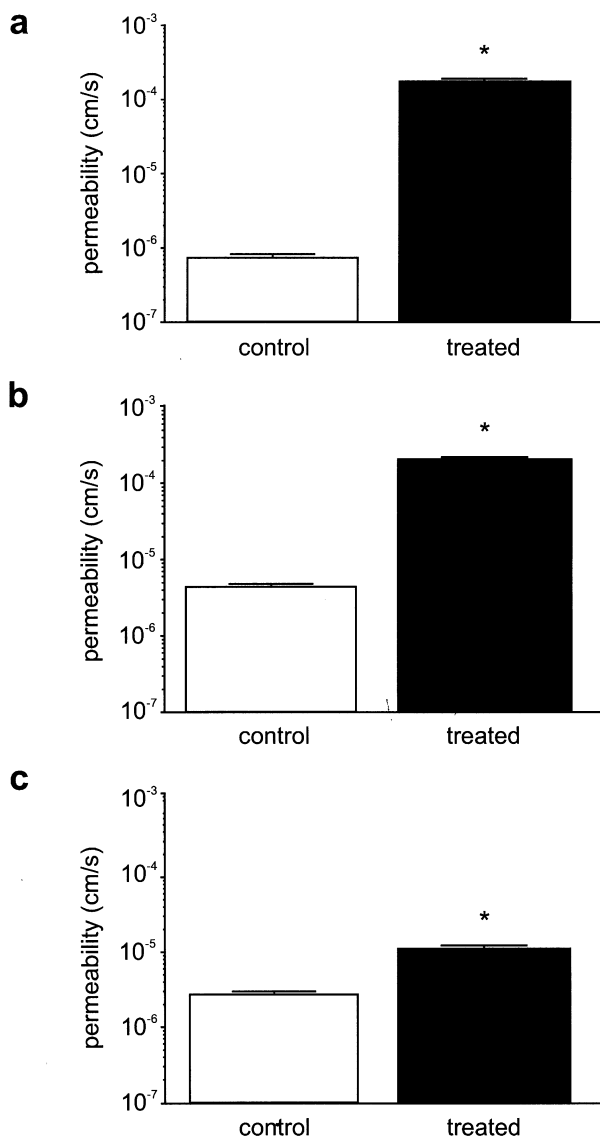


**Fig. 5.** Effect of HDMFP serine peptidases on the permeability of epithelial monolayers to mannitol. Monolayers of (a) MDCK cells, (b) Calu-3 cells, and (c) 16HBE14o-cells were treated for 3 h before permeability was measured. Data are shown as the mean  $\pm$  SE mean in 5 experiments. Asterisks indicate significant differences ( $P < 0.05$ ) from control values.

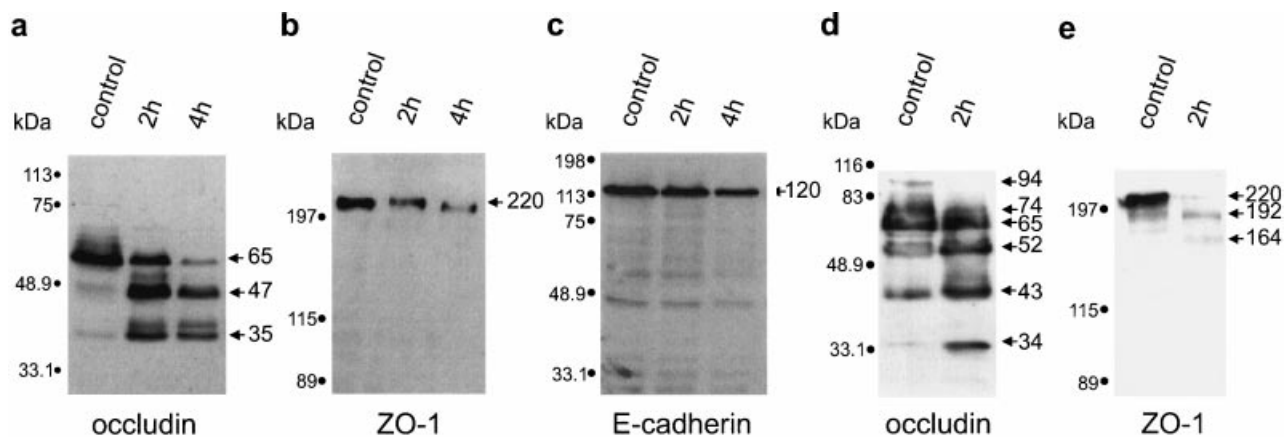


**Fig. 6.** Cleavage of occludin, ZO-1 and E-cadherin in epithelial cells exposed to HDMFP serine peptidases. Immunoblots of MDCK cells (a, b, c) or Calu-3 cells (d, e) under control conditions and following 2 h or 4 h exposure. Filled circles indicate the mobility of molecular mass markers. Arrows designate bands of calculated molecular mass. Serine peptidase activity was 1360 nmol N-benzoyl-Phe-Val-Arg-*p*-nitroanilide degraded/min.

cell adhesion might be transduced by activation of tethered ligand receptors for serine peptidases, we undertook experiments to investigate the effects of PAR-activating peptides on TJ morphology. Initially, we confirmed that the airway epithelial cells expressed PARs 1–4 (Fig. 9). Both the 16HBE14o- and Calu-3 human bronchial epithelial cell lines expressed transcripts for all four receptors. Expression of PAR-1 and PAR-2 receptor proteins was confirmed by immunostaining in 16HBE14o- cells (Fig. 9). The cellular distribution of both receptors was diffuse, with some immunostaining being intracellular, either perinuclear or associated with endoplasmic reticulum. Figure 10 shows that treatment of 16HBE14o- cell monolayers with putative ligands for PARs 1–4 had no significant effects on the morphology of TJs over a time course where HDMFP serine peptidases resulted in junctional cleavage and loss of barrier function. However, the concentrations of PAR peptides used in these experiments are sufficient to transduce cytokine release from airway epithelial cells (not shown).

## Discussion

The concept that TJs are important targets in allergic sensitization represents not only the first identification of a mechanism for transepithelial delivery of an allergen, but is also the first direct association between a clinically significant process and a specific molecular event in TJs. Our original formulation of this hypothesis was based on studies with the HDMFP cysteine peptidase, Der p 1 [9]. As there have been no systematic molecular studies of the susceptibility of TJ adhesion proteins to proteolysis there are no *a priori* reasons to assume that other families of proteolytic enzymes mimic the action of Der p 1. The present study

establishes for the first time that HDMFP serine peptidases lead to specific degradation of TJs and that cleavage sites exist in the extracellular domains of occludin, claudin-1 and, by inference, other members of the claudin family. As the mixture of serine peptidases in HDMFP constitutes ~80% of the proteolytic activity in house dust [6], the effects of the serine peptidases on intercellular junctions may be more important than the actions of Der p 1.

Ordinarily, TJs provide an effective permeability barrier by occluding the apical end of paracellular diffusion paths of epithelia [17,18]. Occludins [21,28] and claudins [29,30] are transmembrane adhesion proteins of TJs, whereas ZO-1 is one of several intracellular proteins associated with TJ plaques [22]. The contiguous expression of TJ complexes at the apical perimeter of epithelial cells creates adhesive rings that form tight seals with neighbouring cells. The effect of these TJ rings is to prevent the ingress of foreign proteins, such as airborne allergens, by denying them access to paracellular permeation routes that lead to dendritic antigen presenting cells [6,9]. Cleavage of TJs by HDMFP serine peptidases and, as previously reported, Der p 1 [9] provides a mechanism to account for transepithelial delivery of intact allergen into lung tissue.

Previously, it has been hard to reconcile the efficiency of the airways epithelium as a barrier with the known potency of some airborne allergens to promote sensitization. Certain respiratory tract infections have been implicated as risk factors for asthma, leading to speculation that host responses to these infections create *inter alia* conditions that result in allergen delivery to dendritic cells [6]. Whilst it is easy to hypothesize that some infections might themselves modify epithelial permeability by some unknown mechanism, there is no direct support for this concept at present. However, our present data add substantially to the accumulating

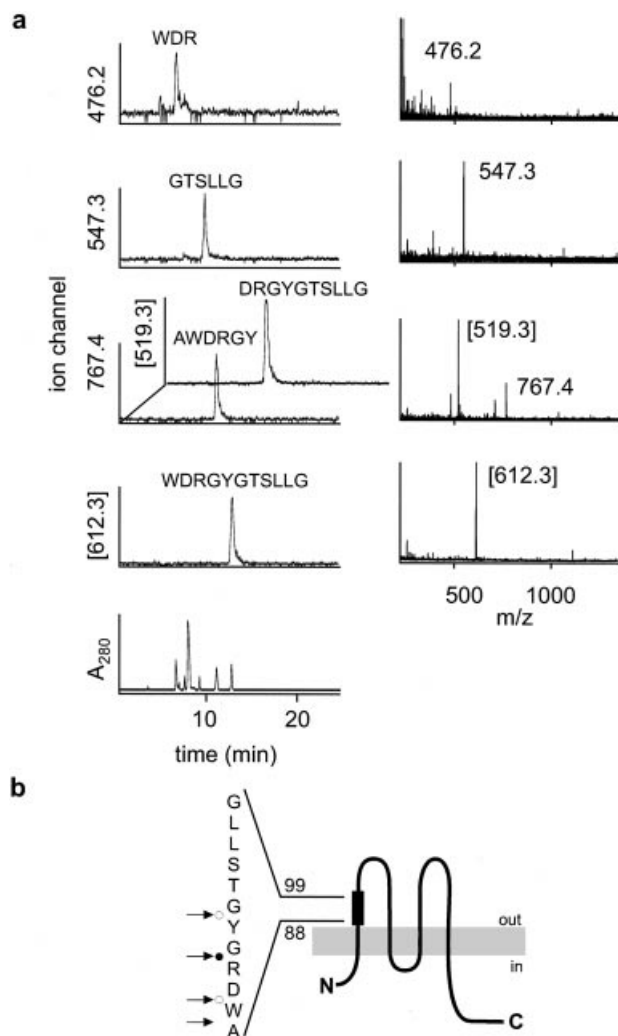


**Fig. 7.** (a) HPLC ion chromatograms and electrospray mass spectra of selected fragments following the degradation of occludin extracellular loop peptide ( $^{88}$ AWDRGYGTSLG $^{99}$ ) by HDMFP serine peptidases.  $A_{280}$  ultra-violet (UV) absorbing fragments include WDRGYGTSLG (13 min, doubly charged ion at  $m/z$  612.3), AWDRGY (11.2 min,  $M + H^+$   $m/z$  767), DRGYGTSLG (11.1 min, doubly charged ion at  $m/z$  519.3), and GTSLG ( $M + H^+$   $m/z$  547.3) which does not exhibit UV absorbance. There was no evidence for undigested substrate. Other peptide fragments were detected, but with reduced ion intensity. Doubly charged ions are bracketed. (b) Diagrammatic representation of suggested HDMFP serine peptidase cleavage sites in relation to the predicted membrane topography of occludin computed using the program SOSUI [36]. Arrows denote cleavage sites found in synthetic peptide, filled and open circles denote theoretical cuts for enzymes with tryptic and chymotryptic specificity, respectively. Serine peptidase activity was 2.7 nmol N-benzoyl-Phe-Val-Arg-*p*-nitroanilide degraded/min. This is equivalent to approximately 40 HDMFP.

experimental evidence that transepithelial delivery of allergens is directly facilitated by allergens that have proteolytic activity capable of degrading TJs. Given the balance of evidence it appears that allergen delivery could be accounted for by the intrinsic properties of certain allergenic proteins alone. Whilst we do not exclude an interaction at the level of allergen delivery between allergens and infections, it is more likely that the latter constitute predisposing factors for asthma if they drive the subsequent host immune response into pro-allergic pathways.

The pattern of immunostaining revealed by the TJ protein antibodies was altered following exposure to HDMFP serine peptidases. Specifically, TJ continuity was reduced and the amount of ZO-1 and occludin in TJs was decreased. The initial increase in occludin levels observed uniquely in Calu-3 cells prior to the reduction in junctional protein may result from the formation of non-sealing TJ strands arranged perpendicular to the junctions. A similar formation on non-sealing TJ strands occurs with trypsin [31]. The increase in cytoplasmic fluorescence that accompanied TJ fragmentation implies that the TJ proteins (or immunoreactive fragments derived therefrom) had been redistributed from the junctions into a cytoplasmic compartment. The increase in cytoplasmic staining was not seen in MDCK cells, but this does not necessarily indicate that their response mechanism was different. Since the amounts of TJ proteins involved are small and liberation into cytoplasm constitutes dilution into a larger cellular compartment, small redistributions into the cytoplasm may not provide a resolvable signal above the cytoplasmic fluorescence 'noise'. Further work will be required to describe the detailed intracellular dynamics of TJ proteins under these conditions, but such studies are outside the scope of the present investigation.

Immunoblotting and studies with synthetic peptides suggested that the HDM serine peptidases have the potential to cleave residues in at least one of the extracellular domains of occludin. We chose to concentrate our studies on the residue region 88–99 of occludin and two segments of the extracellular regions of claudin-1 because we have previously shown that they contain cleavage sites for Der p 1 [9]. Furthermore, the extracellular segment of occludin that we studied is accessible from the apical epithelial surface because TJs can be immunostained in non-permeabilized cells using antibodies that recognize this peptide segment [32]. However, these may not be the only sites at which the HDM serine peptidases could attack TJs. Inspection of the complete extracellular loop sequences for occludin suggests the putative existence of 2 tryptic and 27 chymotryptic cleavage sites (Fig. 11). Since the HDM serine peptidase allergens have tryptic-like (Der p 3), chymotryptic-like (Der p 6) and collagen-degrading (Der p 9)

serine peptidase activity, there are potentially multiple substrate opportunities within the extracellular domain of this TJ adhesion protein. Even if some of these sites were to be sterically hindered when TJs are intact, they may become progressively exposed as TJs break down.

Phosphorylation of occludin into high molecular weight forms has been suggested to regulate TJ function [21,33],

although the pathways that regulate its phosphorylation states have not been identified. Particularly in Calu-3 cells, it was noted that high molecular weight forms of occludin disappeared following serine peptidase treatment. We are not able to determine at present whether these 'heavy' forms of occludin are distinct isoforms or whether they are produced by postranslational modifications such as

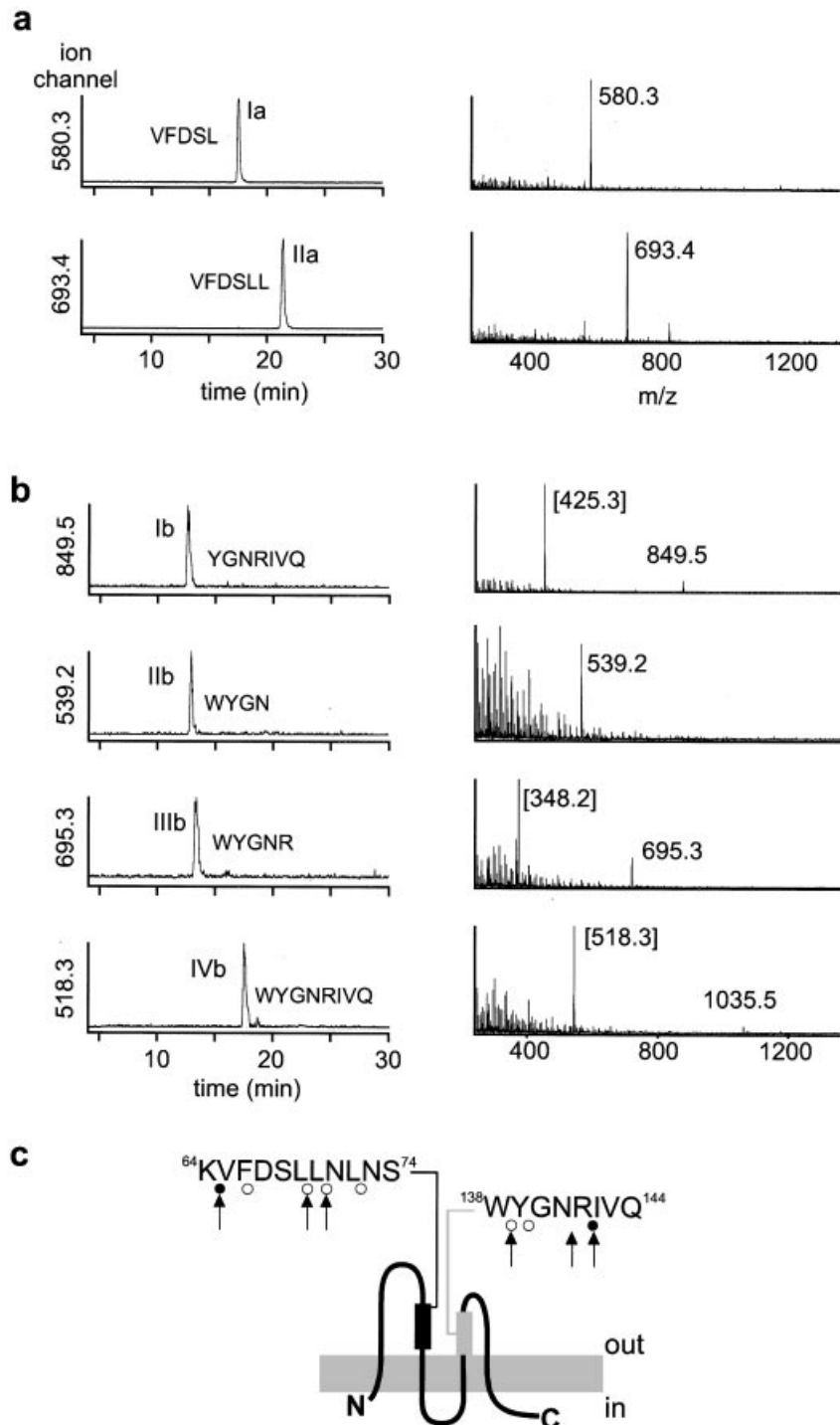


**Fig. 8.** HPLC-electrospray mass spectrometry showing effects of HDMFP serine peptidases on claudin-1 peptide (a) KVFDSSLNLNS, and (b) WYGNRIVQ. Selected ion chromatograms are shown on the left, with the corresponding positive ion electrospray mass spectra on the right. Note that doubly charged ions are bracketed. In panel (a) two major fragments of KVFDSSLNLNS are VFDSL (peak Ia,  $M + H^+$   $m/z$  580.3) and VFDSL (peak IIa,  $M + H^+$   $m/z$  693.4). In panel (b), WYGNRIVQ was digested into a number of major fragments including YGNRIVQ (peak Ib,  $M + H^+$   $m/z$  849.5), WYGN (IIa,  $M + H^+$   $m/z$  539.2), and WYGNR (IIIb,  $M + H^+$   $m/z$  695.3). Doubly charged ions are bracketed. Free tryptophan (not shown) and unchanged WYGNRIVQ (IVb,  $M + H^+$   $m/z$  1035.5) were also present. (c) Shows diagrammatically the suggested HDMFP serine peptidase cleavage sites in relation to the predicted membrane topography of claudin-1 computed using the program SOSUI [36]. Arrows denote cleavage sites found in synthetic peptide, filled and open circles denote theoretical cuts for enzymes with tryptic and chymotryptic specificity, respectively. Serine peptidase activity was 2.7 nmol N-benzoyl-Phe-Val-Arg-*p*-nitroanilide degraded/min. This is equivalent to approximately 40 HDMFP.

phosphorylation. However, the functional consequence of their loss, either by proteolysis of the phosphoprotein itself or by concerted dephosphorylation and proteolysis, is that epithelial permeability increases.

A peptide that mimics the second extracellular loop of occludin has been shown to open TJs and increase epithelial permeability [34]. Although claudin family proteins are significant components of TJ adhesion [29], the effect of the occludin peptide suggests that either occludin plays a key role in regulating paracellular permeability, or that disruption of one TJ transmembrane protein, such as occludin, is sufficient to perturb others, such as the claudins. Our results, which clearly establish that occludin is broken down *in situ* when cells are exposed to HDMFP serine peptidases, suggest that this cleavage alone might open TJs. Nevertheless, we were interested to know whether HDM serine peptidase allergens might

attack claudins. We restricted our study to extracellular regions of murine claudin-1 in which we have previously identified cleavage sites for Der p 1 [9], one of which is strongly conserved throughout the claudin family. Several putative cleavage sites for HDMFP serine peptidases, differing from the profile attacked by Der p 1 [9], were identified within these short sequences. This result suggests that HDM serine peptidase allergens have an extensive range of potential cleavage opportunities within TJs, a view supported by the number of predicted chymotryptic and tryptic cleavage sites in other claudin family proteins (Fig. 11). A significant corollary from these findings is that HDM allergens probably exert the greatest number and diversity of attacks on TJ proteins when they are encountered as the complex mixture of products from intact HDMFP.

It was found that the HDM serine peptidases also converted ZO-1 from a 220-kDa to a 197-kDa form. A

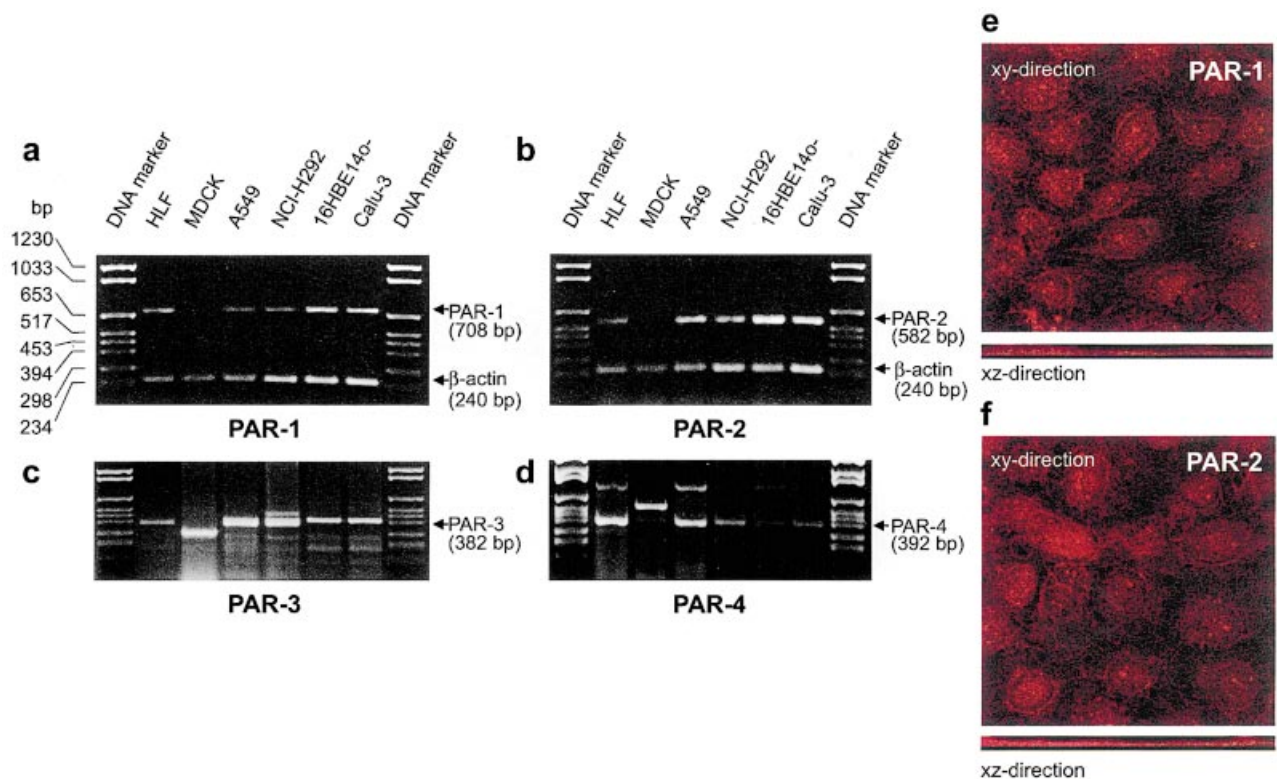


**Fig. 9.** Experiment to detect PAR-1, -2, -3 and -4 mRNA in human airway epithelial cell lines by RT-PCR and to demonstrate PAR immunostaining. RNA was extracted from a variety of cell lines and after reverse transcription amplified with specific primers for PARs and β-actin (as housekeeping gene). (a) PAR-1 detection in human lung fibroblasts (HLF) which were used as control, and in A549, NCI-H292, 16HBE14o- and Calu-3 cells which are all epithelial cell lines derived from human lung. In all cases, amplification resulted in a specific 708 bp product which sequencing confirmed as being PAR-1. Note that MDCK cells do not produce a signal for PAR-1 since the primer pair is specific for the human receptor. (b) Detection of PAR-2 mRNA as a 582-bp amplification product in human lung cells. (c) Detection of PAR-3 mRNA as a 382-bp amplification product in human lung cells. (d) Detection of PAR-4 as a 392-bp amplification product in human lung cells. Outer lanes of each agarose gel show DNA ladders of the indicated size range. (e) Extended focus (thickness 8 μm) confocal microscopy image demonstrating PAR-1 immunostaining in 16HBE14o-cells. (f) Extended focus (thickness 6 μm) confocal microscopy image demonstrating PAR-2 immunostaining in 16HBE14o-cells.

similar process was reported in epithelial cells exposed to Der p 1 [9]. As ZO-1 is an intracellular protein, its cleavage by exogenous proteolytic enzymes implies that an endogenous cascade of proteolysis is initiated by cleavage of TJ adhesion [9].

Consistent with the opening of TJs, an increased access of the peptidases to paracellular spaces resulted in effects on other lateral cell adhesions. E-cadherin of adherens junctions was cleaved in time-dependent fashion, but the extent of cleavage was much less than for the TJ proteins. However, it is not possible to deduce whether this represents differential substrate susceptibility (although there are theoretically several cleavage sites for serine peptidases in E-cadherin), or if it occurs because of the greater diffusional pathway that peptidases must negotiate to act upon adherens junctions.

Desmosomes are highly resilient cell adhesions that are particularly abundant in tissues prone to mechanical stress [35]. Although HDMFP serine peptidases had no effect of the number of desmosomal puncta, there were significant increases in their fluorescence intensity. The mechanism

underlying the increased staining of desmosomal puncta is unknown, but it is tempting to suggest that the changes seen may be reciprocally linked to events in TJs and adherens junctions. This increase in desmosomal staining may be part of an adaptive cellular response to the trauma caused by cleavage of TJs and adherens junctions, and thus essential to prospects for recovery and repair of the epithelium. We have observed that if the erosion of cell adhesion by HDM peptidase allergens is arrested, then epithelial barrier function can be recovered [9]. However, continued assault of epithelial cells results in irretrievable loss of the cells indicating that any adaptive/pre-emptive repair responses can ultimately be overwhelmed (unpublished observations).

Accumulating evidence suggests that HDMFP serine peptidases exert several effects on epithelial cells, raising the possibility that either some TJ disruption could arise indirectly, or that TJ cleavage could act as a trigger for other cellular responses. For example, serine peptidases from HDMFP induce apoptosis in epithelial cells [16], but this mechanism seems unconnected to TJ opening.

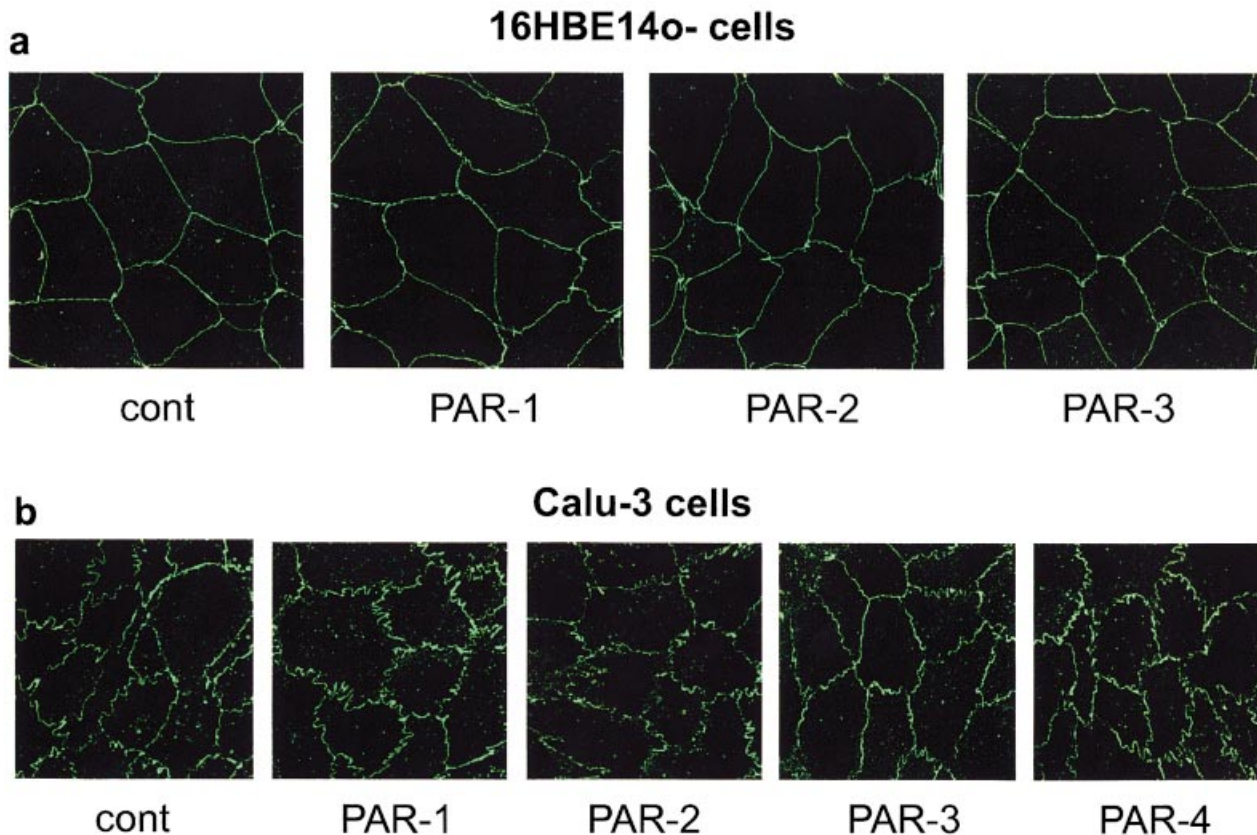


**Fig. 10.** Lack of effect of PAR activating peptides on TJs in 16HBE14o- cells. Cell monolayers were treated with peptides that activate PAR-1, PAR-2, PAR-3 or PAR-4 at a final concentration of 300  $\mu$ M over a 5-h period. Incubations were performed at 37 °C. Images acquired by confocal microscopy are shown in extended focus (thickness 6  $\mu$ m) and display the fluorescent antibody staining of occludin in control cells (cont) and after treatment with the relevant peptide.

Furthermore, Der p 9 induces the release of GM-CSF, IL-6 and IL-8 from the human airway epithelial cell line BEAS2-B [15]. However, these cytokine responses cannot be transduced by TJ cleavage because this particular cell line does not normally form contiguous TJs at cell borders (our unpublished observations).

Although the precise mechanism of this up-regulation of cytokine release is not understood, it is possible that it involves receptor-dependent mechanisms. Whilst serine peptidases act principally as degradative enzymes, some members of this diverse family are known to act as

signalling molecules that regulate cellular functions. Relevant to the action of serine peptidase allergens in epithelial cells is the G-protein linked family of PAR receptors for serine peptidases [26]. PAR-1 and PAR-3 are receptors for thrombin [26], PAR-2 is a receptor for trypsin and tryptase [26] and PAR-4 is responsive to thrombin and trypsin [27]. Activation of these receptors involves proteolytic cleavage of the extracellular N-terminus of the receptor to form a new N-terminal sequence that behaves as a tethered ligand capable of activating extracellular sites of the cleaved receptor.

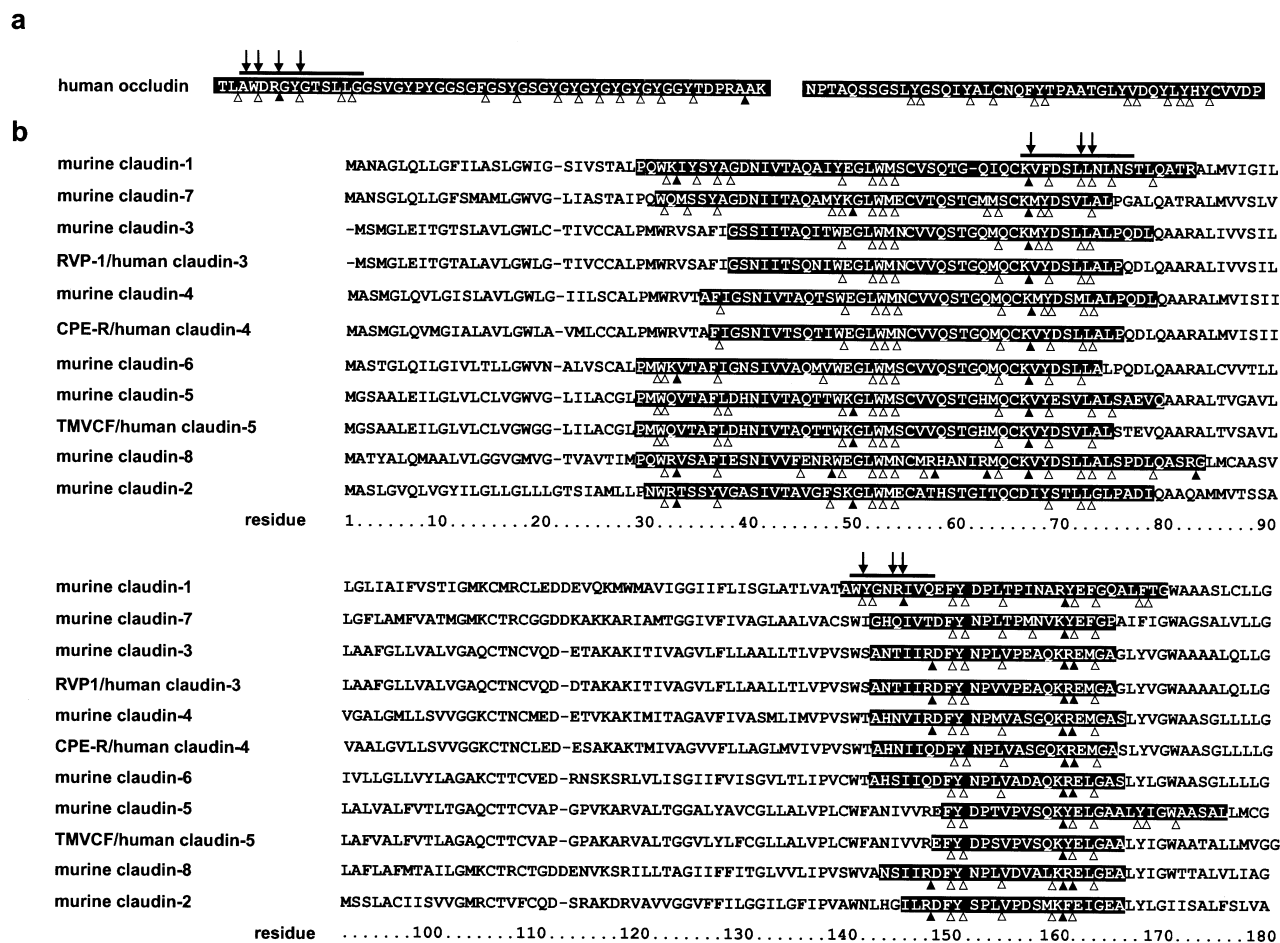


**Fig. 11.** Putative tryptic and chymotryptic cleavage sites in the extracellular loops of human occludin, murine claudins and three human homologues, RVP1, CPE-R and TMVCF. Occludin and claudin sequences were retrieved from the EMBL database (accession numbers for human occludin U49184, murine claudins 1–8 AF072127, AF072128, AF087822, AF087823, AF087824, AF087825, AF087826 and the three human homologues AB000714, AB000712 and AF000959, respectively). For each entry, putative transmembrane alpha helix locations were plotted from the translated amino acid sequence using the program SOSUI [36]. (a) Shows the putative cleavage sites in human occludin. For clarity, only the extracellular domains are illustrated (residues 85–138 and 198–241). (b) Shows the putative cleavage sites of claudins. For the claudins, membrane topography was predicted as above and a multiple sequence alignment constructed for the family members using the program CLUSTALX [37]. For clarity, the intracellular COOH-terminal region has been omitted from this diagram. In both sections of the figure, amino acids are in single letter notation, with white lettering on black backgrounds showing putative membrane spanning segments. Filled triangles denote potential tryptic cleavage sites, open triangles are potential chymotryptic cleavage sites. Underlined segments show the location of oligopeptides used for substrate studies and arrows denote the existence of cleavages detected by experiment using these peptides.

The existence of receptor-mediated responses to serine peptidases prompted us to investigate whether receptor coupled events were linked to TJ disruption. The human airway epithelial cells used in this study expressed mRNA for PAR-1, -2, -3 and, weakly, for PAR-4. We also confirmed that PAR-1 and -2 receptors were expressed in 16HBE14o- cells (we were unable to test for PAR-3 and PAR-4 because antibodies were not available). However, no changes in TJ morphology were seen when cells were treated with activating peptides at concentrations that evoke cytokine release. These results suggest that the simplest interpretation of our TJ cleavage data is that the responses are directly mediated by proteolysis of the adhesion proteins, although it is of course impossible to exclude the theoretical possibility that unknown PARs exist which are coupled to TJ modulation.

In summary, we have demonstrated that serine peptidase activity in an environmentally significant source of domestic allergens creates conditions that would favour delivery of any allergen to antigen presenting cells. Like the recently discovered action of Der p 1 [9], this process involves cleavage of TJs that regulate paracellular permeability. Taken together with our previous work, the present findings are consistent with HDM peptidase allergens inducing discrete responses of epithelial cells that converge to drive an adaptive immunological response which results in the synthesis of specific IgE. On the basis of this accumulating evidence, we propose that modulation of interactions between allergens and epithelial surfaces may represent a novel strategy for treating allergy.

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