House Dust Mite Allergens Induce Proinflammatory Cytokines from Respiratory Epithelial Cells: The Cysteine Protease Allergen, Der p 1, Activates Protease-Activated Receptor (PAR)-2 and Inactivates PAR-1


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House Dust Mite Allergens Induce Proinflammatory Cytokines from Respiratory Epithelial Cells: The Cysteine Protease Allergen, Der p 1, Activates Protease-Activated Receptor (PAR)-2 and Inactivates PAR-1

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In previous studies, we demonstrated that allergenic house dust mite proteases are potent inducers of proinflammatory cytokines from the respiratory epithelium, although the precise mechanisms involved were unclear. In this study, we investigated whether this was achieved through activation of protease-activated receptor (PAR)-1 or -2. Pretreatment of A549 respiratory epithelial cells with the clinically important cysteine protease allergen, Der p 1, ablated subsequent PAR-1, but not PAR-2 agonist peptide-induced IL-6 and IL-8 release. HeLa cells transfected with the plasmid coding for PAR-2, in contrast to PAR-1, released significant concentration of IL-6 after exposure to Der p 1. Exposure of HeLa cells transfected with either PAR-1/enhanced yellow fusion protein or PAR-2/enhanced yellow fusion protein to Der p 1 caused receptor internalization in the latter cells only, as judged by confocal microscopy with re-expression of the receptor within 120-min postenzyme exposure. Der p 1-induced cytokine release from both A549 and transfected HeLa cells was accompanied by changes in intracellular Ca2+ concentrations. Desensitization studies showed that Der p 1 pretreatment of the A549 cells resulted in the abolition of both trypsin- and PAR-2 agonist peptide-induced Ca2+ release, but not that induced by subsequent exposure to either thrombin or PAR-1 agonist peptide. These data indicate for the first time that the house dust mite allergen Der p 1-induced cytokine release from respiratory epithelial cells is, in part, mediated by activation of PAR-2, but not PAR-1. The Journal of Immunology, 2002, 169: 4572–4578.
in this manuscript, we describe studies aimed at determining whether the clinically important mite allergen, Der p 1, induced cytokine release from respiratory epithelial cells by activation of either PAR-1 or PAR-2. Our studies show for the first time that PAR-2, but not PAR-1, is involved in allergen-induced cytokine release.

Materials and Methods

Materials

Synthetic agonist peptides, as well as control PAR peptides, were synthesized with amidated C terminus (purity >85%; Protein Facility, University of Western Australia, Perth, Australia). Given that the human PAR-1 agonist peptide at high concentration has been shown to activate PAR-2, the more specific frog PAR-1 agonist peptide was substituted (24). The sequences of the active and control peptides, respectively, used were: PAR-1, TFLLR-NH2 and FTLLR-NH2; PAR-2, SLIGKV-NH2 and LSIGKV-NH2 (22). Thrombin was purchased from CSL (Melbourne, Australia) and Sigma-Aldrich (St. Louis, MO). Tissue culture reagents were purchased from Life Technologies (Melbourne, Australia). General chemicals were purchased from either BDH (Kilsyth, Victoria, Australia) or Sigma-Aldrich, unless otherwise stated. The human pulmonary epithelial type II A549 cell line HaLa, COS-7, Chinese hamster ovary, and HEK cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Purification of Der p 1 cysteine protease

Der p 1 was isolated from fecally enriched spent growth medium essentially as described previously (17). In brief, spent growth medium was extracted twice with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) for 2 h, and the supernatant obtained after centrifugation was applied to a monoclonal (25) anti-Der p 1 affinity chromatography column prepared using Ab kindly provided by M. Chapman (Indoor Biotechnol- ogy, Charlottesville, VA). Unbound material from the column was eluted with phosphate buffer containing 0.5 M NaCl, and material bound then was eluted after re-equilibration of the column with PBS using water adjusted to pH 11.0 with ammonia. The eluate was then neutralized and applied to a soybean trypsin inhibitor affinity column to remove contaminating serine proteases (26). The unbound eluate was adjusted to pH 8.0 and applied to an anion exchange column (DEAE 52; Whatman, Maidstone, Kent, U.K.), and chromatofocusing was performed. Der p 1 elution was monitored by protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Alexandria, Australia) in the presence and absence of 5 mM cysteine. The Der p 1-containing fractions were pooled, dialyzed against 0.05 M ammonium bicarbonate, and freeze dried. The protein content of Der p 1 was then determined spectrophotometrically.

Protease activity determinations

Trypsin activity was expressed in molar terms after determining the percentage of active enzyme using the active site titrant, p-nitrophenyl-p'-guanidinobenzoate, as described previously (22), whereas thrombin activity was expressed in U ml−1, as determined by the manufacturer. However, the activity of thrombin was confirmed using N-benzyloxycarbonyl-arginine-p-nitroanilide, as described previously (22), before experimentation. The thrombin supplied by CSL (0.5 nM p-nitroaniline released min−1 mg−1) was found to be 50-fold less active on this basis than that supplied by Sigma-Aldrich (23.4 nM p-nitroaniline released min−1 mg−1). Thrombin from this source was used in cell culture experiments, whereas thrombin from the latter was used in Ca2+ flux studies. The proteolytic activity of Der p 1 was determined as described above, and percentage of activatable enzyme was determined by titration using n-(3-carboxyoxorilene-2-carbon- yl)-leucyl-aminino(4-guanido) butane E64 and expressed in molar terms using a m.w. of 25,000. Briefly, dilutions of E64 (starting concentration, 10.5 μM) were added to a fixed amount of activated Der p 1 in a microfuge tube in triplicates, and Azocoll substrate was added and samples were incubated at 37°C. ODs were then determined, and the concentration of activated Der p 1 was calculated by plotting percentage of inhibition against E64 concentrations.

Cell culture

The A549 and HeLa cells were cultured as described previously (22, 28). For initial growth, cells were seeded into 75 cm2 tissue culture flask (Nunc, Nalgene, IL) and grown to confluence in Ham’s F12 Kaight’s Modifi- cation (Life Technologies) and DMEM, respectively, supplemented with 10% (v/v) FCS and antibiotics. For subsequent experiments, cells from the flasks were trypsinized and seeded into 24-well tissue culture plates (Nunc) at a density of 5 × 104 cells well−1 and grown to 80% confluency. At this time, the cells were then incubated in basal medium for a further 24 h. At all stages of culture, cells were maintained at 37°C in 5% (v/v) CO2.

PAR-1 and PAR-2/enhanced yellow fusion protein (EYFP) expression constructs

PAR-1 and PAR-2 plasmids in pRK7 vector constructs were obtained from L. Brass (University of Pennsylvania, Philadelphia, PA) (29) and J. Sundelin (Lund University, Lund, Sweden) (30), respectively. The plasmids were amplified using a 5′ primer annealed to the Sp6 promoter 5′-GAT TTA GGT GAC ACT ATA G-3′, and a 3′ primer that annealed to the 3′ end of the receptor sequence, and mutated out the stop codon by transforming it into a XhoI recognition site. The PAR-1 and PAR-2 primers used were 5′-GAG TTC CTC GAG AAT TAA CAG C-3′ and 5′-GAC CTG GAA CTC GAG AAT GGA GGT C-3′, respectively. The PCR products were digested with HindIII (site already present in the pRK7) and XhoI, and cloned into pcDNA3/EYFP, as described previously (28).

Transient transfection of HeLa cells

HeLa cells were split and seeded into 100-mm tissue culture dishes (Falcon) at a cell density of 1 × 105 cells/plate, and cells were grown overnight to 60% confluence in DMEM supplemented with 10% (v/v) FCS. Transient transfections were performed the following day with 3 μg DNA (deter- mined to be optimal by experimentation) using Superfect (Qiagen, Melbourne, Australia), as described previously (31). Cells were then split and seeded onto 12-well dishes or glass coverslips 24 h after transfection. The transfected cells were then used in assays to determine the functional sig- nificance of PAR receptors 48 h posttransfection.

Confocal imaging

HeLa cells transfected with PAR-1/EYFP and PAR-2/EYFP constructs were plated onto poly-L-lysine-coated eight-well chamber slides 24 h after transfection, and treatment regimes were performed 48 h posttransfection. After treatment, cells were fixed in 4% (v/v) paraformaldehyde, mounted in Fluoroguard (Bio-Rad, Melbourne, Australia), and sealed with a glass cov- erslip. Cells were excited at 488 nm and examined using a Bio-Rad con- focal laser microscope under an oil immersion objective ×60 with light filtered in the green channel from 500 to 550 nm, and data were collected.

Stimulation of epithelial cells and HeLa cells

Epithelial cells were grown in appropriate serum-free basal medium for 24 h, as described previously, and exposed to varying concentrations of Der p 1 activated with 5 mM cysteine, or PAR agonist peptides. In experi- ments designed to study Der p 1- or PAR-induced cytokine secretion at different times during culture, cells were exposed to the optimal concentrations of Der p 1 or peptide, as described previously (22). Culture superna- tants were collected, centrifuged at 12,000 × g for 5 min at 4°C, and stored frozen. At the conclusion of each experiment, cells were detached, and viability and cell number were determined by trypan blue exclusion. Epithelial cells exposed to the various additives were subsequently analyzed for poten- tial cytotoxic reactions by measuring the release of lactate dehydrogenase using a spectrophotometric assay, as described previously (22).

Determination of IL-6 and IL-8 production

IL-6 and IL-8 production was determined using specific ELISA, as de- scribed previously (20). Briefly, 96-well plates (Maxisorp; Nunc) were coated with 100 μl/well of the appropriate Ab (250 ng ml−1 in 0.1 M NaHCO3/NaCO3 buffer, pH 9.6) and incubated overnight at 4°C. The plates were then washed three times with washing buffer (PBS, pH 7.5, containing 0.5% (v/v) Tween 20) and blocked by incubating with 100 μl blocking buffer/well (washing buffer containing 1% (w/v) BSA) at room temperature for 1 h. The plates were washed three times with washing buffer before 100 μl test or standards were added to the wells. Plates were incubated overnight at 4°C, washed, and incubated with biotinylated sec- ondary Ab (100 μl stock in blocking buffer/well) at room temperature for 1 h. After washing, 100 μl peroxidase-labeled streptavidin (125 ng ml−1; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well, incubated at room temperature for 30 min, and, after washing, incubated with 100 μl peroxidase substrate (K-Blue ELISA sub- strate; Graphic Scientific, Brisbane, Australia) per well. Reactions were terminated by the addition of 100 μl 1 M phosphoric acid/well, and OD was determined using a microplate reader (250; Molecular Devices, Sunnyvale, CA) at 450 nm. The concentrations of cytokines in each sample were determined by interpolation from the standard curve using the SoftMax-Pro software (Molecular Devices) and expressed as pg
2.5 × 10^5 cells⁻¹. Second Abs used in ELISA were obtained from BD PharMingen (San Diego, CA).

**Measurement of cytosolic free calcium (Ca²⁺)**

Changes in cytosolic Ca²⁺ concentration were measured using the fluorescent Ca²⁺ indicator, Indo-1 acetoxymethylester (AM; Teflabs, Austin, TX), as described previously (20). The ratio of fluorescence emission at 405 and 490 nm was measured using a spectrophotometer (Cairn, Faverham, Kent, U.K.) attached to an inverted microscope (Nikon, Tokyo, Japan), configured for epifluorescence. The excitation wavelength of 340 nm was provided by a variable monochromator system (Cairn). A549, transfected and nontransfected HeLa cells were grown on a coverslip to 80% confluency and loaded with Indo-1 by incubation in RPMI (medium free of phenol red) containing 6 μM membrane-permeable form of the Ca²⁺ indicator, Indo-1 AM, and 0.1% (w/v) Pluronic F-127 (Molecular Probes, Eugene, OR) for 45 min at room temperature. After incubation, cells were washed with two changes of RPMI to remove the excess dye and then exposed to the various test substances after establishing baseline. The ratio of the emission intensities was used as a measure of changes in cytosolic calcium fluorecence. The excitation wavelength of 340 nm was measured for epifluorescence (Fig. 2). Nonactivated Der p 1 did not cause any significant cytokine release (Fig. 2). Activated Der p 1 caused significant IL-6 and IL-8 release, in response to Der p 1 stimulation at the 80-nM Der p 1 dose (Fig. 2).

**Results**

**Der p 1 induces cytokine release from A549 cells**

Activated Der p 1 caused significant release of IL-6 and IL-8 from A549 cells in a dose-dependent manner over a 24-h period (Fig. 1, A and B) when compared with medium controls and nonactivated allergens. The mean maximum concentrations of IL-6 and IL-8 released in response to Der p 1 were obtained with 0.8 and 8 nM, respectively, with the exception of IL-6, but not IL-8. Release was observed at 0.008 and 0.08 nM (Fig. 1, A and B). Der p 1-induced IL-6 and IL-8 production was reduced at peptide concentrations higher than those shown to be optimal (Fig. 1, A and B). Both IL-6 and IL-8 release, in response to Der p 1 exposure, increased with time and, for Der p 1-induced IL-6 and IL-8, maximum production was observed at 24 h (Fig. 1, C and D), but had not reached a plateau.

**Pretreatment of A549 cells with Der p 1 results in inhibition of PAR-1-mediated cytokine release**

Pretreatment of A549 cells with Der p 1 for 2 h at 37°C, followed by exposure with either PAR-1 or PAR-2 agonist peptides at 400 μM resulted in little or no IL-6 production after PAR-1 agonist peptide stimulation at all concentrations tested (Fig. 2A), but only significantly reduced IL-6 production with PAR-2-stimulated cells at the 80-nM Der p 1 dose (Fig. 2B). Nonactivated Der p 1 did not cause any significant cytokine release (Fig. 2).

**Der p 1 induces calcium flux in A549 cells, via PAR-2, but not PAR-1 activation**

Der p 1 (80 nM) induced changes in intracellular Ca²⁺ in the A549 cells (Fig. 3A) that were inhibited by pretreatment of the enzyme with the cysteine protease inhibitor for Der p 1, E64 (Fig. 3B). Exposure of the A549 cells to 80 nM Der p 1 before either thrombin (Fig. 3C) or trypsin (Fig. 3D) treatment resulted in desensitization to the latter, but not the former. Similarly, desensitization was obtained when cells were treated with Der p 1, followed by PAR-2 agonist peptide (Fig. 3F), but not PAR-1 (Fig. 3E). Exposure of cells to thrombin (Fig. 3G) or trypsin (Fig. 3H) before Der p 1 resulted in desensitization to the latter only.

**Statistical analyses**

Unless stated otherwise, data are expressed as mean ± SEM. Statistical significances between means were determined using ANOVA or the Student’s t test using Microsoft Excel for Macintosh. Bonferroni’s correction was used for multiple test comparisons.

**PAR-2, but not PAR-1 DNA-transfected HeLa cells secrete cytokines in response to PAR agonist peptides and Der p 1**

To confirm that Der p 1 activated PAR-2, but not PAR-1 receptors on the A549 cells, transfection studies were undertaken. A range of suitable cell lines including COS-7, Chinese hamster ovary, HEK, and HeLa cell lines was exposed to a range of Der p 1 and PAR agonist peptides, and cytokine responses were determined. However, with the exception of the HeLa cell line (Fig. 4A), the other cell lines produced cytokines in response to both Der p 1 and PAR agonist peptide treatment (data not shown). HeLa cells were, therefore, used in the transfection studies. Cells transfected with optimal concentration of PAR-2, but not PAR-1 (data not shown) DNA secreted significant concentration of IL-6 in response to PAR-2 agonist peptides, and 0.4 nM trypsin and 8 nM Der p 1 (Fig. 4B). These agonists had little significant influence on cytokine release from HeLa cells transfected with vector alone (Fig. 4C), although constitutive production appeared elevated compared with nontransfected cells. Examination of medium from
cells exposed to either peptidase or peptides for lactate dehydrogenase activity indicated that PAR treatment did not result in cell damage (data not shown).

Changes in intracellular calcium induced by Der p 1 and PAR-2 agonist peptides on PAR-2-, but not PAR-1-transfected HeLa cells

Neither the PAR-2 agonist peptide nor Der p 1 induced changes in intracellular [Ca$^{2+}$] in the nontransfected or vector alone-transfected HeLa cells, as judged by the fluorescence ratio (Fig. 5A–D). In contrast, both stimuli caused an increase in intracellular [Ca$^{2+}$] in the PAR-2-transfected HeLa cells (Fig. 5, E and F) within 20 min of exposure. Changes in [Ca$^{2+}$] were not observed when HeLa cells transfected with PAR-1 plasmid were exposed to PAR-1 agonist peptide or 80 nM Der p 1 (data not shown).

Expression and internalization of tagged PAR-2 receptors on HeLa cells

Confocal microscopy showed that HeLa cells transfected with either PAR-1/EYFP (Fig. 6A–D) or PAR-2/EYFP cDNA (Fig. 6E–H) expressed protein on the plasma membrane. When the cells were exposed to 80 nM Der p 1, internalization of PAR-2, but not PAR-1 (Fig. 6, B and C) was observed. With regard to PAR-2, this occurred within 30 min of adding the activated Der p 1 (Fig. 6, F and G). Nonactivated Der p 1 did not result in internalization (data not shown). After removal of the enzyme, PAR-2 receptors reappeared on the HeLa cell membrane within 120 min (Fig. 6H). Control experiments performed using agonist peptide showed PAR-1 receptors were capable of internalization, which occurred within 30 min of exposure (Fig. 6D).

Discussion

We have extended our previous studies on mite peptidase allergen-induced cytokine responses (10) and investigated the role of PAR using the mite cysteine peptidase, Der p 1, and the A549 respiratory epithelial cell line. The data obtained confirmed the ability of this peptidase to release both IL-6 and IL-8, with maximal responses being obtained with 0.8 and 8 nM, respectively, although diminished responses were obtained with higher concentrations, suggesting susceptibility of cytokines to proteolysis, particularly IL-6 (10). We showed that cytokine release involved activation of PAR-2, but not PAR-1, and obtained evidence to indicate that the latter receptor was inactivated on exposure to the mite allergen. Although others have reported the effects of the mite serine peptidase allergens Der p 3 and Der p 9 on PAR using 15-mer peptides comprising either PAR-1 or PAR-2 activation sites (23), these studies represent the first to demonstrate the direct interaction between peptidase allergens and functional PAR receptors.

The involvement of PAR in these responses was investigated by pretreating A549 cells with activated enzyme before treatment with agonist peptide. We chose a 2-h pretreatment on the basis of data showing that in mouse lung, regeneration of PAR-2 receptors on the cell surface after trypsin treatment was completed within this time frame (32). The results obtained showed that Der p 1 pretreatment appear to ablate PAR-1, but not PAR-2 agonist peptide responses, suggesting cleavage of PAR-1 downstream from the thrombin cleavage site, data consistent with those derived using a range of endogenous peptidases (33–36). For example, pretreatment of endothelial cells with cathepsin G, human neutrophil elastase, or proteinase 3 for 5 min has been shown to render PAR-1

![FIGURE 2.](https://example.com/figure2.png)

 activated Der p 1 pretreatment of A549 cells inhibits PAR-1, but not PAR-2 agonist peptide-induced cytokine release. Cell monolayers were cultured for 24 h with basal medium devoid of serum or additives, then stimulated with increasing concentrations of Der p 1 for 2 h at 37°C. Cells were then washed and incubated with either PAR-1 or PAR-2 agonist peptides or PAR-1 and PAR-2 control peptides at a final concentration of 400 μM for 24 h. Supernatants were harvested, and the presence of IL-6 was measured by ELISA. Data are expressed as mean pg IL-6/pg 10$^5$ cells$^{-1}$ SEM from three independent experiments performed in quadruplicate. Significant differences in means between Der p 1 pre-treatment and PAR agonist peptide-induced and control peptide-induced responses were determined using ANOVA and the Student’s t test incorporating Bonferoni’s correction where necessary; *, p < 0.05.

![FIGURE 3.](https://example.com/figure3.png)

 Desensitization of PAR in A549 cells by treatment with Der p 1. A549 cells were loaded with Indo-1 AM and incubated with Der p 1 (80 nM) (A–F). Cells were then incubated with either thrombin (20 U/ml) (C) or trypsin (0.4 nM) (D) or with 400 μM of either PAR-1 (E) or PAR-2 (F) agonist peptides after a return to baseline. B. Shows the effect of the cysteine protease inhibitor E64 (10 μM) on activated Der p 1-induced Ca$^{2+}$ responses. A549 cells were also pretreated with thrombin (20 U/ml) (G) or trypsin (0.4 nM) (H), followed by Der p 1. Arrows indicate the addition of the second stimulus. Responses were measured over 400 s and expressed as a fluorescence ratio (405/485 nm).
refractory to subsequent activation by thrombin. However, the findings that the PAR-1 agonist peptide activated the receptor despite pre-exposure to peptidase appear to contrast with our data in which a lack of agonist peptide activation of PAR-1 was demonstrated. It is likely these differences reflect the use of the human PAR-1 agonist peptide rather than the frog agonist peptide because the human version was used at concentrations known to activate PAR-2, a receptor known to be present on endothelial cells (37).

The finding that Der p 1 pretreatment appeared to inactivate PAR-1, but activate PAR-2, formed the basis for further studies, and, to this end, both Ca$^{2+}$ flux and transfection studies were used to confirm our previous observations. We showed that activated, but not E64-inhibited Der p 1 induced changes in cytosolic [Ca$^{2+}$] in the A549 cell line, and that allergenic peptidase pretreatment abolished PAR-2 agonist peptide and trypsin-induced [Ca$^{2+}$] flux, but not that induced by PAR-1 agonist peptide or thrombin. These findings indicate that PAR-1 remains sufficiently intact after Der p

**FIGURE 4.** Der p 1 and PAR-2, but not PAR-1 agonist peptides induce cytokine release from PAR-2-transfected HeLa cells. Nontransfected (A), PAR-2-transfected (B), or vector-transfected (C) cell monolayers were cultured with basal medium devoid of serum or additives for 24 h, then stimulated with either trypsin, PAR-1, or PAR-2 agonist peptide (400 μM), Der p 1 (8 nM), or medium alone (Control) for 24 h. Supernatants were harvested, and the presence of IL-6 was measured by ELISA. Data are expressed as mean pg 2.5 × 10⁵ cells⁻¹ ± SEM from three independent experiments performed in quadruplicate. Significant differences in means between medium-induced and peptidase- or PAR-2 agonist peptide-induced responses were determined using Student’s t test; *, p < 0.05.

**FIGURE 5.** Der p 1- and PAR-2 agonist peptide-induced cytosolic Ca$^{2+}$ responses in transfected cells. Nontransfected HeLa cells (A, B), vector only-transfected (C, D), or PAR-2-transfected HeLa cells (E, F) were loaded with Indo-1 AM and incubated with either 400 μM agonist peptide or Der p 1 at 80 nM. Responses were measured over 180 s and expressed as a fluorescence ratio (405/485 nm).

**FIGURE 6.** Der p 1 causes internalization of EYFP-tagged PAR-2, but not PAR-1 in HeLa cells. PAR-1/EYFP-transfected HeLa cells (A) were exposed to activated Der p 1 (80 nM) for 10 (B) and 30 min (C). Transfected cells were also exposed to PAR-1 agonist peptide (400 μM) for 30 min (D) without any pre-exposure to Der p 1. PAR-2/EYFP-transfected HeLa cells (E) were exposed to activated Der p 1 for 5 (F) and 30 min (G). Der p 1 was removed after 30-min exposure, and cells were washed and then incubated in medium for further 120 min (H).

**FIGURE 7.** Potential extracellular cleavage sites in PAR-1 and PAR-2, based on data obtained with claudin (crooked arrow), occludin (arrow with line beneath) (16), α1-antiprotease (dotted arrow) (35), CD23 (double arrow) (36), as well as synthetic substrates with C-terminal arginine or lysine (bent arrow) (24, 37). The thrombin/trypsin cleavage sites required for activation of PAR-1 (top line of each pair of sequences) and PAR-2, respectively, are marked with #. The underlined regions indicate the residues corresponding to the agonist peptide region (APR), and the NH₂-terminal extracellular residues (ECD) and extracellular loop residues (ECL) required for activation (reviewed in Refs. 38 and 39). Sequence alignments were generated using ClustalW3.21 (45) and Boxshade program.
1 treatment to respond appropriately to agonist peptide, in contrast to data obtained in the cytokine studies in which pretreatment ablated subsequent PAR-1-mediated responses. However, these differences are likely to reflect either the relatively short exposure times or Der p 1 concentrations used, or both.

Before embarking on transfection studies, we tested a number of cell lines for their suitability, but only HeLa were found to be appropriate, and these data are consistent with those reported by others (38). Transfection studies confirmed that Der p 1 activated PAR-2, but not PAR-1, as judged by both cytokine production and Ca$^{2+}$ flux, and similar conclusions were drawn from studies performed with HeLa cells transfected with EYFP-tagged PAR-1 and PAR-2. We showed that Der p 1 treatment caused internalization of tagged PAR-2, but not PAR-1, and that complete internalization was obtained within 5 min of exposure. Our studies also indicated that PAR-2 receptors were re-expressed on the transfected HeLa cell surface within 2.5 h post-Der p 1 treatment. These data are similar to those obtained with green fluorescent protein-tagged PAR-2 receptor in Kirsten murine kidney epithelial cells (39), in which complete internalization of the receptor was apparent within 10 min posttrypsin exposure, and receptor re-expression was observed within 4 h of enzyme removal.

Our data suggest that Der p 1 inactivates PAR-1 by specific cleavage of the receptor. Although detailed knowledge of the cleavage specificity of Der p 1 is lacking, a number of susceptible residues have been determined using synthetic peptides representing sections of oocullin (between L-L, G-T, Y-G) and claudin (between L-L, N-L, L-N, Y-G) (18), natural protein substrates such as $\gamma$-antiprotease (between (13$D$-13$T$, 6$D$-7$A$) (40) and CD23 (between 296$E$-299$S$, 155$S$-156$S$) (41), and synthetic substrates containing C-terminal R or K (26, 42). Using this information, several cleavage sites on PAR-2 receptor in Kirsten murine kidney epithelial cells (39), in which complete internalization of the receptor was apparent within 10 min posttrypsin exposure, and receptor re-expression was observed within 4 h of enzyme removal.

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References

19. Allergen-induced modulation of respiratory epithelial cell function has the potential to be exaggerated in this condition in the absence of appropriate control mechanisms.


