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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[1]

Nithiananthan Asokananthan,* Peter T. Graham,* David J. Stewart,* Anthony J. Bakker,* Karin A. Eidne,† Philip J. Thompson,‡ and Geoffrey A. Stewart[2]*

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 Ca^{2+} 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 Ca^{2+} 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.**

Asthma is characterized by marked inflammation with either reversible or fixed airway obstruction, bronchial hyperreactivity, and atopy. In this regard, inflammation induced by interactions between IgE and allergen seems to be central to its development, and allergy to the house dust mite has been shown to be an independent risk factor for the development of this disease (1). The inflammatory responses observed in asthma are complex, but the epithelium has emerged as a critical tissue in its development because it plays a prominent role in regulating, orchestrating, and localizing airway inflammation, as well as being central to repair and remodeling (2, 3). A variety of stimuli are known to induce proinflammatory cytokine and mediator release from airway epithelium, all of which have the potential to contribute to the disease process (4–7).

Recently, we and others have shown that allergens from house dust mite and fungi, but particularly those possessing peptidase activity, are also significant modulators of epithelial function (8–12). The peptidases originating from the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* include the group 1 cysteine proteases (13), the group 3 trypsins (14), the group 6 chymotrypsins (15), and the recently identified group 9 collagenolytic serine proteases (16). These peptides, either individually or in combination, have been shown to increase permeability of epithelial cells in vitro due to their ability to degrade the tight junction proteins ZO-1 and desmoplakin (17, 19), and stimulate airway epithelial cells directly or synergize with IL-4 and IL-13 (12) to produce cytokines such as IL-6 and IL-8. These activities may not only facilitate access of inhaled allergens to dendritic cells below the epithelial layer, but also modulate the function of immune and structural cells in the airway mucosa. With regard to more peptidase-induced cytokine release, the cellular mechanism remains unclear, although members of a recently identified G protein-coupled family of cell surface receptors, designated protease-activated receptors (PARs),[3] have been implicated (10, 20–23), based on biochemical similarities between mite allergens and known endogenous activators of PAR such as thrombin and trypsin. We showed that endogenous peptidases caused the release of cytokines through the activation of PAR receptors on the respiratory epithelium, and that all four members of the PAR family were expressed on respiratory epithelial cells (22). In preliminary experiments designed to investigate this possibility, we showed that activation of PAR-1, PAR-2, and PAR-4, but not PAR-3, was associated with cytokine production as well as prostanoid mediator production and combined, these data provide a robust theoretical framework upon which to investigate the role of PAR in mite allergen modulation of epithelial function. Therefore,

*School of Biomedical and Chemical Sciences, †Western Australian Institute for Medical Research, and ‡Asthma and Allergy Research Institute and School of Medicine and Pharmacology, University of Western Australia, Crawley, Perth, Western Australia

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[2] Address correspondence and reprint requests to Dr. Geoffrey A. Stewart, Department of Microbiology, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009. E-mail address: geoffrey@cyllene.uwa.edu.au

[3] Abbreviations used in this paper: PAR, protease-activated receptor; AM, acetoxymethylester; EYFP, enhanced yellow fusion protein.

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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 termini (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-NH_2 and FTLLR-NH_2; PAR-2, SLIGKV-NH_2 and LSIGKV-NH_2 (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 HeLa, 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 Biotechnology, Charlottesville, VA). Unbound material from the column was eluted with phosphate buffer containing 0.5 M NaCl, and bound material then was eluted after re-equilibration of the column with PBS using water adjusted to pH 11.0 with ammonia. The elute 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 proteolysis using the Azocoll substrate (Calbiochem, 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*′-guanidino benzoate**,** 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*-benzoyl-DL-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 the former source was used in cell culture experiments, whereas that from the latter was used in Ca^{2+} 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-carboxyoxirane-2-carbonyl)-leucyl-amino(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-cm^2 tissue culture flask (Nunc, Naperville, IL) and grown to confluence in Ham's F12 Kaighn's Modification (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 5×10^4 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) CO_2.

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 *Xho*I recognition site. The PAR-1 and PAR-2 primers used were 5′-CAG TCC CTT CTC GAG AGT TAAA CAG-3′ and 5′-GAC CTG GAA CTC GAG ATA GGA GGT C-3′, respectively. The PCR products were digested with *Hin*dIII (site already present in the pRK7) and *Xho*I, 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 1×10^6 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 (determined 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 significance 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 coverslip. Cells were excited at 488 nm and examined using a Bio-Rad confocal 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 experiments designed to study Der p 1- or PAR-induced cytokine secretion at different times during culture, cells were exposed to the optimum concentrations of Der p 1 or peptide, as described previously (22). Culture supernatants 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 potential 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 described previously (20). Briefly, 96-well plates (Maxisorp; Nunc) were coated with 100 μl/$well^{-1}$ of the appropriate Ab (250 ng ml^{-1} in 0.1 M $NaHCO_3/NaCO_3$ 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/$well^{-1}$ (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 secondary Ab (100 μl 250 ng ml^{-1} 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 substrate; 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 (Spectramax 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 (Ca^{2+})

Changes in cytosolic Ca^{2+} concentration were measured using the fluorescent Ca^{2+} 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, Faversham, 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 Ca^{2+} 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 Ca^{2+}. In experiments designed to measure receptor specificity, A549 cells loaded with Indo-1 dye were initially exposed to either peptidases or peptides and then exposed to the second stimulus after ensuring that the calcium flux reached a baseline, or after 3 min if changes in Ca^{2+} were not observed.

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. Bonferoni's correction was used for multiple *t* test comparisons.

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 allergen. 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, although significant 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 peptidase 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. 2*A*), but only significantly reduced IL-6 production with PAR-2-stimulated cells at the 80-nM Der p 1 dose (Fig. 2*B*). 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 Ca^{2+} in the A549 cells (Fig. 3*A*) that were inhibited by pretreatment of the enzyme with the cysteine protease inhibitor for Der p 1, E64 (Fig. 3*B*). Exposure of the A549 cells to 80 nM Der p 1 before either thrombin (Fig. 3*C*) or trypsin (Fig. 3*D*) 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. 3*F*), but not PAR-1 (Fig. 3*E*). Exposure of cells to thrombin (Fig. 3*G*) or trypsin (Fig. 3*H*) before Der p 1 resulted in desensitization to the latter only.

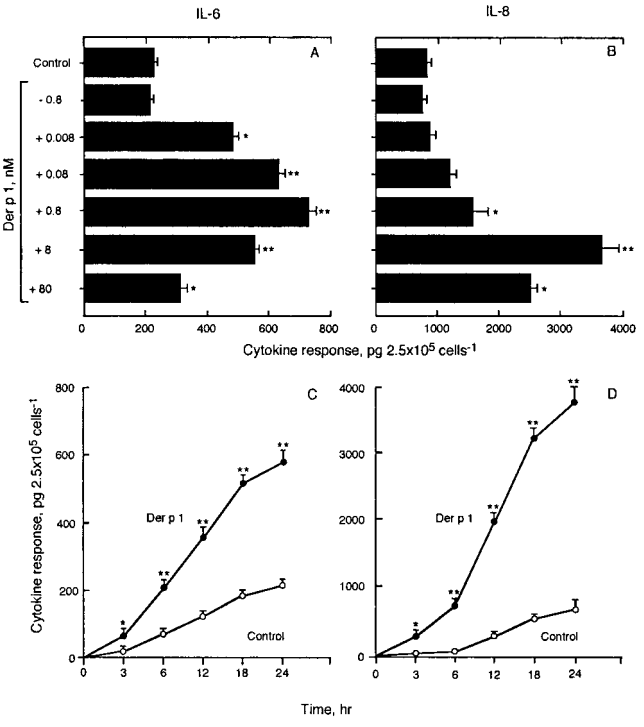

FIGURE 1. Activated Der p 1 promotes cytokine release from the human respiratory epithelial cell line, A549, in a dose (*A*, *B*)- and time-dependent manner (*C*, *D*). In *A* and *B*, cell monolayers were cultured for 24 h with basal medium devoid of serum or additives and then stimulated with increasing concentrations of activated Der p 1. The presence of IL-6 and IL-8 in supernatants was measured by ELISA, and data were expressed as mean pg 2.5×10^5 cells^{-1} ± SEM from three independent experiments performed in quadruplicate. Significant differences in mean Der p 1-induced cytokine responses compared with medium alone (Control) were determined using the Student's *t* test incorporating Bonferoni's correction; *, $p < 0.05$; **, $p < 0.001$. In *C* and *D*, cell monolayers were cultured for 24 h with basal medium devoid of serum or additives and then stimulated with (●) or without (○) Der p 1 (8 nM). Supernatants were collected at 3, 6, 9, 12, 18, and 24 h poststimulation, and cytokine assays were performed. Data are expressed as above from three independent experiments performed in quadruplicate. The concentrations of cytokine secreted in response to Der p 1 at all time points tested were significantly different from the medium-induced responses at the corresponding time points, as determined using ANOVA and the Student's *t* test incorporating Bonferoni's correction; *, $p < 0.05$; **, $p < 0.001$.

PAR-2, but not PAR-1 cDNA-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. 4*A*), the other cell lines produced cytokines in response to both Der p 1 and PAR-2 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) cDNA 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. 4*B*). These agonists had little significant influence on cytokine release from HeLa cells transfected with vector alone (Fig. 4*C*), although constitutive production appeared elevated compared with nontransfected cells. Examination of medium from

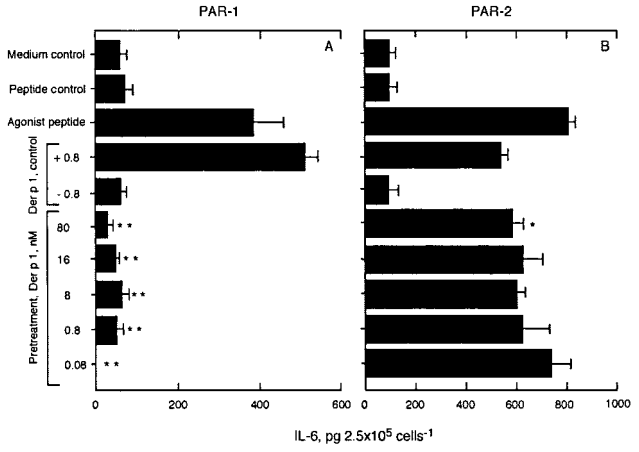

FIGURE 2. Activated Der p 1 pretreatment of A549 cells inhibits PAR-1, but not PAR-2 agonist peptide-induced cytokine release. Cell monolayers were cultured for 24 h with basal medium devoid of serum or additives, then stimulated with increasing concentrations of Der p 1 for 2 h at 37°C. Cells were then 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 2.5×10^5 cells^{-1} $\pm$ SEM from three independent experiments performed in quadruplicate. Significant differences in means between Der p 1 pretreatment 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; $*$, $p < 0.05$.

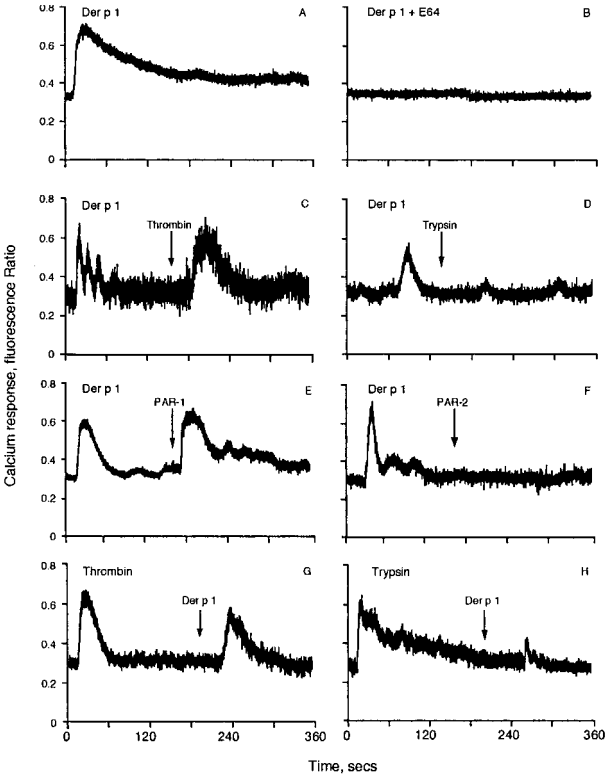

FIGURE 3. Desensitization of PAR in A549 cells by treatment with Der p 1. A549 cells were loaded with Indo-1 AM and incubated with Der p 1 (80 nM) (*A–F*). Cells were then incubated with either thrombin (20 U ml^{-1}) (*C*) or trypsin (0.4 nM) (*D*) or with 400 μM of either PAR-1 (*E*) or PAR-2 (*F*) 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^{-1}) (*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).

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. 5*A–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 a few seconds. 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. 6*A–D*) or PAR-2/EYFP cDNA (Fig. 6*E–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. 6*H*). Control experiments performed using agonist peptide showed PAR-1 receptors were capable of internalization, which occurred within 30 min of exposure (Fig. 6*D*).

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

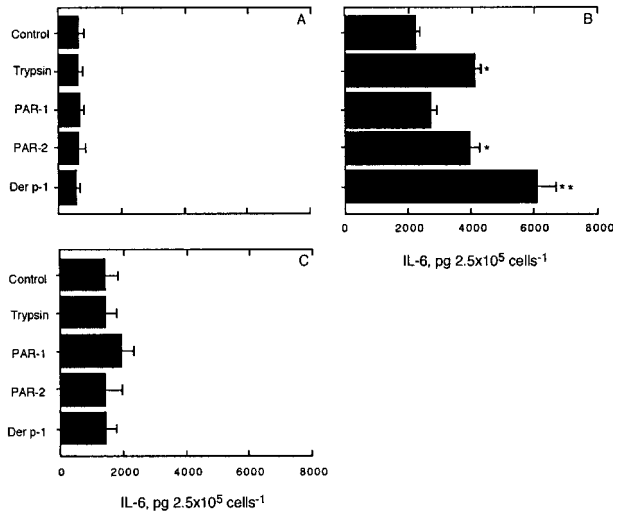

FIGURE 4. Der p 1 and PAR-2, but not PAR-1 agonist peptides induce cytokine release from PAR-2-transfected HeLa cells. Nontransfected (*A*), PAR-2-transfected (*B*), or vector-transfected (*C*) cell monolayers were cultured with basal medium devoid of serum or additives for 24 h, then stimulated with 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 $\times$ 10^5 cells^{-1} $\pm$ 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; $*$, $p < 0.05$.

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

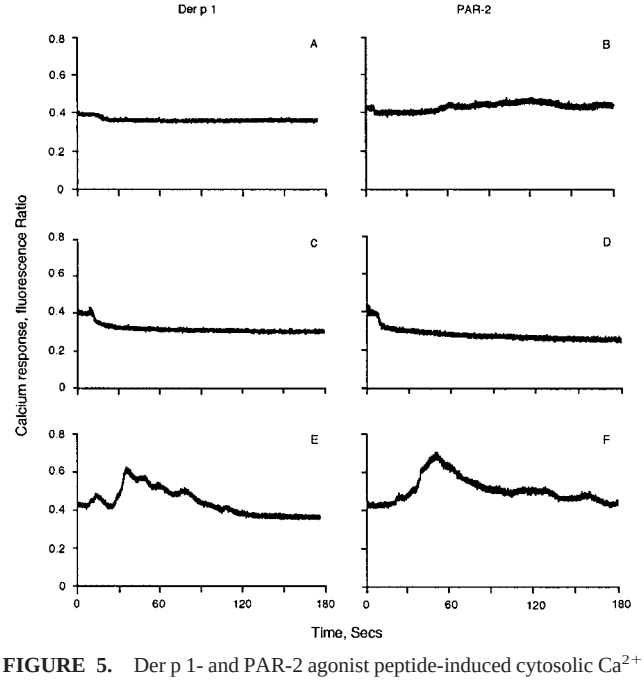

FIGURE 5. Der p 1- and PAR-2 agonist peptide-induced cytosolic Ca^{2+} responses in transfected cells. Nontransfected HeLa cells (*A*, *B*), vector only-transfected (*C*, *D*), or PAR-2-transfected HeLa cells (*E*, *F*) were loaded with Indo-1 AM and incubated with either 400 μM agonist peptide or Der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

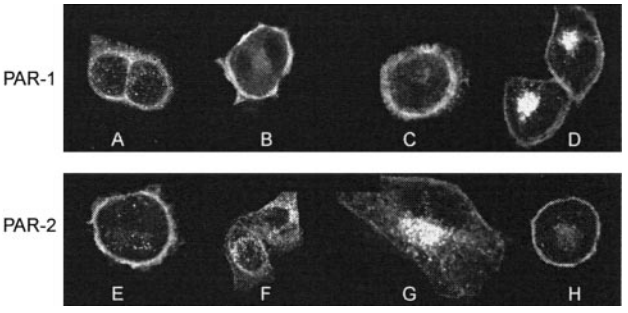

FIGURE 6. Der p 1 causes internalization of EYFP-tagged PAR-2, but not PAR-1 in HeLa cells. PAR-1/EYFP-transfected HeLa cells (*A*) were exposed to activated Der p 1 (80 nM) for 10 (*B*) and 30 min (*C*). Transfected cells were also exposed to PAR-1 agonist peptide (400 μM) for 30 min (*D*) without any 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*).

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

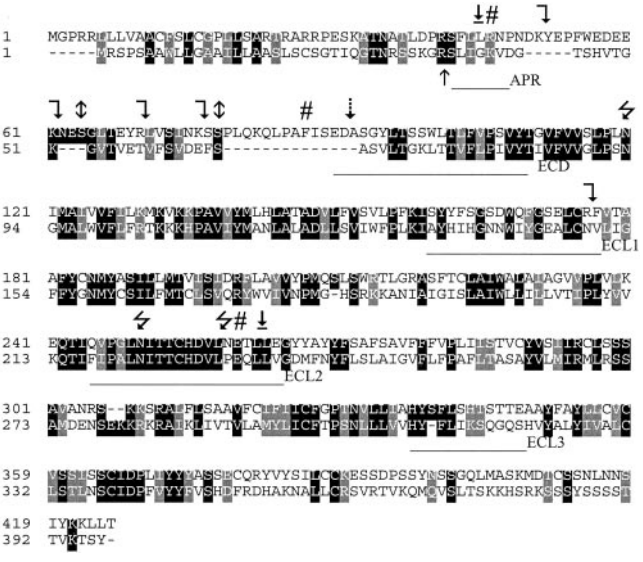

FIGURE 7. Potential extracellular cleavage sites in PAR-1 and PAR-2, based on data obtained with claudin (crooked arrow), occludin (arrow with line beneath) (16), α_1-antiprotease (dotted arorow) (35), CD23 (double arrow) (36), as well as synthetic substrates with C-terminal arginine or lysine (bent 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 η, and the critical residues involved in activation are marked with #. The underlined regions indicate the residues corresponding to the agonist peptide region (APR), and the NH_2-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 occludin (between L-L, G-T, Y-G) and claudin (between L-L, N-L, L-N, Y-G) (18), natural protein substrates such as α_1-antiprotease (between (^{12}D-^{13}T, ^{6}D-^{7}A) (40) and CD23 (between ^{298}E-^{299}S, ^{155}S-^{156}S) (41), and synthetic substrates containing C-terminal R or K (26, 42). Using this information, several possible sites in the extracellular NH_2-terminal domain, and first and second extracellular loop regions of PAR-1 known to be involved in the activation process can be identified (reviewed in Refs. 43 and 44) (Fig. 7). In this regard, point and deletion mutation studies have highlighted the importance of the region comprising residues ^{83}Q to ^{89}S in NH_2-terminal domain (43, 45), suggesting that Der p 1 cleavage may involve, at the very least, ^{91}D-^{92}A. The ECL1 and 2 regions of PAR-1 also contain several Der p 1 cleavable sites, one of which namely, ^{258}L-^{259}N, is absent from PAR-2 and, if cleaved in PAR-1, would result in the direct loss of the essential ^{256}D (43) as well as, perhaps, altering the receptor conformation in the region of ^{260}E, a residue also thought to be important in the activation process (46).

In conclusion, we have demonstrated that the Der p 1-induced IL-6 and IL-8 release from A549 cell line was associated with PAR-2, but not PAR-1 activation using a range of techniques. Further, we showed that the enzyme appeared to inactive PAR-1, and we currently are determining the susceptibility of peptides representing appropriate regions of PAR-1 to confirm our observations. At this stage, it is not clear whether Der p 1 also modulates PAR-3 or PAR-4 known to be present on respiratory epithelium, but given the involvement of PAR-4 in cytokine release (22), such studies are warranted. Our data, and those showing that mite serine protease allergens Der p 3 and Der p 9 are capable of cleaving at the activation sites of PAR-1 and PAR-2 (23), suggest that PAR-mediated cytokine release from respiratory epithelium may be induced by all known mite peptidases. However, the physiological consequences of either PAR-2 activation or PAR-1 inactivation by mite allergenic peptidases are unclear at present, but our findings, together with those showing degradation of epithelial cell tight junction proteins (18, 47), indicate the potential of mite and similar enzymes to modulate a variety of epithelial cell functions when inhaled and, thus, contribute to the sensitization or inflammatory processes in diseases such as asthma. In this regard, we have demonstrated that PAR-2 expression is elevated in bronchial biopsies from asthmatic subjects compared with control donors (21), suggesting that allergen-induced modulation of respiratory epithelial cell function has the potential to be exaggerated in this condition in the absence of appropriate control mechanisms.

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