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The House Dust Mite Allergen *Der p*1 Catalytically Inactivates α_1-Antitrypsin by Specific Reactive Centre Loop Cleavage: A Mechanism That Promotes Airway Inflammation and Asthma

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*Der p*1, a cysteine proteinase derived from the house dust mite (HDM) *Dermatophagoides pteronyssinus,* is a major component of the allergic immune response in HDM atopic individuals. Recent evidence suggests that cysteine proteinase activity is important in the disease process as it increases the permeability of the allergen in the respiratory tract and disrupts the regulation of IgE synthesis. *Der p*1 is found in high concentrations in the faecal pellets of mites which are aerosolised and inhaled via the respiratory tract. The serine proteinase inhibitor, α_1-antitrypsin, protects the lower respiratory tract against damage by proteinases released in the lung during inflammation. *Der p*1 catalytically inactivates α_1-antitrypsin by a thiol-dependent mechanism involving specific cleavage of the reactive centre loop and we propose that this mechanism may be important in the pathogenesis of asthma.

The house dust mite (HDM) is a major contributor to the rising incidence of asthma. *Der p*1, a cysteine proteinase derived from the species *Dermatophagoides pteronyssinus* is a major component of the allergic immune response in HDM atopic individuals (1,2). *Der p*1 is present in the faecal pellets of mites and the protein is inhaled into the respiratory tract (1). It is at this site that the allergen is thought to exert its major effect. Enzyme activity appears to be important in the disease process. This is supported by recent observations which demonstrate increased permeability of the allergen in the respiratory tract (3,4) and cleavage of the low affinity receptor for IgE, resulting in a soluble form of the receptor which potentially enhances the synthesis of IgE (5), the hallmark of an allergic reaction.

The serine proteinase inhibitor (serpin) α_1-antitrypsin, protects the lower respiratory tract against damage by proteinases released during inflammation (6). α_1-antitrypsin deficiency is associated with predisposition to developing chronic pulmonary disease (7) and childhood asthma (8). We have explored the interaction of *Der p*1 with α_1-antitrypsin.

MATERIALS AND METHODS

Materials. 4C1 monoclonal antibody to *Der p*1 from Indoor Biotechnologies Ltd., Deeside, United Kingdom; dust mite extracts from SmithKline Beecham, England; E-64c from Sigma Chemical Co., Poole, Dorset, UK. 2,2-Dipyridyl disulphides (Aldrich) was purified and characterised as described previously (9). α_1-antitrypsin was prepared by 50 and 75% ammonium sulphate fractionations followed by thiol and anion exchange as described previously (10). α_1-antichymotrypsin was isolated using a modification of a published method (11). In both cases the purified protein migrated as a single species on SDS and non-denaturing polyacrylamide gel electrophoresis and was greater than 85% active as an inhibitor of bovine α-chymotrypsin.

Der p1 purification. 40 mg of monoclonal antibody to *Der p*1, 4C1 was coupled to 2 g cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (12). The affinity column was washed with 100 ml of phosphate

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buffered saline containing 0.5 M NaCl, pH 7.4. Lyophilised mite extract containing a total of 10 g of protein was resuspended in the same buffer and applied to the column. Elution was performed with 5 mM glycine in 50% ethylene glycol; pH 10.0 (the pH was adjusted with 1 M NaOH). Fractions were collected and the absorbance monitored at 280 nm. The purity of the preparation was confirmed by SDS-PAGE and by N-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Foster City, CA: the sequence obtained (TNACSINGNA) matches the published sequence (13)).

Der p1 serpin incubations. Reaction mixtures containing *Der p*1, α_1-antitrypsin and α_1-antichymotrypsin (in varying amounts from 0.05 to 1:1 mol/mol) were incubated at 37°C in 10 mM phosphate buffer pH 6.8 or 7.4 for 2 hours in the presence of 20 mM cysteine and analysed. All subsequent experiments were performed with enzyme α_1-antitrypsin ratios of 0.1 to 1. The irreversible inhibitors E-64 and E64c (100 μM) were pre-incubated with 2 μM of *Der p*1 prior to incubation with either α_1-antitrypsin or α_2-antichymotrypsin and enzyme activity was monitored in the presence and absence of inhibitor. The concentration of active enzyme was determined by the measurement of protein concentrations using a Pierce Bichinchoninic Acid microassay kit and active site titration with E-64.

Studies using 2,2′-dipyridyl disulphide. Inhibition was carried out using 100 μM inhibitor and reactivation was achieved using 20 mM cysteine, both reactions being carried out at pH 7.4.

RESULTS AND DISCUSSION

The structure of the serpin α_1-antitrypsin is based on a dominant A β-pleated sheet and a mobile reactive centre loop that interacts with and inhibits cognate enzymes (14). *Der p*1 efficiently inactivated antitrypsin after incubation at 0.1:1 (w/w) ratios at 37°C for two hours (Fig. 1). Following incubation with *Der p*1 α_1-antitrypsin migrated with an electrophoretic mobility of the reactive loop cleaved protein (Fig. 1) and no complexes were seen on SDS polyacrylamide gels even after silver staining. The absence of complexes precludes a stoichiometric interaction and favours a catalytic mechanism for loop cleavage. Figure 2 shows the reactive loop residues. N-terminal sequencing of cleavage fragments separated by HPLC (15) identified the reactive loop cleavage sites P_4–P_5 and P_3–P_4 in a molar ratio of approximately 4:1. Cleavage site were also identified at the N-terminus as follows: ^{12}Asp ↓ Thr-Ser-His-His-Asp and ^{6}Asp ↓ Ala-Ala-Gln-Lys-Thr in a ratio of about 2.5:1. Definitive evidence that the cleavage of the reactive loop by *Der p*1 is due to cysteine proteinase activity is provided by the inhibition/reactivation cycle using 2,2′-dipyridyl disulphide followed by 20 mM cysteine (16,17) and this is supported by the irre-

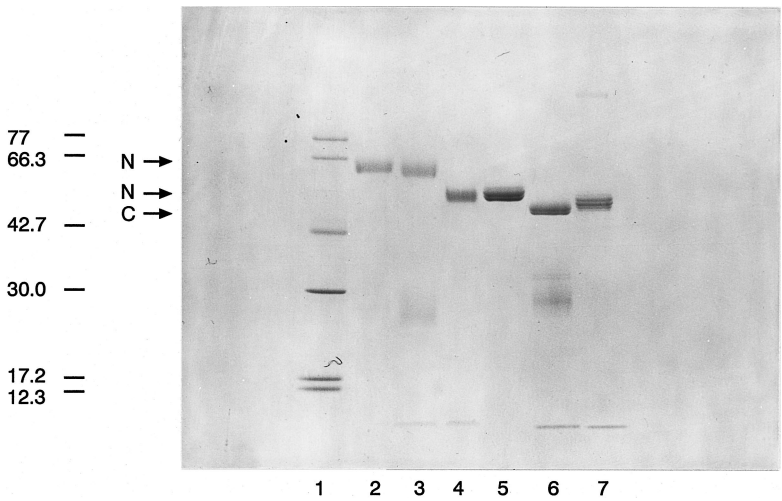

FIG. 1. 7.5–15% w/v SDS-PAGE to show the cleavage of antitrypsin by the house dust mite antigen *Der p*1. α_1-antitrypsin, or α_1-antichymotrypsin, was incubated with *Der p*1 at 1: 0.1 w/w ratios at 37°C for two hours in phosphate buffered saline and 20 mM cysteine. All lanes contain 5 μg protein. Lane 1, molecular weight markers; lane 2, antichymotrypsin control; lane 3, antichymotrypsin with *Der p*1; lane 4, antichymotrypsin cleaved at reactive centre loop with snake venom 5 μg; lane 5, antitrypsin control; lane 6, antitrypsin incubated with *Der p*1; lane 7, antitrypsin cleaved at P_4–P_5 of the reactive centre loop with *Staph aureus* V8 proteinase.

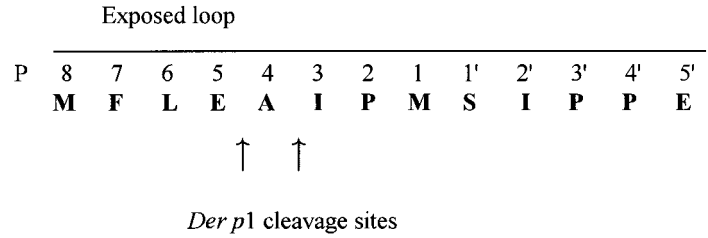

FIG. 2. Reactive centre loop sequence of α_1-antitrypsin and *Der p*1 cleavage sites.

versible inhibition observed using E-64c (16). The major cleavage site of α_1-antitrypsin was identical to that observed with *Staph aureus* V8 proteinase which cleaves at P_4–P_5 (18) Reactive loop cleavage did not occur with a closely related protein, α_1-antichymotrypsin, suggesting that this reaction is highly specific.

These studies have important physiological consequences, as the concentration of dust mite allergen *Der p*1 can exceed 10 μg/gram of dust, and consequently the concentration in the lung could result in significant inactivation of α_1-antitrypsin. Moreover, reactive loop cleaved α_1-antitrypsin has been shown to be chemotactic for human neutrophils *in vivo* (19). The significance of these results is underscored by recent observations that patients with fatal asthma have a predominantly neutrophil, rather than an eosinophil, mediated inflammatory response (20). Inactivation of the major natural inhibitor of neutrophil enzymes, α_1-antitrypsin, will exacerbate tissue damage and inflammation and so accentuate asthma.

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