

Short paper

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Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23)

The nature of the proteases that cleave CD23 *in vivo* is of considerable interest, but remains unknown. Here, we demonstrate that *Der p I*, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, cleaves CD23 from the surface of cultured human B cells (RPMI 8866 B cell line). The cleavage of the receptor from the B cell surface was associated with a parallel increase in soluble CD23 (sCD23) in the culture supernatant. Furthermore, the proteolytic effect of *Der p I* was specific for CD23, since none of the other B cell markers tested (CD20, HLA-DR, CD71 and CD49d) were affected. Labeled antibody experiments and protease inhibition assays clearly demonstrate that *Der p I* is a cysteine protease that directly cleaves a 25-kDa fragment of CD23. These data suggest that the cysteine protease *Der p I*, in addition to being highly immunogenic, may up-regulate IgE synthesis by virtue of its ability to cleave CD23.

1 Introduction

The low-affinity receptor for IgE (FcεRII), also known as CD23, is a 45-kDa marker for activated B cells. It is also found on other cells, including eosinophils, follicular dendritic cells, neutrophils, macrophages and platelets. CD23 is a calcium-dependent type II integral membrane protein which belongs to the lectin family of adhesion molecules. The extracellular portion of this receptor consists of a lectin homology domain flanked on the N-terminal side by an α-helical coiled-coil stalk which mediates trimer formation. IgE binds to CD23 via the lectin domain [1].

Soluble fragments of CD23 (sCD23) are thought to be involved in stimulating the growth and differentiation of the precursors of plasma cells, T cells and basophils, thereby initiating both humoral and cellular immune responses [1]. The expression of CD23 on B lymphocytes is of particular importance, since it is involved in the regulation of IgE synthesis [2–5]. The modulatory effect of CD23 on IgE synthesis has been demonstrated *in vivo* in experimental animals treated with anti-CD23 antibodies [2] and in those genetically deficient in CD23 [3–5]. When CD23 is not occupied by IgE, it is susceptible to proteolytic cleavage, releasing sCD23 fragments which are known to up-regulate IgE synthesis in an autocrine fashion [6]. The binding of IgE to CD23 apparently not only protects the receptor against proteolytic cleavage [7], but also allows membrane CD23 to deliver a negative IgE regulatory signal to the B lymphocyte [3, 8].

Enzymatic proteolysis of membrane CD23 first leads to the formation of unstable 37-kDa and 33-kDa fragments

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which are subsequently cleaved to form a stable 25-kDa product [9]. Although it has been suggested that CD23 has autoproteolytic activity [10], we are still unaware of what proteases cleave membrane CD23 *in vivo*. By virtue of its ability to bind IgE, CD23 is exposed to a high concentration of allergens, many of which, such as those of house dust mite *Dermatophagoides pteronyssinus*, are potent proteases [11]. However, no attempts have so far been made to link the proteolytic activity exhibited by such allergens to CD23 cleavage.

Here, we demonstrate that *Der p I*, a major allergen of the house dust mite, cleaves CD23 from the surface of cultured human RPMI 8866 B cells.

2 Materials and methods

2.1 Antibody reagents

FITC-labeled mouse monoclonal anti-CD23 antibody Bu38 was obtained from The Binding Site (Birmingham, GB) and antibody EBVCS1, which was generated by Dr. B. Sugden (Madison, WI), was a kind gift from Prof. J. Gordon (Birmingham, GB). FITC-labeled anti-CD20 (L27), anti-HLA-DR (L243) (Becton Dickinson, Oxford, GB), anti-CD71 (Ber-T9) (Dako, Buckinghamshire, GB), anti-CD49d (HP2.1) (Immunotech, Westbrook, ME) and ExtrAvidin (Sigma, Poole, GB) were purchased.

2.2 *Der p I* preparation

Der p I was purified by affinity chromatography using monoclonal anti-*Der p I* antibody (4C1, Indoor Biotechnologies, Clwyd, GB) as described [12]. The purity of the preparation was assessed by silver-stained SDS-12% PAGE analysis, and its identity confirmed by N-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Foster City, CA): the sequence obtained (TNACSSINGNA) matches the published sequence of *Der p I* [13].

2.3 Monitoring CD23 expression on RPMI 8866 B cells

FITC-labeled mouse monoclonal anti-CD23 antibody preparation (2 µg/ml; Bu38 or EBVCS1) were used to assess CD23 expression on human RPMI 8866 B cells (a kind gift from Prof. J. Gordon). Bu38 recognizes the lectin domain and EBVCS1 recognizes the stalk region between the 25-kDa and 37-kDa fragments. Thus, Bu38 recognizes the intact molecule and all soluble fragments [14], while EBVCS1 recognizes the intact molecule and the residual membrane bound portion after cleavage of the 25-kDa fragment (sCD23) (J. Gordon, personal communication). The expression of other B cell surface markers (CD20, HLA-DR, CD71 and CD49d) was monitored using appropriate FITC-labeled antibodies. FITC-labeled mouse monoclonal anti-KLH (Becton Dickinson, Oxford, GB) was used as an isotype (IgG1) control for Bu38.

Cells were analyzed on a FACScan (Becton Dickinson, Oxford, GB) with a linear fluorescence setting of 660 volts. The fluorescence (FL1) profile versus forward scatter (FSC) was used to monitor the cells, and the amplification scale was altered according to the level of fluorescence. For each sample, 4000 events were collected and then analyzed using the flowMATE programme (Dako).

2.4 CD23 cleavage assay

Der p I was pre-incubated (15 min at 37°C) with or without 5 mM cysteine and added to $2 \times 10^5 - 3 \times 10^5$ RPMI

8866 cells in a total volume of 200 µl RPMI 1640 + 10 mM Hepes. The mixture was then incubated (1 h at 37°C) and the cells, collected by centrifugation, were washed in RPMI 1640 + 10 mM Hepes and incubated (30 min at room temperature) with FITC-conjugated anti-CD23 monoclonal antibody. The supernatant was collected from cultured RPMI 8866 cells treated with *Der p* I and diluted 1/5 for sCD23 determination by ELISA (The Binding Site). In this ELISA, there was no cross-reactivity between *Der p* I and sCD23.

2.5 Enzyme inhibition assay

Der p I (100 µl; 5 µg/ml) was pre-incubated (30 min at 37°C) with 10 µl either E64 [*L*-trans-epoxysuccinyl-leucylamido (4-guanidino) butane; Sigma] or α 1-anti-trypsin (a kind gift from Dr. D. Lomas, Cambridge, GB and Prof. N. Kalsheker, Nottingham, GB) and then added to the RPMI 8866 cells as described above. The activity of the α 1-anti-trypsin preparation was ascertained by active-site titration against bovine chymotrypsin (D. Lomas, personal communication). To demonstrate total cleavage of α 1-anti-trypsin, 0.25 µg *Der p* I was incubated for 2 h at 37°C with 5 µg α 1-anti-trypsin in a total volume of 10 µl.

3 Results and discussion

We have affinity-purified *Der p* I from dust mite extract and confirmed its identity by N-terminal sequencing. The

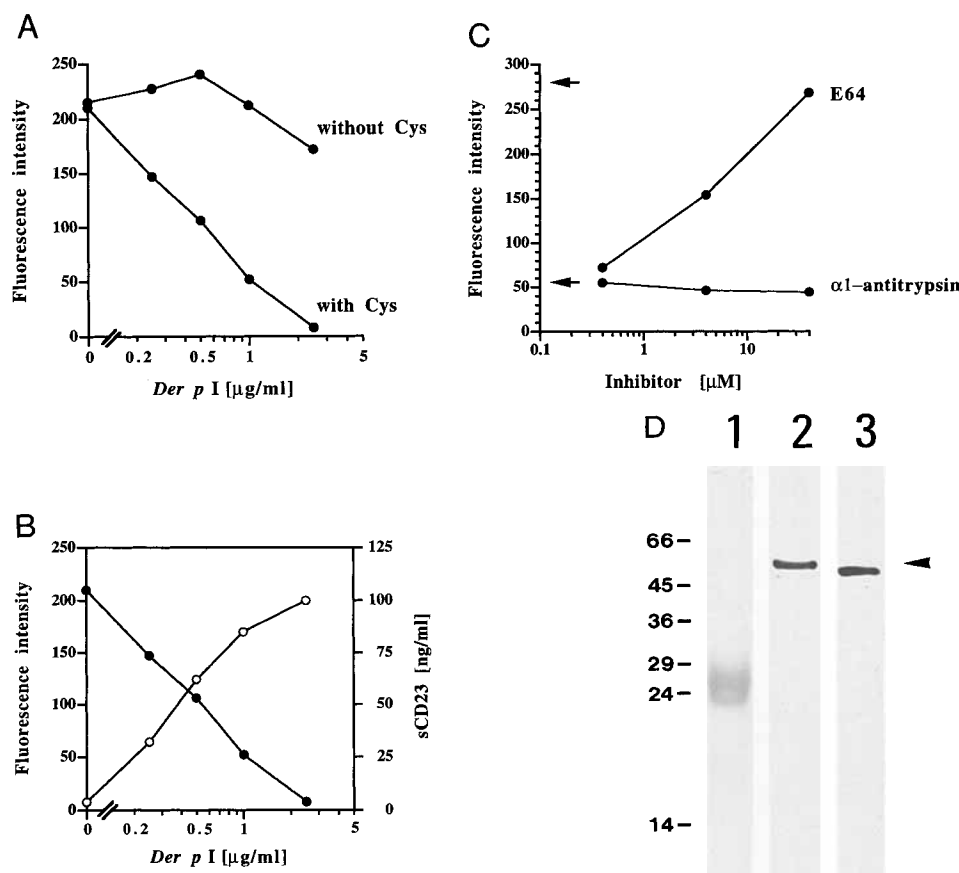


Figure 1. CD23 expression on RPMI 8866 human B cells using FITC-labeled mouse monoclonal anti-CD23 antibody Bu38. Data presented are representative of three replicate experiments; in A to C, the standard error was less than 5%. (A) Dose and cysteine dependency of CD23 cleavage by *Der p* I. (B) Cleavage of membrane CD23 was associated with a parallel dose-dependent release of sCD23 in culture supernatant. (C) Class-specific inhibitor of cysteine proteases prevents membrane CD23 cleavage by *Der p* I. E64 completely inhibits cleavage of CD23 by *Der p* I, whereas no such inhibitory effect was demonstrable with α 1-anti-trypsin. The arrows indicate the level of CD23 expression in the absence (upper arrow) and presence (lower arrow) of *Der p* I. (D) Silver-stained SDS-PAGE analysis of the *Der p* I preparation, human α 1-anti-trypsin and the effect of *Der p* I on α 1-anti-trypsin. The gel shows single bands for *Der p* I (lane 1) and α 1-anti-trypsin (lane 2). Incubation of *Der p* I with α 1-anti-trypsin results in the cleavage of a large fragment (arrow) from α 1-anti-trypsin (lane 3). The molecular mass standards are indicated on the left.

Der p I preparation was tested for its ability to cleave proteolytically CD23 expressed on cultured RPMI 8866 B cells, using FITC-labeled monoclonal anti-human CD23 (Bu38). The data show that in the presence of 5 mM cysteine, *Der p I* cleaves membrane CD23 in a dose-dependent manner, thereby releasing sCD23 into the culture supernatant (Fig. 1A, B). More than 60 % of this cleavage occurred in the first 30 min of the reaction time. The proteolytic activity of *Der p I* was inhibited by E64, a cysteine protease inhibitor [15], but not by α 1-anti-trypsin (a serine protease inhibitor), thereby confirming the cysteine protease nature of *Der p I* (Fig. 1C). We have in fact demonstrated that *Der p I* completely cleaves α 1-anti-trypsin (1:10 molar ratio) to yield a degradation pattern (Fig. 1D) similar to that generated by papain [16], a well-characterized cysteine protease. This observation has serious implications *in vivo*, since proteolytic cleavage of α 1-anti-trypsin by *Der p I* will weaken innate immunity on mucosal surfaces.

To investigate the enzymatic specificity of *Der p I* for CD23, we monitored the expression of other B cell markers following treatment with 2.5 μ g/ml (final concentration) *Der p I*. At this *Der p I* concentration, which has been shown to give maximum cleavage of CD23 (Fig. 1A), there was no significant loss of CD20, HLA-DR, CD71 and CD49d expression (Fig. 2).

To gain insight into the *Der p I* cleavage site on CD23, we monitored the proteolytic cleavage process using Bu38 and EBVCS1 monoclonal anti-CD23 antibodies, which are directed against the lectin domain and the stalk region, respectively. Thus, Bu38 detects all fragments down to 25 kDa [14], whereas EBVCS1 recognizes only fragments larger than 25 kDa (J. Gordon, personal communication). The results show that *Der p I* cleaves CD23 at a site close to the lectin domain, since EBVCS1 antibody was still capable of binding to the residual membrane-bound portion of the receptor (Fig. 3). However, at a *Der p I* concentration of greater than 1 μ g/ml, there also appeared to be

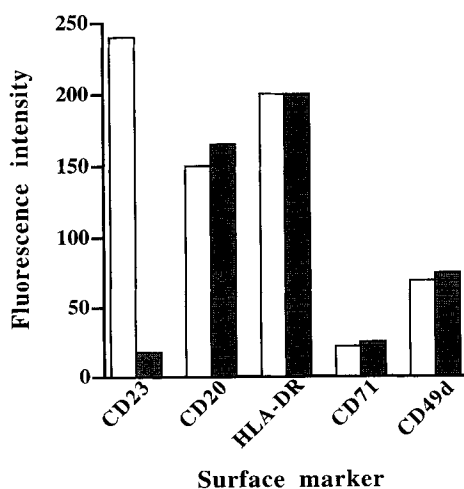


Figure 2. The proteolytic effect of *Der p I* is specific for CD23. Paired results represent the expression of markers in the absence (open bars) and presence (solid bars) of *Der p I*. Data presented are representative of three replicate experiments; the standard error was less than 5 %.

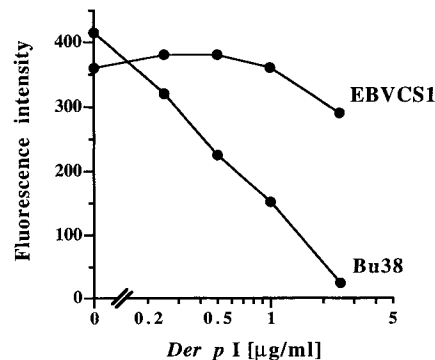


Figure 3. *Der p I* preferentially cleaves CD23 at a site close to the lectin domain. RPMI 8866 cells were treated with *Der p I*, and the expression of membrane CD23 was monitored using Bu38 and EBVCS1 monoclonal anti-CD23 antibodies. The experiment demonstrates that *Der p I*, at concentrations of up to 1 μ g/ml, preferentially releases a 25-kDa fragment of CD23. Data presented are representative of three replicate experiments; the standard error was less than 5 %.

some cleavage of CD23 fragments larger than 25 kDa. Since the highest concentration of *Der p I* (2.5 μ g/ml) resulted in complete loss of Bu38 binding and only partial loss of EBVCS1 binding, the preferred site of initial cleavage of CD23 by *Der p I* appears to be close to the lectin domain.

The demonstration that *Der p I* proteolytically cleaves membrane CD23 raises the question of the role of IgE in this process. We have found that prior occupancy of CD23 by IgE protects it from proteolytic attack by *Der p I* (data not shown), a finding which is in line with the notion that IgE protects CD23 from enzymatic cleavage [7]. On the other hand, IgE specific to *Der p I* could target *Der p I* to B lymphocytes and other CD23-bearing cells (e.g. eosinophils), thereby helping to build a high concentration of this allergen on the cell surface. Cleavage of CD23 by *Der p I* may lead to up-regulation of IgE synthesis through both sCD23 release [6] and disruption of the negative IgE regulatory signal delivered by membrane CD23 to the B lymphocyte [3, 8].

4 Concluding remarks

Soluble CD23, reported to induce IgE-producing B cells to become plasma cells [17, 18], is one of the signals required for IgE synthesis. In this paper, we demonstrated that *Der p I*, an exogenous cysteine protease, cleaves membrane CD23. *Der p I* elicits IgE antibody responses in 80 % of patients suffering from dust mite allergy [19, 20], and there is evidence that such patients have high circulating levels of sCD23 *in vivo* [21, 22]. This ubiquitous inhaled allergen is clearly highly immunogenic; however, its immunogenicity may also be due in part to its enzymatic activity. It has indeed been demonstrated that the allergenicity of papain, a cysteine protease showing sequence homology with *Der p I* [13], is highly related to its enzymatic activity [23].

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