Biochimica et Biophysica Acta xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Relationship between the magnitude of IgE production in mice and conformational stability of the house dust mite allergen, Der p 2

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ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 20 March 2016
Accepted 17 April 2016
Available online xxxx

Keywords:
ADAs (anti-drug antibodies)
Allergen
Conformational stability
Der p 2
Immunogenicity

ABSTRACT

Background: Protein antigens are degraded by endosomal protease in antigen presentation cell. T cells recognize peptides derived from antigen proteins bound to class II major histocompatibility complex molecules. We previously reported that an increase in the conformational stability of an antigen depressed its immunogenicity. However, there is little information on antigens with differences in molecular properties such as net charges and molecular weight.
Methods: Denaturation experiments against guanidine hydrochloride. The serum IgE levels to protein antigens at 35 days after the first immunization analyzed using ELISA.
Results: The Der p 2 mutations in which Ile13 is mutated to Ala (I13A) and Ala122 is mutated to Ile (A122I) were shown to have lower and higher conformational stability than the wild-type, respectively, by denaturation experiments. The amount of IgE production by the less stable I13A mutant was higher and that of the stable A122I mutant was lower than that of the wild-type.
Conclusion: Our results suggest that the increased conformational stability of Der p 2 depressed the IgE production in mice.
General significance: These findings should provide a milestone for the engineering of allergen vaccines.
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1. Introduction

Various therapeutic proteins—including erythropoietin, insulin proteins, growth hormones and cytokines—have been shown to provide unique and effective treatments for human diseases [1,2]. In addition, several therapeutic immunoglobulins are currently proving to be blockbuster drugs in the pharmaceutical industry. Nonetheless, repeated administration of protein therapeutics, whether natural or recombinant, often leads to the induction of undesirable anti-drug antibodies (ADAs), which can neutralize the effect of the drug [3–6]. In the mammalian immune system, antigen-specific $CD4^+$ T-cells recognize antigen-derived peptides. The antigen peptides are produced by lysosomal protease and efficient lysosomal degradation favors the production of peptides for MHC class II molecules [7–11]. Proteins in solution usually exist in equilibrium between a native and denatured state. Proteases preferentially digest proteins in an unfolded state rather than those in a folded state [12–15]; thus, the conformational protein stability may be related to intracellular antigen processing. In previous studies using modified hen egg lysozyme (HEL) as a model protein antigen, we found that the T-cell triggering response was governed by the degree of conformational stability of the protein antigen [16,17]. Moreover, it was demonstrated that the amounts of IgG production against HEL with different stabilities in mice were inversely and linearly correlated with the free-energy changes of protein unfolding [18]. These findings were supported by other studies using snake toxin [19].

There are many antigens with differences in molecular properties, such as net charges and molecular weight. Therefore, we still need to verify the relation between the conformational stability of protein antigens and the immune response using various antigens. House dust mites are a major source of aeroallergens for patients with allergic asthma [20]. Der p 2, which has a molecular weight of about 14 kDa, is one of the major allergens of *Dermatophagoides pteronyssinus* (Der p). Since it has structural homology with MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signaling complex [21,22], Der p 2 induced allergy in MD-2-deficient mice [23]. In this study,

Abbreviations: Der p, *Dermatophagoides pteronyssinus*; HEL, hen egg lysozyme; CD, circular dichroism.

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http://dx.doi.org/10.1016/j.bbagen.2016.04.014
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Please cite this article as: H. Nakamura, et al., Relationship between the magnitude of IgE production in mice and conformational stability of the house dust mite allergen, Der p 2, Biochim. Biophys. Acta (2016), http://dx.doi.org/10.1016/j.bbagen.2016.04.014

we examined whether the relationship between the magnitude of IgE production and conformational stability was applicable to the house dust mite allergen, Der p 2.

2. Materials and methods

2.1. Construction of the expression vector

The *Der p 2* gene was obtained by PCR using long primers and was inserted between the *NdeI* and *EcoRI* sites of the pET22b vector (Novagen, Madison, WI), just downstream of the T7 promoter. Site-directed mutagenesis of the *Der p 2* gene was performed by PCR, according to previously described methods [24]. Mutations were confirmed by DNA sequence analysis.

2.2. Expression, refolding, and purification of Der p 2

Der p 2 was overexpressed in *Escherichia coli* BL21 cells harboring a recombinant plasmid constructed using a pET22b expression vector (Novagen). The transformant cells were grown at 37 °C in 1 L of LB medium containing 100 μg/mL ampicillin. The culture was allowed to grow until mid-log phase (OD_{600} = 0.5–1.0) and the expression of Der p 2 was induced by addition of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and incubation for 3 h. The cultured cells were harvested by centrifugation for 7 min at 8000 rpm. The pellets were suspended in 50 mL of 0.1 M Tris–HCl buffer, pH 8.5, containing 10 mM EDTA, and then sonicated twice for 2 min each time in an ice-water bath. The mixture was centrifuged for 20 min at 12,000 rpm. The precipitates were resuspended in 7 mL of 0.1 M Tris–HCl buffer, pH 8.5, containing 10 mM EDTA. After addition of DNase I (final concentration of 5 μg/mL), RNase A (final concentration of 20 μg/mL) and $MgCl_2$ (final concentration of 10 mM), the solution was incubated at 40 °C for 1 h to decompose nucleic acids. Then sodium chloride was added to the solution to a final concentration of 0.8 M, and the mixture was centrifuged. The precipitates were suspended in 160 mL of 6 M guanidine solution (0.1 M Tris–HCl buffer, pH 8.5, containing 10 mM EDTA and 6 M guanidine hydrochloride) and incubated at 4 °C for 12 h. The folding of Der p 2 was performed by dialyzing against 4 L of 20 mM Tris–HCl buffer, pH 8.5, containing 1 mM EDTA without guanidine hydrochloride at 4 °C, three times for 4 h each. After the refolding reaction, the protein solution was dialyzed against 50 mM sodium acetate buffer, pH 4.0, and applied to a cation exchange (SP-Toyopearl) column (2 cm × 25 cm). The column was eluted with a gradient of 300 mL of 50 mM sodium acetate buffer, pH 4.0, and 300 mL of the same buffer containing 1.5 M NaCl. The protein fraction was collected and lyophilized after dialysis against 1% acetic acid.

2.3. CD spectra of the folded wild-type and mutant Der p 2

The folded and purified wild-type and mutant Der p 2 were dialyzed against ultrapure water and prepared at a concentration of 0.1 mg/mL with ultrapure water. Then, the CD spectra were obtained with a Jasco-J 720 spectropolarimeter.

2.4. Measurement of the stabilities of the wild-type and mutant Der p 2 against guanidine hydrochloride

Measurement of the stabilities of Der p 2 against guanidine hydrochloride was performed with a Hitachi F2000 fluorescence spectrophotometer thermostated at 37 °C. Der p 2 was incubated overnight at 37 °C in 50 mM sodium acetate buffer, pH 4.0 containing 6 M guanidine hydrochloride, due to slow relaxation in the unfolding-refolding process of Der p 2. The tryptophyl fluorescence of Der p 2 at 330 nm and 370 nm was measured by using 280 nm light for excitation with various concentrations of guanidine hydrochloride. The protein concentration was 0.2 μM.

2.5. Protease digestion of the wild-type and mutant Der p2

The wild-type and mutant Der p 2 dissolved 1% aqueous acetic acid were exhaustively dialyzed against 50 mM phosphate buffer at pH 7.5. To these protein solutions (final concentration of 1 mg/mL), 10 μg of subtilisin BPN' (Sigma-Aldrich, St. Louis, MO) was added three times every 15 min. Meanwhile, respective solutions were incubated at 37 °C. After 15 min of the final addition of subtilisin BPN', in which total incubation time at 37 °C is 1 h, 10% aqueous acetic acid was added to the protein solutions in order to stop respective protease reactions. Respective reactants was applied to the cation-exchange resin (1 × 3 cm, CM-Toyopearl) equilibrated with 50 mM sodium acetate buffer at pH 4.0, and then eluted with the same buffer containing 1.0 M NaCl. Each elution was monitored by absorbance at 280 nm. On the other hand, the respective proteins (1 mg/mL) unreacted with protease were eluted as was the same. Protease digestion ratios for the wild-type and mutant Der p 2 were obtained by the ratio of respective absorbances from the wild-type and mutant Der p 2 reacted with subtilisin BPN' to those unreacted with subtilisin BPN'.

2.6. Immunization protocol

Six-week-old female BALB/c mice were obtained from SLC Inc. (Japan). Five mice were immunized by repeated intraperitoneal injections (i.p.) with 0.1 mL of phosphate buffered saline (PBS) emulsified 1:1 in Imject Alum adjuvant (Thermo Scientific, Rockford, IL) containing the wild-type Der p 2 or Der p 2 mutants (50 μg/mouse) on day 0, 7, 21, and 28. Blood samples were drawn from the orbital sinus into capillary tubes.

2.7. Measurement of serum IgE levels

Antigen-specific IgE antibody titers were measured by sandwich ELISA. 96-well ELISA plates were coated with anti-mouse IgE (10 μg/mL) (LO-ME-2; Technopharm Biotechnology, Paris, France) in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4 °C. Plates were washed three times with wash solution (0.02% Tween 20, 0.15 M NaCl, 0.02% NaN_3; TBS-Tween) and blocked for 1 h with blocking buffer (TBS-Tween containing 2% non-fat dry milk) at room temperature. After washing, serum diluted 1/50 in blocking buffer was added and the plates were incubated overnight at 4 °C. Der p 2 modified with biotin (10 μg/mL) (Sulfo-NHS-LC Biotin; Thermo Scientific) was then added to each well followed by incubation overnight at 4 °C. Binding of the biotinylated Der p 2 was subsequently probed by the avidin–biotin alkaline phosphatase complex method using a Vectastain ABC-AP Standard Kit (Vector Laboratories, Burlingame, CA). p-Nitrophenyl phosphate was used as a substrate for alkaline phosphate and, following development, the absorbance at 405 nm of each well was recorded using an ImmunoMini NJ-2300 plate reader (InterMed, Tokyo, Japan).

3. Results

3.1. Preparation of the functional Derp2

Recombinant Der p 2 was overexpressed in *E. coli* and the expression of the protein was assessed by SDS-PAGE as shown in Fig. 1. Der p 2 was produced as an inclusion body and cell debris was collected by centrifugation after sonication of the harvest cells. The refolding of Der p 2 from the inclusion body was performed according to the method described previously [25]. Mueller et al. reported that the inclusion body was dissolved in one-fifth of the culture volume of 6 M guanidine hydrochloride and dialyzed against 20 mM Tris–HCl, pH 8.5, 1 mM EDTA, with the result that Der p 2, which has three disulfide bonds (Cys21–Cys27, Cys73–Cys78, Cys8–Cys119), could be refolded without oxidizing and reducing reagents. Unfortunately, however, by this method most of the Der p 2 was aggregated during the dialysis in the

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