Fold stability during endolysosomal acidification is a key factor for allergenicity and immunogenicity of the major birch pollen allergen

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Background: The search for intrinsic factors, which account for a protein's capability to act as an allergen, is ongoing. Fold stability has been identified as a molecular feature that affects processing and presentation, thereby influencing an antigen's immunologic properties.
Objective: We assessed how changes in fold stability modulate the immunogenicity and sensitization capacity of the major birch pollen allergen Bet v 1.
Methods: By exploiting an exhaustive virtual mutation screening, we generated mutants of the prototype allergen Bet v 1 with enhanced thermal and chemical stability and rigidity. Structural changes were analyzed by means of x-ray crystallography, nuclear magnetic resonance, and molecular dynamics simulations. Stability was monitored by using differential scanning calorimetry, circular dichroism, and Fourier transform infrared spectroscopy. Endolysosomal degradation was simulated *in vitro* by using the microsomal fraction of JAWS II cells, followed by liquid chromatography coupled to mass spectrometry. Immunologic properties were characterized *in vitro* by using a human T-cell line specific for the immunodominant epitope of Bet v 1 and *in vivo* in an adjuvant-free BALB/c mouse model.
Results: Fold stabilization of Bet v 1 was pH dependent and resulted in resistance to endosomal degradation at a pH of 5 or greater, affecting presentation of the immunodominant T-cell epitope *in vitro*. These properties translated *in vivo* into a strong allergy-promoting T_H2-type immune response. Efficient T_H2 cell activation required both an increased stability at the pH of the early endosome and efficient degradation at lower pH in the late endosomal/lysosomal compartment.
Conclusions: Our data indicate that differential pH-dependent fold stability along endosomal maturation is an essential protein-inherent determinant of allergenicity. (J Allergy Clin Immunol 2016;137:1525-34.)

Key words: *Allergic sensitization, Bet v 1, structural stability, endolysosomal degradation, antigen processing and presentation, molecular allergology*

Among the plethora of environmental antigens to which human subjects are regularly exposed, only a few display a high propensity to act as allergens triggering type I allergic diseases (ie, T_H2-biased immune responses characterized by aberrant levels of IgE production). Allergens encompass a diverse group of molecules that address innate immune pathways through protease activity, lipid binding, engagement of pattern recognition receptors, molecular mimicry of Toll-like receptor signaling molecules, or provision of complex carbohydrate structures.[1] Interestingly, allergens can be found in only 2% of all known protein families, and the distribution of allergens is highly biased toward a few of these protein families.[2] Hence it is obvious to speculate that beyond the abovementioned particular intrinsic biological functions, common structural and biochemical determinants of allergenicity might exist that have yet to be determined.

Bet v 1.0101 (Bet v 1), the major allergen found in birch pollen, belongs to family 10 of the plant pathogenesis-related proteins and is the most intensely studied allergen. Its structure,[3] as well as its T- and B-cell epitopes,[4-7] are known. The hydrophobic cavity of Bet v 1 has been demonstrated to bind a variety of ligands,[8] including pollen-derived quercetin,[9] an iron-binding catechol derivative. Recently, the apo-form of Bet v 1 (loaded with catechol in the absence of iron) has been suggested to have a T_H2-promoting potential.[10] Moreover, binding of the model ligand deoxycholate has been implicated in compaction and rigidification of the Bet v 1 molecule[11] and stabilization of its conformational epitopes.[12] These data indicate that the structural stability of Bet v 1 represents a determinant of its allergenicity.

To investigate solely the effect of conformational stability on the sensitization capacity of Bet v 1, we used an approach lacking any immunologically relevant confounders, such as artificial ligands or immunization with adjuvants. We generated variants of

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Abbreviations used
CD: Circular dichroism
DSC: Differential scanning calorimetry
FTIR: Fourier transform infrared spectroscopy
moDC: Monocyte-derived dendritic cell
NMR: Nuclear magnetic resonance
RBL: Rat basophil leukemia
SEC-MALS: Size exclusion chromatography coupled to multiangle light scattering

Bet v 1 with enhanced fold stability by means of introducing 1, 2, 3, or 4 sequential point mutations. Mutations were selected based on a recently developed *in silico* approach that uses normalized knowledge-based energy potentials for predicting the influence of single point mutations or combinations thereof on protein stability.[13] In-depth comparison of the Bet v 1 mutants with the wild-type protein revealed increased thermal and chemical stability, reduced backbone flexibility, and enhanced resistance to degradation by proteases. In the absence of adjuvants, the wild-type molecule induced only marginal immune responses, whereas the stabilized mutants triggered significant serum IgG and IgE titers and IL-4 production. Allergenicity and immunogenicity of the mutant proteins strongly correlated with protease resistance at slightly acidic pH and efficient proteolytic processing at lower pH, resembling the early and late endosomal environment, respectively. Based on our findings, we propose that differential, pH-dependent fold stability in the early versus late endosomal compartment is a protein-inherent key determinant of allergenicity.

METHODS

Expression and purification of recombinant proteins

Wild-type Bet v 1 and the 4 mutants were expressed from pET28b constructs in *Escherichia coli* strain BL21 Star (DE3; Invitrogen, Carlsbad, Calif), as described in the Methods section in this article's Online Repository at www.jacionline.org.

Protein characterization and structure determination

Protein folding in solution and thermal and chemical denaturation were monitored by using differential scanning calorimetry (DSC), circular dichroism (CD), and Fourier transform infrared spectroscopy (FTIR). The monomeric state was measured by using size exclusion chromatography coupled to multiangle light scattering (SEC-MALS). Structure was determined by means of x-ray crystallography. Protein flexibility was assessed by using molecular dynamics simulations, nuclear magnetic resonance (NMR) spectroscopy, and hydrogen/deuterium exchange. These methods are described in detail in the Methods section in this article's Online Repository.

Mice and immunizations

Female 6- to 10-week-old BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained at the animal facility of the University of Salzburg according to local guidelines for animal care. Animal experiments were approved by the Austrian Ministry of Science (permit no. BMWFW-66.012/0008-WF/II/3b/2014). Mice (n = 5) were immunized 5 times with 20 μg of Bet v 1 or one of the mutant proteins in sterile PBS by means of intradermal injection into the ear pinnae on days 0, 14, 28, 42, and 81. A total volume of 80 μL was divided between the 2 sides. Blood samples were taken at regular intervals. Mice were killed on day 88 for preparation of splenocytes.

Antibodies and cytokines

Levels of IgG_1 and IgG_{2a} subclass antibodies in sera measured on day 88 were evaluated by using a luminometric ELISA[14] at serum dilutions of 1:300 and 1:100, respectively, lying within the linear range of the assay.

The IgE cross-linking capacity of the mutant proteins was assessed by using a β-hexosaminidase release assay, as described in the Methods section in this article's Online Repository.

The amount of cell-bound IgE was measured by using a basophil activation test, as previously described.[15] Heparinized whole blood taken on day 84 of the experimental schedule was diluted 1:2 with RPMI 1640 and incubated with 10 ng/mL Bet v 1 or the respective mutant or left untreated as a control for 2 hours at 37°C and 5% CO_2. Cells were washed, and surface staining was performed with anti-CD200 R (clone OX110; eBioscience, San Diego, Calif), anti-IgE (clone RME-1; BioLegend, San Diego, Calif), anti-CD4 (clone GK1.5, BioLegend), and anti-CD19 (clone 6D5, BioLegend). Subsequently, cells were washed with PBS/1% BSA/2 mmol/L EDTA and analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, Calif).

Splenocytes from immunized mice were prepared and cultured, as previously described,[14] in the presence of Bet v 1 or the mutants (20 μg/mL) to determine the number of IL-4 and IFN-γ producers per 2×10^5 cells by using ELISpot (Millipore, Bedford, Mass).

Endolysosomal degradation simulations

Endolysosomal degradation assays were performed, as previously described.[16] Briefly, 5 μg of protein substrates (Bet v 1 and Bet_mut1 to Bet_mut4) was mixed with 8 μg of isolated microsomal fraction from the JAWS II cell line in 50 mmol/L citrate buffer (pH 5.9, pH 5.2, or pH 4.5) and 2 mmol/L dithiothreitol.

The pool of peptides generated from Bet v 1 and the mutants in the degradation assay at pH 5.2 and pH 4.5 was assessed by using mass spectrometry with a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, Mass) with nanoelectrospray and nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific). For details, see the Methods section in this article's Online Repository.

Uptake and processing of Bet v 1 and Bet variants by monocyte-derived dendritic cells and stimulation of Bet v 1–specific T cells

Experiments involving human cells were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Samples of human origin were obtained from blood donations, and use of residual cells was approved by the Institutional Review Board of Upper Austria. Monocyte-derived dendritic cells (moDCs) were generated from buffy coats from healthy anonymous donors (provided by the Red Cross Transfusion Service of Upper Austria), as described in the Methods section in this article's Online Repository. For investigating the uptake kinetics of Bet v 1 and its variants, moDCs were incubated with DyLight488-labeled proteins at 20 μg/mL for 30, 60, and 120 minutes, respectively, and uptake was determined by means of flow cytometry.

moDCs from HLA-DRB1*07:01–restricted donors were incubated for 30 to 480 minutes with Bet v 1 or the mutants at 20 μg/mL. Subsequently, moDCs were washed and cocultured with Jurkat T cells expressing a Bet v $1_{142\text{-}153}$–specific T-cell receptor and harboring a human IL-2 promoter/enhancer controlling luciferase expression[17] for 6 hours. Cells were lysed in 50 μL of lysis buffer (100 mmol/L potassium phosphate, 0.1% Triton X-100, and 1 mmol/L dithiothreitol), and luciferase activity was measured in a Tecan Infinite 200 Pro microplate reader with 50 μL of luciferase substrate (Promega, Madison, Wis).

RESULTS

Generation of Bet v 1 mutants with increased fold stability

We have previously demonstrated the use of knowledge-based potentials for generating destabilized allergens.[13]