



Inhibitory mechanism of anti-allergic peptides in RBL2H3 cells

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Abstract

Here we report the identification and functional characterization of several anti-allergic peptides identified through biopanning of pooled sera of patients with various allergies. Several peptides, including LSYLLWRSRLP (LSY), LVAHVGAGGV (LVA), RVSSCRGRNHIV (RVS), ETIGARWVRIE (ETI), TDGVTYTNDCL (TDG), RVVRYDADFVI (RVV), GFWCRRSGLVGV (GFW), were further characterized. These peptides inhibited the release of histamine from antigen-stimulated mast cells isolated from lung tissues of guinea pigs. Furthermore, the peptides inhibited calcium influx in ovalbumin-stimulated mast cells isolated from lung tissues of guinea pigs. Likewise, the peptides inhibited the release of β -hexosaminidase from antigen-stimulated rat basophilic leukemia (RBL2H3) cells and decreased calcium influx and intracellular reactive oxygen species production as well. We found that the peptides significantly decreased phosphorylation of extracellular regulated kinase (ERK) and that this was responsible for the decreased calcium influx and β -hexosaminidase in antigen-stimulated RBL2H3 cells, suggesting that ERK plays an important role in allergic reactions. The peptides identified in this study also affected upstream signaling of allergic inflammation. In other words, these peptides decreased phosphorylation of Lyn, PKC α , and δ . Lyn and PKC are known to be responsible for the phosphorylation of Fc ϵ RI, in response to receptor aggregation. The peptides inhibited interaction between IgE and Fc ϵ RI, suggesting that these peptides exert anti-allergic effects by inhibiting receptor cross-linking. These peptides also inhibited interaction between Fc ϵ RI and PKC δ . Taken together, these data suggest that peptides exert anti-allergic effect through the inhibition of upstream signaling, involving receptor cross-linking, and downstream multiple signaling.

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1. Introduction

Type I hypersensitivity is an immune disorder that is based on the production of immunoglobulin E (IgE) in response to allergens, which include egg whites (Urisu et al., 1997), dust mites (Thomas and Smith, 1998), soybeans (Quirce et al., 2002; Ogawa et al., 2000), bird nests (Ou et al., 2001), and peanuts (Pons et al., 2002) have been identified as allergens. Allergies to egg white is one of the most common causes of food allergy in infants and young children, with atopic dermatitis being the most frequent symptom associated with the allergy. The specific

allergens in egg whites include ovomucoid, ovotransferrin, and lysozyme. The major epitopes of ovomucoid were identified previously (Zhang and Mine, 1998). In addition, the major allergens for the mite *Dermatophagoides* (Eriksson et al., 2001), include tropomyosin, paramyosin, and fatty acid-binding proteins. Lastly, allergens of *Lepidoglypus destructor* have been identified from whole lysates via Western blot analysis (Johansson et al., 1994).

Currently, the only effective treatment for Type I hypersensitivity is specific immunotherapy; however, when allergen extracts are used for specific immunotherapy, the induction of new IgE reactivity to previously non-IgE-binding allergen components is a common complication (van Ree et al., 1996). The fact that specific immunotherapy results in *de novo* IgE synthesis highlights the unpredictable responses resulting from exposure to unknown components of allergen extracts. The

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induction of food allergy during specific immunotherapy can be solved by employing recombinant allergen components (Valenta et al., 1999), which should preclude the development of specific immunotherapy-induced allergy against previously unknown allergens. Furthermore, in order to exclude the potential enhancement of unexpected allergic reactions, it is reasonable to consider the use of epitope-defined immunotherapy. For example, B-cell epitope-derived peptides have been used to induce and direct immunoglobulin G (IgG) to IgE domains for allergens in which the IgE epitopes were known (Ball et al., 1999). The most efficient epitopes for treatment of allergies should be directed against the same epitope-specific IgE while lacking to capability to bind the IgE receptor itself. Phage display techniques have been used to identify epitopes that can inhibit the IgE-allergen interaction, and these techniques are useful for the identification of peptide structures that mimic the antibody-binding site of an antigen and do not correspond to the linear sequence of the cognate antigen. These peptides, known as mimotopes, have been identified for the major birch pollen allergen Bet V (Jensen-Jarolim et al., 1998), the major house dust mite allergen Der p (Furmonaviciene et al., 1999), and grass pollen allergen (Suphioglu et al., 2001).

Mast cells and basophils play essential roles in the pathogenesis of allergic reactions such as atopic dermatitis and asthma. RBL2H3 cells are mucosal mast cells that express the immunoglobulin Fc epsilon receptor I (FcεRI). Stimulation of IgE-sensitized RBL2H3 cells with specific antigen triggers a cascade of events leading to degranulation, mediator release, activation of mitogen activated protein kinase (MAPK), tyrosine kinase, and phospholipase C, increase in reactive oxygen species production, calcium influx, and cytokine production (Reth, 1989; Cambier, 1995; Wange and Samelson, 1996). This cascade of events suggests that mast cells are ideal targets for controlling various allergic diseases, including atopic dermatitis, allergic rhinitis, and asthma (Kobayashi et al., 2000; Williams and Galli, 2000; Matsuda et al., 1997; Pawankar et al., 1997).

Here, we employed bacterial surface-displayed random peptide library to identify epitopes binding to immunoglobulins in the sera of allergic patients (biopanning). We identified a multitude of peptides that bind to sera of patients with allergies against egg white, soy, and dust mites. The functional role of these peptides in allergic inflammation was determined using RBL2H3 cells and mast cells isolated from lung tissues of guinea pigs. Eight peptides identified in this study significantly inhibited β-hexosaminidase secretion, histamine release, calcium influx, and intracellular reactive oxygen species production from antigen-stimulated cells. Furthermore, these peptides also inhibited the activation of signaling molecule such as extracellular regulated kinase (ERK).

2. Materials and methods

2.1. Cell lines and cell culture

RBL2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified

Eagle's medium containing heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, San Diego, CA). Cultures were maintained in 5% CO₂ at 37 °C.

2.2. Materials

The primers used in this study were commercially synthesized by Bionex (Seoul, Korea). Dinitrophenyl-specific human serum albumin (DNP-HSA), Dinitrophenyl (DNP)-specific IgE, p-nitrophenyl N-acetyl-beta-D-glucosamine, 2'-amino-3'-methoxyflavone (PD98059), N-acetyl-L-cysteine (NAC), dichlorodihydrofluorescein diacetate (DCFHDA), and ketotifen fumarate were purchased from Sigma Chemical Company. The FliTrix panning kit and random peptide library (12-mer) were purchased from Invitrogen (San Diego, CA). Superscript reverse transcriptase was purchased from Life Technologies Inc. Trizol was purchased from Invitrogen. Anti-ERK, anti-phospho ERK, anti-Lyn, anti-phospho-Lyn^{Y507}, anti-PKCα, anti-phospho-PKCα^{T638/641}, anti-PKCδ, anti-phospho-PKCδ^{T505}, anti-FcεRIγ, anti-p38 MAPK, and anti-phospho p38 MAPK antibodies were purchased from R&D Company (Minneapolis, MN). Anti-Jun N-terminal kinase (JNK), anti-phospho JNK, and anti-TGase II antibodies were purchased from Cell Signaling (Beverly, MA). The yT&A cloning vector kit was purchased from Dongin Biotech (Seoul, Korea). The peptides used in this study were synthesized commercially by the Pepton Company (Daejeon, Korea). *Thermophilus aquaticus* (Taq) DNA polymerase was purchased from Takara (Seoul, Korea).

2.3. Human sera used in biopanning

The patients involved in this study were enrolled at the allergy clinic in the Department of Pediatric Medicine, at Samsung hospital. Human serum was obtained from patients with egg white allergy (109), soy allergy (60), dust mite allergy (120), and milk allergy (70). The criteria for selection were: (1) a positive skin prick test response to each of those allergens; and (2) a specific IgE level to each of those allergens greater than 1 kilounit (KU)/l, as determined by the Pharmacia ImmunoCAP Ig E specific blood test. Written informed consent was obtained from all selected patients and control subjects.

2.4. Biopanning

Sepharose containing mouse IgG was mixed with mouse anti-human IgE antibody (5 μg) in phosphate buffered saline (PBS) buffer containing 1% bovine serum albumin (BSA). The beads were incubated end over end at room temperature for 1 h, washed twice with PBS buffer, and resuspended in blocking buffer (1× PBS, 0.05% Tween 20, 2% BSA). After 1 h of blocking and two washes, the beads were resuspended in the pooled sera of allergic patients and incubated at room temperature for 1 h. After incubation, the beads were washed twice and were mixed with 100 μl of random peptide library (12-mer peptides) and incubated for 1 h. The beads were then

washed once with PBS buffer followed by five washes with 5 mM Tris–HCl, pH7.5, 150 mM NaCl and 0.1% Tween 20, and finally twice with water. Bound peptides were eluted twice with elution buffer (0.1 M HCl, 0.1% BSA, pH2.2). The eluates were neutralized with 2 M Tris base and were plated out onto Luria broth (LB)/agar plates. The next day, colony polymerase chain reaction (PCR) was performed to detect the presence of peptides.

Primers Rsr A (sense, 5'-ATTACCTGACTGACGAC-3') and Rsr B (antisense, 5'-CCCTGATATTCGTCAGCG-3') were used. Polymerase chain reaction (PCR) was performed for 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min.

For sequencing of inserts containing peptide, the band containing the insert was excised and purified according to standard procedures. DNA obtained from the band was cloned into a pT&A vector and ligation was performed at 4 °C overnight. Following ligation the reaction mixture was transformed into *E. coli* and plasmid DNA was isolated from each colony. The sequences of the isolated clones were analyzed from Applied Biosystems 377 (ABI377) automatic DNA sequencer using M13 universal sequencing primers.

2.5. Cell enzyme-linked immunosorbent assay (ELISA)

To detect the binding of biopanned clones to the sera of patients with food allergies, clones containing peptide-thio fusion constructs were incubated in RM base medium at 37 °C for 12–16 h. Forty µl of the cells then were transferred into IMC induction medium containing tryptophan (20 µg/ml), and incubation continued overnight at 37 °C. Cells were centrifuged at 16,600 ×g and the pellets were resuspended in PBS buffer. Next, 10 µl of serum of each allergic patient was added and incubated for additional 25 min. After incubation, centrifugation at 16600 ×g was followed. The resulting cell pellets were resuspended in PBS buffer, combined with 0.5 µl of biotin-conjugated mouse anti-human IgE antibody (Ab), and incubated for 25 min. After incubation and centrifugation as described above, cell pellets were resuspended in PBS buffer, mixed with 0.5 µl of streptavidin-horse radish peroxidase (HRP), and incubated for 25 min. After incubation, equal numbers of cells were split into a 96-well plate and 100 µl 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution was added. Finally, 2 M phosphoric acid was added to stop the reaction and optical density was measured at 450 nm. Positivity was defined as an optical density (OD) value higher than the mean value (for the sera of normal donor) + 2 S.D. The lines denote the cut-off point.

2.6. β -hexosaminidase secretion assay

Degranulation was determined by measuring the release of β -hexosaminidase. RBL2H3 cells were grown on 2% (w/v) BSA-coated 96 well plates (2×10^5 cells/well) and sensitized for 16 h with DNP-specific IgE (100 ng/ml). The growth medium was replaced with Tyrodes' assay buffer (119 mM NaCl, 4.74 mM KCl, 2.5 mM CaCl₂, 1.19 mM MgSO₄, 10 mM 4-2-hydroxyethyl-1-piperazineethane sulfonic acid (HEPES), 5 mM

glucose, 0.1% (w/v) BSA, pH7.3). Next, IgE-sensitized cells were preincubated with the above buffer for 15 min and then treated with anti-allergic drug or peptides for 15 min. After treatment, the cells were stimulated with DNP-HSA (100 ng/ml) for 1 h. Following stimulation, the supernatant was incubated with an equal volume of substrate solution (1 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosamine in 0.05 M citrate buffer, pH 4.5) for 1 h at 37 °C. The enzyme reaction was stopped by the addition of 0.05 M sodium bicarbonate buffer (pH 10.0) and the reaction product was measured at 450 nm. To determine the total amount of β -hexosaminidase released, the remaining cells were lysed by assay buffer containing 1% (v/v) Triton X-100 prior to incubation with substrate using the same procedure as for determination of activity in the supernatant. The inhibitory effects of peptides on β -hexosaminidase secretion were compared with that of ketotifen fumarate. To determine the effect of signaling molecule inhibitors on β -hexosaminidase secretion, the IgE-sensitized cells were then incubated with PD98059 (10 µM) for various time intervals. After treatment, cells were stimulated with DNP-HSA (100 ng/ml) and β -hexosaminidase secretion assay was performed as described above. Optimal conditions for β -hexosaminidase were predetermined.

2.7. Active sensitization protocol (anti-ovalbumin production)

Ten outbred female guinea pigs were first immunized by foot pad injections of a mixture of 50 µg ovalbumin and complete Freund's adjuvant. One week after immunization, the animals were injected intradermally with 100 µg ovalbumin in one side of the back and with 200 µg ovalbumin in the other side. Animals were sacrificed one week later, and serum samples were stored in aliquots at –70 °C until required. Serum antibody titers were determined by passive cutaneous anaphylaxis (PCA) as described previously (Ro et al., 2000). IgG₁ antibody from guinea pig serum was applied and separated by anti-IgG₂ affinity column chromatography, which was then washed with 0.1 M citric acid (pH 2.1). IgG₁ antibody was passed by 0.1 M citric acid, and the absorbed IgG₂ antibody was eluted with 0.2 M sodium carbonate (pH 11.3). The separated IgG₁ was concentrated using an Amicon device (Millipore, MA). The titers of anti-ovalbumin in serum were in the range 1600–3200. Sera were used to prepare passively sensitized mast cells, as previously described (Ro et al., 2000).

2.8. Guinea pig lung mast cell preparations

Guinea pig lung mast cells were isolated and purified using techniques similar to those previously reported (Ro et al., 2000). In brief, the lungs obtained from 16 unsensitized guinea pigs (48 g/each lung) were each perfused with 50 ml of modified Tyrode buffer (TGCM) consisting of (millimolar): NaCl, 137; NaH₂PO₄, 0.36; KCl, 2.6; CaCl₂, 1; MgCl₂, 1.5; NaHCO₃, 25; glucose, 1 g/l; gelatin, 1 g/l, pH 7.4 (TGCM buffer). After removing of large airways and blood vessels the lungs were minced using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd, Gomershall, England). The

pooled tissue was treated three times with 125 U/g tissue of collagenase and 5 U/g tissue of elastase. The lung fragments were treated with those enzymes for 15, 15, and 25 min, respectively. Freed cells were separated from residual tissue by filtration through metal mesh and a Nytex mesh (100 μ m). The cells were then washed with CaCl_2 and MgCl_2 -free Tyrode buffer containing gelatin (TG buffer), layered over gradients consisting of 10 ml of Percoll (density, 1.045 g/ml), and centrifuged at 1000 $\times g$ for 25 min. Pelleted cells (containing mast cells) were then resuspended in TG buffer, and purified further using a discontinuous Percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was centrifuged at 1000 $\times g$ for 25 min ($\sim 3.5 \times 10^7$ cells/gradient). The cell band between the 1.09 and 1.10 g/ml densities contained the highest purity and number ($1\text{--}2 \times 10^7$) of mast cells. This band was removed, washed with TGCM buffer, and designated the purified mast cell preparation. Mast cell counts were obtained by alcian blue staining, and cell viability was determined by trypan blue exclusion. The cell viability was consistently above 98%, and the purity range of the mast cells obtained was 80%–90%.

2.9. Mediator release from mast cells

The purified mast cells (4×10^5) were passively sensitized with anti-ovalbumin antibody (1 ml/ 10^6 cells) for 45 min at 37 $^\circ\text{C}$ in a shaking water bath. The cells were then washed, resuspended in TGCM buffer (hereafter sensitized mast cells), challenged with 1.0 $\mu\text{g/ml}$ of ovalbumin for 10 min. The reaction was subsequently terminated by placing tubes in an ice bath. The supernatants obtained by centrifugation were used for histamine determinations. LVA (100 μM), RVV (5 μM), LSY (100 μM), TDG (100 μM), GFW (5 μM), RVS (100 μM), and PHV (100 μM) were added to mast cells 10 min before ovalbumin challenge (1.0 $\mu\text{g/ml}$).

2.10. Histamine release assay

Histamine was analyzed using an automated fluorometric method (with dialyzer) as previously described (Ro et al., 2000). The sensitivity of the assay was approximately 1–20 ng/ml of histamine. The amount of histamine released was expressed as a percentage of the total histamine present in unstimulated cells.

2.11. The effects of peptides on calcium influx

RBL2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml) on 2% (w/v) BSA-coated 6 well plates. The growth medium was then replaced with assay buffer, and the cells were exposed to 4 μM fluo-3-Acetoxymethyl (AM) esters in the presence of 1% (v/v) fetal bovine serum and 0.04% Pluronic F-127 in the dark for 45 min. The fluo-3-loaded cells were washed three times with assay buffer, treated with the peptides or ketotifen fumarate for 5 min, and then immediately stimulated with DNP-HSA (100 ng/ml). The change in fluorescence of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

2.12. Measurement of $[\text{Ca}^{2+}]_i$ Level

Sensitized mast cells (4×10^5) were challenged 1.0 $\mu\text{g/ml}$ ovalbumin for 10 min, and then reaction terminated by placing tubes in an ice bath. The cells were then incubated with fluo-3AM esters (5 μM) for 30 min and fixed on glass slides treated with poly-L-lysine. Ovalbumin antigen (0.1 $\mu\text{g/ml}$) was added to the glass slides to stimulate the cells. The intracellular calcium ($[\text{Ca}^{2+}]_i$) levels of mast cells were analyzed using a LSM 510 laser scanning microscopy to quantify fluorescence intensity (Carl Zeiss, Germany). The results were expressed as relative values versus the fluorescence (F_0) of unstimulated cells ($F_0=1$). RVV (5 μM), TDG (100 μM), LVA (100 μM), and LSY (100 μM) peptides were added to the mast cells 10 min prior to the ovalbumin challenge (0.1 $\mu\text{g/ml}$).

2.13. Reactive oxygen species and nitric oxide (NO) measurement

IgE-sensitized cells were incubated with 5 μM of 2, 7-dichlorodihydrofluorescein diacetate for 30 min. DNP-HSA (100 ng/ml) was added to the cells for the last 5 min of the incubation. After incubation, cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss). The samples were excited by a 488 nm Ar laser and a 515 nm long-pass filter. Approximately 20 cells were selected at random from each of three separate experiments for evaluation. To check the effect of the peptides on reactive oxygen species production, cells were sensitized with DNP-specific IgE (100 ng/ml) and incubated with each peptide for 15 min in the presence of 5 μM of 2, 7-Dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA was added, and reactive oxygen species measurement was performed as above. NO production was monitored and quantitated by accumulated supernatant nitrite via Griess assay (Gilchrist et al., 2003).

2.14. Western blot analysis and immunoprecipitation

The IgE-sensitized RBL-23 cells were treated with an anti-allergic drug or peptides for 15 min and stimulated with DNP-HSA (100 ng/ml) for 30 min. For Western blot analysis, cells were solubilized with lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) Sodium dodecyl sulfate, 10% (v/v) glycerol, 50 mM Dithiothreitol, 0.01% (w/v) bromophenol blue, 10 mM sodium fluoride (NaF), 1% (v/v) protease inhibitor cocktail, 1 mM sodium orthovanadate). The samples were boiled for 5 min, and equal amounts of protein (20 $\mu\text{g/well}$) were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE). After electrophoresis, proteins were transferred onto a nitrocellulose membrane and subjected to immunoblotting. The antibodies and dilutions included anti-ERK (1:1,000), anti-phospho ERK (1:1,000), anti-PKC α (1:3,000), anti-phospho-PKC α ^{T638/641} (1:3,000), anti-PKC δ (1:2,000), anti-phospho-PKC δ ^{T505} (1:2,000), anti-Fc ϵ RI γ (1:3,000), anti-jun N-terminal kinase (JNK) (1:1,000), anti-phospho JNK (1:1,000), p38 MAPK (1:2,000), anti-phospho p38 MAPK (1:2,000), and anti-

transglutaminase II (1:1,000). After extensive washing, blots were further incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody at a 1:3,000 for 1 h at room temperature and developed using enhanced chemiluminescence kit (Amersham). For immunoprecipitation, cell lysates were immunoprecipitated with monoclonal anti-human IgE antibody (5 µg) or anti-FcεRIγ antibody (5 µg) on ice for 1 h. Protein G-sepharose (50 µl) was then added and reaction was performed at 4 °C for 2 h on a rotary shaker. Immune complexes were washed three times with lysis buffer, and 2× sample buffer was added to the beads. Boiled samples were loaded for SDS-PAGE. Western immunoblot analysis was performed.

3. Results

3.1. Screening of peptides that bind to the sera of patients with allergies

In an effort to identify anti-allergic peptides, we attempted to identify peptides that were capable of binding to the sera of patients with food allergies against egg white, soy, or dust mites. The majority of patients with food allergies exhibited atopic dermatitis, and those with dust mite allergies had asthma. Tables 1 and 2 describe the clinicopathological characteristics of the allergic patients whose pooled sera were used in biopanning for the purpose of identifying anti-allergic peptides. We employed bacterially displayed random peptide library to identify peptides that bind to sera of allergic patients. The random peptide library displays peptides on the surface of *E. coli* using the major bacterial flagellar protein (Fli C) and thioredoxin. This random peptide library was subjected to five rounds of biopanning using pooled sera of patients that had egg white, soy, or dust mite allergies. After biopanning, plasmid DNAs were prepared and the peptide inserts were sequenced. PCR was performed to detect the presence of inserts and the

resulting PCR product were cloned into a T-vector; restriction digestion of T-vector by EcoRI was performed to confirm the presence of inserts. We determined the sequences of 29, 15, 3, 1 peptide(s) that bind to the sera of allergy patients against egg white, house dust mites, soy, or milk, respectively. The random peptide display technique has been used to define the peptide structures that mimic natural epitopes (Jensen-Jarolim et al., 1998). The peptides identified in this study did not show homologies with linear sequences of the previously identified allergens.

Binding of these peptides to the sera of patients with various food allergies was confirmed using cell-based enzyme-linked immunosorbent assay (ELISA). Results using some of the clones containing peptide inserts are shown (Fig. 1). Two clones containing peptides ETI and RVS demonstrated the ability to bind to sera of patients with allergies to dust mites. Clones containing peptides with sequences LVA, GFW, LSY, and RVV demonstrated the ability to bind to sera of patients with allergies to egg whites. The peptide PHV was found to bind to sera of patients with soy allergies. Peptides that bound to sera of patients with dust mite allergies did not show cross reactivity with sera of patients with food allergies, including egg whites, soy, and milk (data not shown) according to the immunoCAP test. Based on the results from sequencing and cell ELISA we synthesized seven peptides to determine their anti-allergic effects in RBL2H3 cells. We also synthesized peptides TDG and SIL, which are epitopes of well-known allergen ovomucoid (Zhang and Mine, 1998).

3.2. Effects of peptides on β-hexosaminidase secretion

The secretion of β-hexosaminidase is the hallmark of an allergic reaction resulting from allergen exposure. We examined the effects of the synthesized peptides on β-hexosaminidase secretion from antigen-stimulated cells. Antigen

Table 1
Clinical characteristics of patients with egg white allergies whose sera were used in biopanning

No.	Age	Result (total IgE/CAP)	Diagnosis	No.	Age	Result (total IgE/CAP)	Diagnosis
21	1	3041/45.2; egg white 3041/4.13; DF 3041/3.94; DP 3041/4.64; buck-wheat	AD	26	1	1156/101; egg white 1156/0.49; DF 1156/18.8; soy 1156/0.74; DP	AD
22	1	994/101; egg white 994/0.50; milk 994/0; DP 994/0; DF	AD	28	1	6910/101; egg white 6910/2.08; milk 6910/42.8; DF 6910/32.3; tomato	AD
23	0	83.2/21.8; egg white 83.2/0.91; milk 83.2/0; DP 83.2/0; soy	AD	29	0	89/24.1; egg white 89/0; DP 89/0; soy 89/0; milk	AD
24	5	175/24.6; egg white 175/0.40; soy 175/1.0; DP 175/0; wheat	AD	30	1	302/36.6; egg white 302/0; DP 302/0; DF 302/1.44; wheat	AD

AD, atopic dermatitis.

DP, *D. pteronyssius*.

DF, *D. farinae*.

No. denotes patients. Total IgE/CAP denotes kilounit U/ml.

Table 2
Clinical characteristics of patients with dust mite allergies

No.	Age	Result (total IgE/CAP)	Diagnosis	No.	Age	Result (total IgE/CAP)	Diagnosis
1	5	305/101; DF 305/25.8; DP 305/0; soy 305/0.49; egg white	AR	12	4	539/101; DF 539/60.4; DP 539/negative; grass pollen mix 539/negative; tree mixture	Asthma
3	8	60.1/16.9; DF 60.1/6.52; DP 60.1/0; soy 60.1/0; milk	Asthma	14	6	1235/101; DF 1235/101; DP 1235/0; soy 1235/0.59; milk	Asthma
4	6	1235/101; DF 1235/101; DP 1235/0; soy 1235/0.59; milk	Asthma	17	6	1144/101; DF 1144/56.7; DP 1144/0; soy 1144/0.81; milk	Asthma
5	6	1144/101; DF 1144/56.7; DP 1144/0; soy 1144/0.81; milk	Asthma	19	5	220/76.2; DF 220/16.1; DP	Asthma

AR, allergic rhinitis; DP, *D. pteronyssinus*; DF, *D. fairnae*.

No. denotes patients. Total IgE/CAP denotes kilounit/ml.

stimulation led to an increased secretion of β -hexosaminidase from RBL2H3 cells (Fig. 2A). We next examined the inhibitory effects of these peptides on β -hexosaminidase secretion. All of the peptides significantly decreased β -hexosaminidase secretion from antigen-stimulated RBL2H3 cells (Fig. 2B–H), doing so in a dose-dependent manner (Fig. 2B–F). Peptide alone,

without antigen stimulation, did not affect β -hexosaminidase secretion (data not shown). The anti-allergic drug, ketotifen fumarate (Gilchrist et al., 2003; Joly et al., 1987) also decreased β -hexosaminidase secretion (Fig. 2B–I). Lastly, the peptide ECE, which did not appear to bind to sera of allergic patients, was added as a negative control (Fig. 3I).

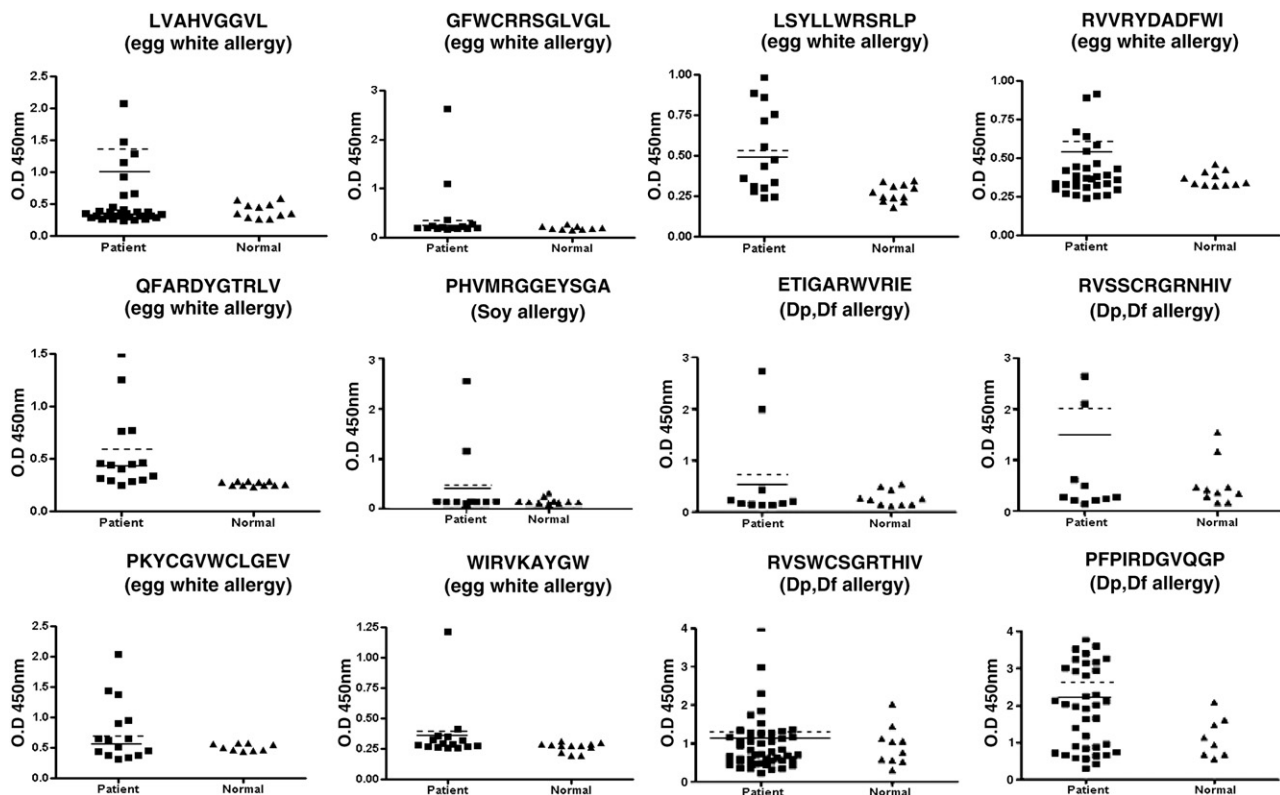


Fig. 1. Reactivity in ELISA of bacterial clones containing peptide-fusion constructs. Bacteria clones containing peptide-thio fusion constructs were grown in medium containing tryptophan (100 μ g/ml) for induction of peptides. After induction, bacterial cells were centrifuged. Thus obtained cell pellets were incubated with biotinylated anti-human IgE Ab (1:2000 dilution) for 25 min. After washing, streptavidin-horse radish peroxidase was added at a 1:2000 dilution. Finally, 2 M phosphoric acid was added to each tube and the optical density was measured at 450 nm. Positivity was denoted by lines to the cut off point.

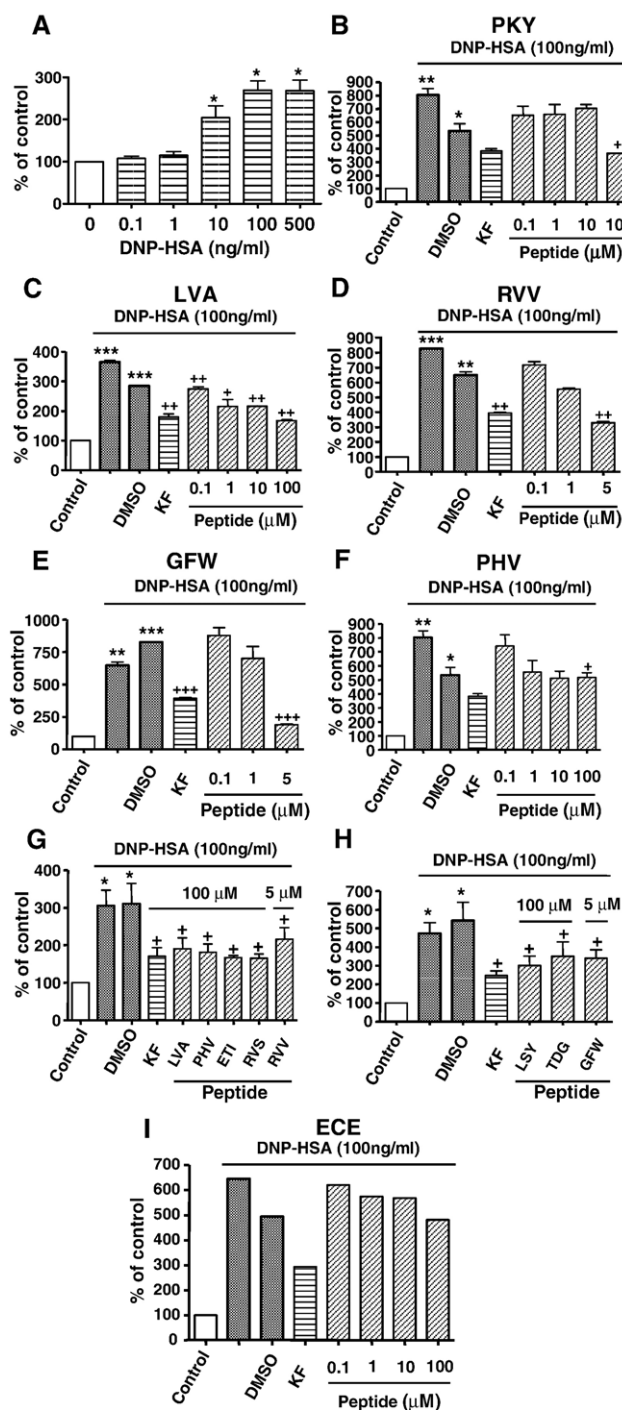


Fig. 2. Effects of peptides on β -hexosaminidase secretion from antigen-stimulated RBL2H3 cells. (A) RBL2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml), and stimulated with various concentrations of DNP-HSA (0, 0.1, 1, 10, 100, 500 ng/ml). The enzyme activities of β -hexosaminidase in the supernatant and solubilized cells were measured with *p*-nitrophenyl *N*-acetyl-beta-D-glucosamine. (B–I) RBL2H3 cells were sensitized 16 h with DNP-specific IgE (100 ng/ml), and treated with ketotifen fumarate (100 μ M), or various concentrations of each peptide (100 μ M) for 15 min. The significance between each control group and experimental group was performed by the student's *t* test. Asterisks denote statistical significance. **P*<0.01 compared without DNP-HSA challenge. +*P*<0.01 compared without peptide treatment.

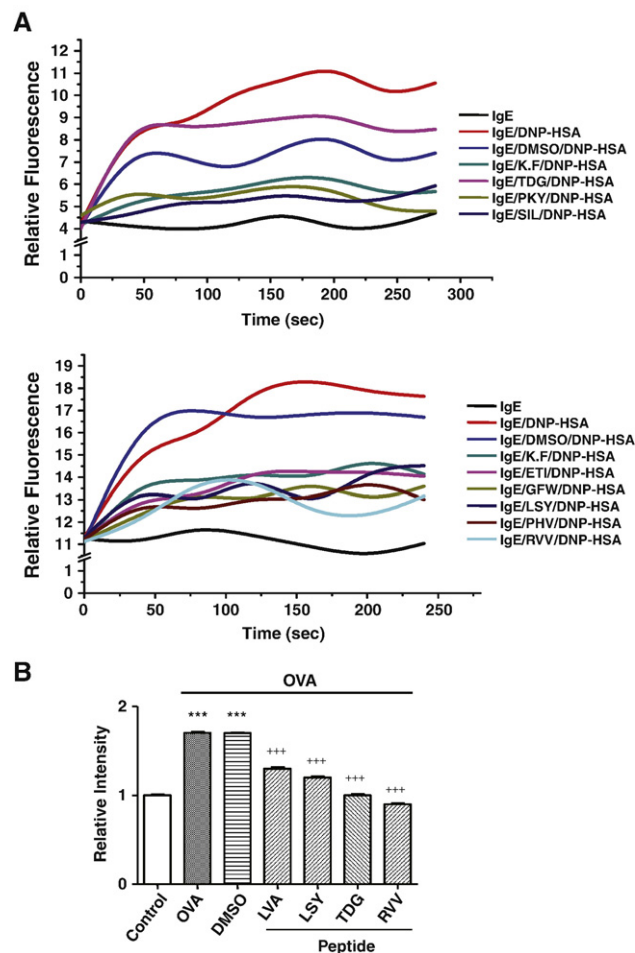


Fig. 3. Effects of peptides on calcium influx. (A) Calcium influx in RBL2H3 cells was measured using fluo-3AM. The IgE-sensitized cells were loaded with fluo-3AM (4 μ M) for 45 min. The fluo-3-loaded cells were exposed to ketotifen fumarate or peptides. The cells were then stimulated with DNP-HSA (100 ng/ml) and the change in fluorescence of fluo-3 AM was monitored. (B) Effect of each peptide on the Ca^{2+} influx in mast cells activated by ovalbumin/anti-ovalbumin reaction. Fluo-3 AM (5 μ M) was added to sensitized and challenged mast cells (4×10^5), and then incubated for 30 min as described in "Materials". Each peptide was added 10 min prior to the ovalbumin challenge. Intracellular Ca^{2+} levels were visualized using a confocal laser scanning fluorescence microscopy. Data are expressed as relative intensity versus the fluorescence values (F_0) of unstimulated cells ($F_0=1$). Experiments were performed in groups of four. Ovalbumin, ovalbumin; DMSO, dimethyl sulfoxide. ****P*<0.001 compared with control; +++*P*<0.01 compared with ovalbumin alone.

3.2. Effects of peptides on mediator releases during mast cell activation

The release of chemical mediator such as histamine plays important role in allergic inflammation (Reth, 1989). Therefore, we examined the effects of these peptides on histamine release. In order to examine if each peptide itself induces histamine release, unsensitized mast cells, isolated from lung tissues of guinea pigs, were challenged with each peptide (30, 50, 100, 150, or 200 μ M), and were challenged by 0.1, 1.0, or 10 μ g/ml ovalbumin. Mast cells sensitized with anti-ovalbumin antibody were challenged with various concentrations of each

Table 3

Effect of peptides on the histamine release from passively sensitized (anti-ovalbumin antibody) lung mast cells activated by ovalbumin ($1.0 \mu\text{g}/4 \times 10^5$ cells)^a

Treatment	Histamine release
Ovalbumin ^a alone	30.4 ± 0.23^b
RVVRYDADFWI (5 μM)	2.5 ± 2.86 (91.8) ^d
GFWCRRSGLVGV (5 μM)	26.5 ± 1.08 (12.9) ^c
LVAHVAGAGGVL (100 μM)	3.3 ± 3.37 (89.0) ^d
LSYLLWRSRLP (100 μM)	13.4 ± 5.16 (56.0) ^d
TDGVTYTNDCL (100 μM)	5.1 ± 4.14 (83.1) ^d
RVSSCRGRNHIV (100 μM)	12.0 ± 4.46 (52.1) ^d
PHVMRGGEYSGA (100 μM)	16.7 ± 2.86 (45.0) ^d
ETIGARWVRIE (100 μM)	20.1 ± 2.07 (33.9) ^d

^aGuinea pig mast cells were isolated, and purified by enzyme digestion and rough and discontinuous percoll density gradient method. Mast cells (4×10^5 cells) were passively sensitized by an anti-ovalbumin antibody, and challenged by $1.0 \mu\text{g}/\text{ml}$ ovalbumin. Each peptide was added 5 min before antigen challenge. Histamine in supernatant was determined by fluorometric analyzer.

^bThe amount of histamine released was expressed as a percentage of the total histamine content. Results in parentheses show a decreasing percentage evoked by pretreatment with each peptide. ^c $P < 0.05$ compared with ovalbumin alone, ^d $P < 0.001$ compared with ovalbumin alone.

peptide (30, 50, 100, 150, or 200 μM) followed by ovalbumin challenge. The results obtained showed that each peptide was not capable of inducing histamine release (data not shown) alone. The dose ($1.0 \mu\text{g}/\text{ml}$ ovalbumin) and time (10 min for histamine) of the ovalbumin challenge were decided after preparing dose-response curves and examining time dependences. The histamine release of mast cells sensitized with anti-

ovalbumin antibody ($1 \text{ ml}/10^6$ cells) and challenged with $0.1 \mu\text{g}/\text{ml}$ ovalbumin after pretreatment with RVV (5 μM), GFW (5 μM), LVA (100 μM), LSY (100 μM), TDG (100 μM), RVS (100 μM), PHV (100 μM), and ETI (100 μM) was $2.5 \pm 2.86\%$, $26.5 \pm 1.08\%$, $3.3 \pm 3.37\%$, $13.4 \pm 5.15\%$, $5.1 \pm 4.14\%$, $14.0 \pm 4.46\%$, and $16.7 \pm 2.86\%$, respectively, whereas ovalbumin alone released $30.4 \pm 0.23\%$ (Table 3). The corresponding degrees of inhibition showed a 91.8%, 12.9%, 89.0%, 56.0%, 83.1%, 52.1%, 42.8%, and 33.9% respectively (Table 3). These results suggest a potential value of the peptides identified in this study as allergy therapeutics.

3.4. Effects of peptides on calcium influx

IgE-mediated release of mediators is associated with an increase in calcium concentration caused by an influx of calcium (Beaven et al., 1984). Therefore, we examined whether the peptides identified through biopanning were capable of inhibiting calcium influx in antigen-stimulated RBL2H3 cells. As expected, stimulation of cells with DNP-HSA induced calcium influx (Fig. 3A), and ketotifen fumarate significantly decreased calcium influx (Fig. 3A). The addition of the peptides to IgE-sensitized RBL2H3 cells resulted in a significant reduction in calcium influx (Fig. 3A), and we also observed similar effects on the calcium influx with the other peptides (data not shown).

Increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is necessary for the degranulation of preformed mediator (histamine) in activated mast cell. Therefore, we examined the effect of each peptide

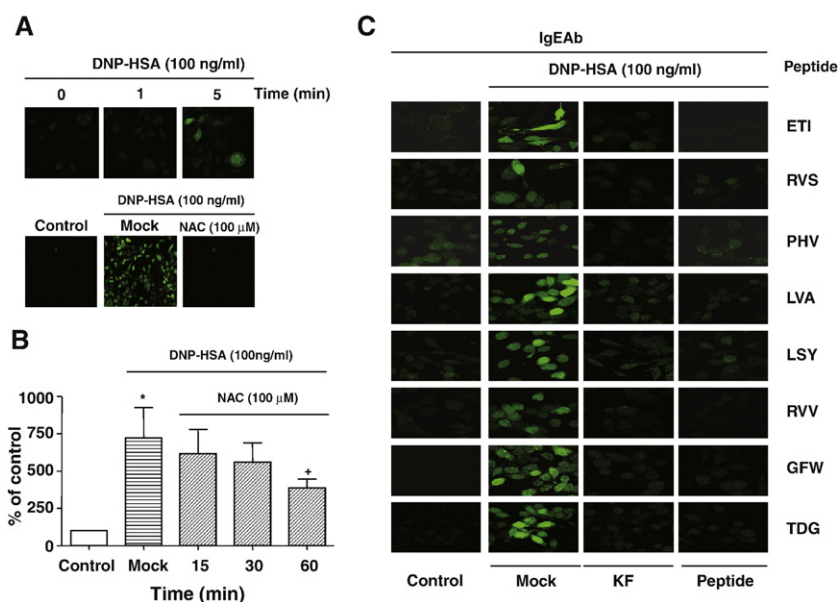


Fig. 4. Peptides decrease reactive oxygen species in antigen-stimulated RBL2H3 cells. (A) RBL2H3 cells were sensitized 16 h with DNP-specific IgE (100 ng/ml). The sensitized cells were then incubated with 5 μM of 2, 7-Dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. After incubation, cells were immediately observed with a laser scanning confocal microscope (LSM410, Carl Zeiss). The IgE-sensitized RBL2H3 cells were treated for 15 min with *N*-acetyl-L-cysteine (100 μM), an inhibitor of reactive oxygen species production. The cells were then incubated with 5 μM of 2, 7-Dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. (B) IgE-sensitized RBL2H3 cells were incubated with *N*-acetyl-L-cysteine (100 μM), for 15 min. After incubation, cells were stimulated with DNP-HSA (100 ng/ml) for 15 min, and a β -hexosaminidase secretion assay was carried out. The data are averages of three independent experiments. (C) IgE-sensitized cells were incubated with 5 μM of 2, 7-Dichlorodihydrofluorescein diacetate in the absence or presence of the peptides for 30 min. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added and the cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss).

on $[Ca^{2+}]_i$ in mast cells, isolated from lung tissues of guinea pigs, by confocal laser scanning microscopy; a relative intensity ratio of 1 was used to describe the fluorescence intensity of unstimulated cells. The $[Ca^{2+}]_i$ level in activated mast cells reached a plateau at 200 sec and sustained for 10 min (from 1.00 ± 0.015 to 1.70 ± 0.021 relative intensity) in the presence of external Ca^{2+} , as compared with unsensitized single mast cells (1.00 ± 0.015 intensity). Each peptide (LVA, LSY, TDG, or RVV) significantly reduced $[Ca^{2+}]_i$ level in each concentration (to 1.3 ± 0.028 at 100 μ M LVA; to 1.2 ± 0.02 at 100 μ M LSY; 1.00 ± 0.024 at 100 μ M TDG; to 0.9 ± 0.019 RVV) (Fig. 4B). Similar effects with the other peptides were also observed (data not shown). These results suggest that the inhibitory effects of these peptides on calcium influx are likely closely related to their effects on the secretion of chemical mediators.

3.5. Reactive oxygen species are responsible for β -hexosaminidase secretion in response to antigenic stimulation

RBL2H3 cells release reactive oxygen species in response to various stimuli (Yoshimaru et al., 2002; Matsui et al., 2000). Mast cells are secretory cells associated with specific and innate immunity (Malaviya et al., 1996; Echtenacher et al., 1996). Mast cells secrete chemical mediators such as histamine, prostaglandin, and cytokines during specific immune responses. In innate responses, mast cells promote phagocytosis resulting in the production of reactive oxygen species such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Hampton et al., 1998). Thus, we looked at the relationship between reactive oxygen species production and β -hexosaminidase secretion from antigen-stimulated cells. Antigen stimulation resulted in reactive oxygen species production (Fig. 4A), and as expected, the reactive oxygen species blocker *N*-acetyl-L-cysteine (NAC) inhibited reactive oxygen species production from antigen-stimulated cells, as expected (Fig. 4A). NAC caused inhibition of β -hexosaminidase secretion, suggesting that reactive oxygen species production is indeed associated with β -hexosaminidase secretion (Fig. 4B). The effect of NO on β -hexosaminidase secretion also was determined. Antigen-stimulated RBL2H3 did not result in nitric oxide (NO) synthesis (data not shown), and N(G)-mono-methyl-L-arginine (L-NMMA), an inhibitor of NO synthesis, did not affect β -hexosaminidase secretion (data not shown). These results indicate that reactive oxygen species, such as H_2O_2 , but NO, are responsible for β -hexosaminidase secretion from antigen-stimulated cells.

3.6. Peptides inhibit reactive oxygen species production in antigen-stimulated RBL2H3 cells

Since reactive oxygen species production is responsible for β -hexosaminidase secretion from antigen-stimulated cells (Fig. 4B), we next determined the effects of the peptides on reactive oxygen species production in antigen-stimulated RBL2H3 cells. Each of the eight peptides led to the inhibition of reactive oxygen species production in antigen-stimulated RBL2H3 cells (Fig. 4C). These results suggested that the inhibition of β -

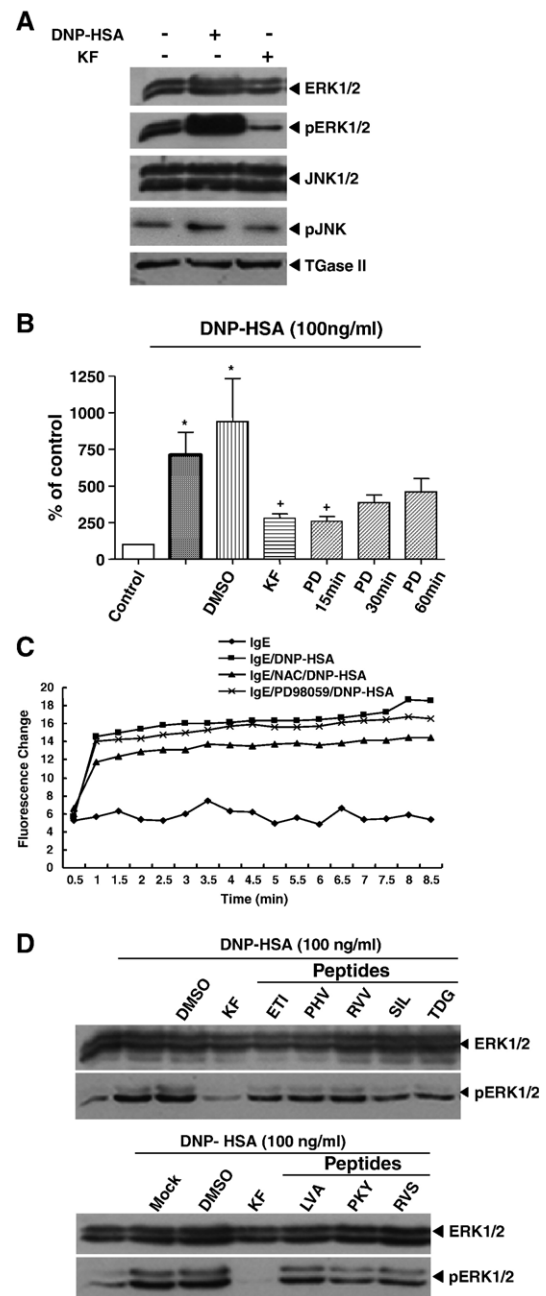


Fig. 5. Peptides decrease phosphorylation of ERK in antigen-stimulated RBL2H3 cells. (A) IgE-sensitized RBL2H3 cells were treated without or with ketotifen fumarate (100 μ M) followed by stimulation with DNP-HSA (100 ng/ml). Cell lysates were then prepared and subjected to Western blot analysis. (B) IgE-sensitized RBL2H3 cells were treated without or with ketotifen fumarate (100 μ M) or PD98059 (10 μ M). Cells were then treated with DNP-HSA (100 ng/ml), and β -hexosaminidase secretion assays were carried out. Asterisks denote statistical significance. * $P < 0.01$ compared without DNP-HSA challenge. + $P < 0.01$ compared with DNP-HSA challenge in the absence of pretreatment with PD98059. (C) The IgE-sensitized cells were loaded with fluo-3 AM (4 μ M) for 45 min, and the fluo-3-loaded cells then exposed to ketotifen fumarate or peptides. The cells were then stimulated with DNP-HSA (100 ng/ml), and the change in fluorescence of fluo-3 was monitored. (D) The IgE-sensitized RBL2H3 cells were treated with ketotifen fumarate (100 μ M) or peptides for 15 min. After treatment, cells were stimulated with DNP-HSA (100 ng/ml), and after stimulation, protein extracts were prepared and subjected to Western blot analysis using anti-ERK, and anti-phospho ERK.

hexosaminidase secretion by these peptides was closely related with their inhibitory effects on reactive oxygen species production.

3.7. Anti-allergic effect of peptides is mediated by ERK

Mast cell activation results in the recruitment of the spleen tyrosine kinase (syk), the activation of phospholipase C, and members of the MAPK family as well (Reischl et al., 1999;

Ogasawara et al., 1986; Oliver et al., 2000). The activation of ERK was observed following antigenic stimulation (Fig. 5A). There was no activation of JNK by antigen stimulation (Fig. 5A). The inactivation of ERK by PD98059 led to the inhibition of β -hexosaminidase secretion from antigen-stimulated cells, suggesting that ERK may have a role in the secretion of β -hexosaminidase (Fig. 5B). Inhibition of reactive oxygen species by NAC and that of ERK by PD98059 did not affect calcium influx in antigen-stimulated RBL2H3 cells (Fig. 5C), suggesting that reactive oxygen species and ERK function downstream of calcium influx. Next, we examined the effect of the peptides on activation of ERK and found that they indeed inhibit the activation of ERK in antigen-stimulated cells (Fig. 5D); however, none of these peptides by themselves were able to affect activation of ERK in antigen-stimulated cells (data not shown). These results suggest that the peptides exert anti-allergic effect by decreasing phosphorylation of ERK.

3.8. Peptides inhibit upstream signaling of allergic inflammation

Allergic inflammation is regulated by aggregation of Fc ϵ RI. Aggregation of Fc ϵ RI leads to the activation of the Src family tyrosine kinase Lyn (Tolar et al., 2004). Several molecules that phosphorylate Fc ϵ RI, in response to receptor aggregation, have been identified. These include Lyn and PKC δ (Eiseman and Bolen, 1992; Germano et al., 1994). Effect of peptides on upstream signaling of allergic inflammation was investigated. Antigen stimulation increased phosphorylation of Lyn, as expected (Fig. 6A). Peptides identified in this study effectively inhibited phosphorylation of the Lyn (Fig. 6A). These peptides also inhibited phosphorylation of PKC α (Fig. 6B) and δ (Fig. 6C). The other four peptides showed same effects (data not shown). Effect of peptides on receptor cross-linking in response to antigen stimulation was examined. For this, immunoprecipitation using anti-IgE antibody was followed by Western blot analysis using anti-Fc ϵ RI γ antibody. Peptides effectively inhibited interaction between IgE and Fc ϵ RI (Fig. 6D). The other four peptides showed same effects (data not shown). Since PKC δ has been shown to interact with Fc ϵ RI (Eiseman and Bolen, 1992), it was necessary to examine whether peptides inhibit interaction between Fc ϵ RI and PKC δ . Peptides effectively inhibited interaction between Fc ϵ RI and PKC δ , but not PKC α (Fig. 6D). These data indicates that peptides exert

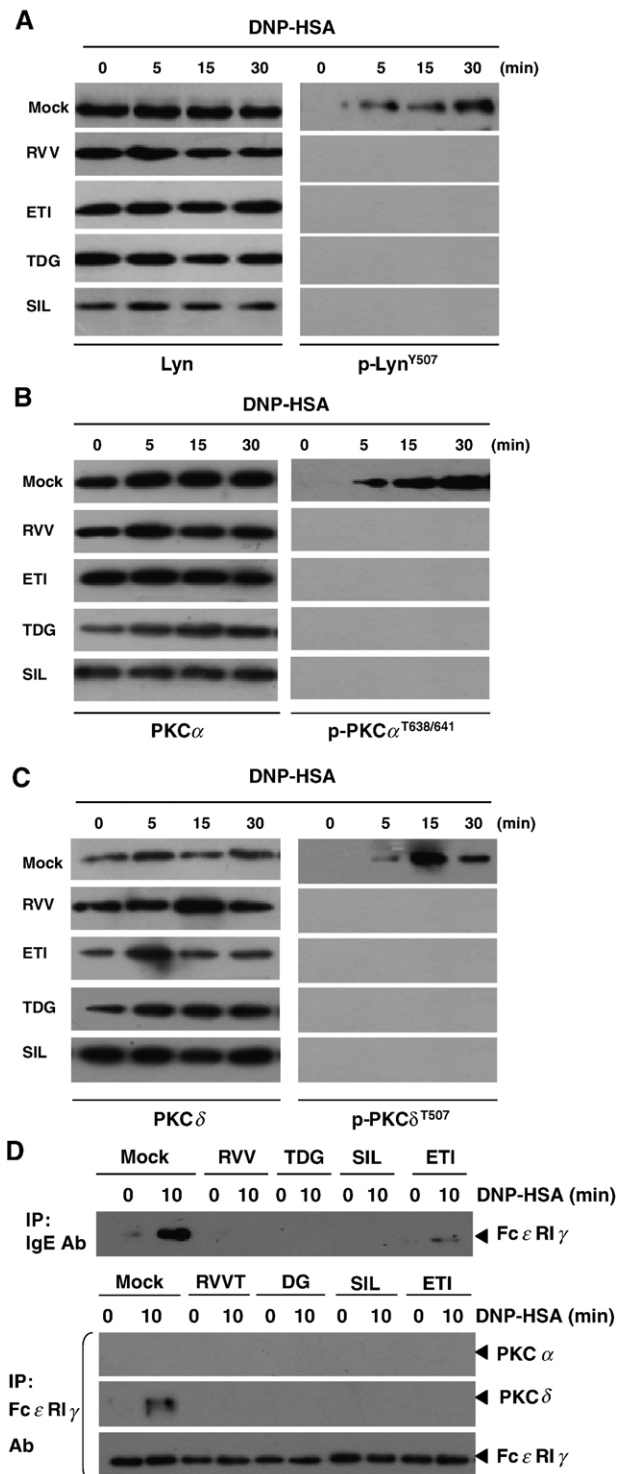


Fig. 6. Peptides decrease phosphorylation of Lyn, PKC α , PKC δ and receptor cross-linking in antigen-stimulated RBL2H3 cells. (A) The IgE-sensitized RBL2H3 cells were pretreated with various peptides for 15 min. After treatment, cells were stimulated with DNP-HSA (100 ng/ml) for various time intervals, and after stimulation, protein extracts were prepared and subjected to Western blot analysis using anti-Lyn, and anti-phospho Lyn^{Y507}. (B) Protein extracts were subjected to Western blot analysis using anti-PKC α and anti-phospho-PKC α ^{T638/641}. (C) Western blot analysis was performed using anti-PKC δ and anti-phospho-PKC δ ^{T507}. (D) The IgE-sensitized RBL2H3 cells were pretreated with various peptides for 15 min. After treatment, cells were stimulated with DNP-HSA (100 ng/ml) for various time intervals, and after stimulation, protein extracts were subjected to immunoprecipitation using anti-IgE antibody or anti-Fc ϵ RI γ antibody. The immunoprecipitates were then subjected to Western blot analysis.

negative effect on allergic inflammation by regulating upstream signaling.

4. Discussion

We employed a bacterial surface-displayed random peptide library to identify peptides capable of binding to the sera of patients with various allergies. This strategy involves the display of peptides on the surface of bacteria using the major bacterial flagellar protein and thioredoxin. To identify IgE-binding epitopes, we performed biopanning using pooled sera of patients allergic to egg white, soy, and dust mites. Using this technique, we identified a number of reactive peptides and their sequences were determined. The binding of these peptides to the sera of allergic patients was confirmed by using a cell-based ELISA. RBL2H3 cells were used to assess the effects of these peptides on allergic reactions. All peptides analyzed were able to inhibit the secretion of β -hexosaminidase from antigen-stimulated cells (Fig. 2B–H), and did so in a dose-dependent manner (Fig. 2B–H). In this experiment, we found that each of these eight peptides exerted a negative effect on histamine release from guinea pig mast cells challenged with ovalbumin (Table 3). The cross-linking of Fc ϵ RI resulted in the release of calcium from intracellular stores. It is known that the released calcium, in combination with PI3 kinase, induces calcium influx (Ching et al., 2001; Hsu et al., 2000), and similar to some anti-allergic drugs that inhibit degranulation via suppression of calcium influx (Matsubara et al., 2004; Paulussen et al., 1998; Berthon et al., 1994), all of the peptides evaluated significantly inhibited calcium influx (Fig. 3A). These peptides also showed similar effects on calcium influx in mast cells stimulated with ovalbumin (Fig. 3B).

NO and H₂O₂ are two of the major reactive oxygen species known to induce histamine release in mast cells (Swindle et al., 2002; Swindle et al., 2004). Hydrogen peroxide (H₂O₂) modulates protein phosphorylation and potassium channel function (Carbajal and Schaeffer, 1998; Suzuki et al., 2003) and stimulates the production of leukotrienes, prostaglandins, and histamine (Harlan and Callahan, 1984; Dent et al., 1997; Matsui et al., 2000). Antigen-stimulated RBL2H3 cells showed increased levels of reactive oxygen species, and NAC specifically blocked it (Fig. 4A). NAC significantly decreased the secretion of β -hexosaminidase from antigen-stimulated cells, suggesting that reactive oxygen species are necessary for secretion of β -hexosaminidase (Fig. 4B). Next, we determined the effects of the peptides on intracellular reactive oxygen species production in antigen-stimulated cells. All of these peptides inhibited reactive oxygen species production in antigen-stimulated cells (Fig. 4C), and we also found that antigen stimulation increased peroxynitrite (ONOO[−]) based on DHR123 staining (data not shown). All of the peptides inhibited peroxynitrite production in antigen-stimulated RBL2H3 cells (data not shown). These results suggest that the inhibitory effects of these peptides on β -hexosaminidase secretion are related to their effects on reactive oxygen species production. We also examined whether NO, another form of reactive oxygen species, had an effect on β -hexosaminidase secretion.

We did not observe any change in NO level in response to antigenic stimulation (data not shown). Based on the fact that antigen stimulation leads to increase in reactive oxygen species production, it was necessary to determine sources of reactive oxygen species. In our preliminary results, protein kinase C (PKC) was responsible for reactive oxygen species production in response to antigen stimulation (data not shown). The inhibition of PKC α by indolocarbazole (Go6976) and that of PKC δ by rottlerin suppressed reactive oxygen species production by antigen stimulation (data not shown). Phorbol myristate acetate (PMA), an activator of PKC, has been shown to increase reactive oxygen species (Kim and Ro, 2005). In this study, we found that antigen stimulation increased expression of p47^{phox} and p67^{phox} (data not shown). Antigen stimulation increases NADPH oxidase activity and the inhibition of NADPH oxidase activity decreases reactive oxygen species production (data not shown). This suggests that NADPH oxidase is mainly responsible for reactive oxygen species production in antigen-stimulated RBL2H3 cells. It is known that Rac1 activity is responsible for reactive oxygen species production in many other cell types (Cheng et al., 2006). It would be necessary to examine whether antigen stimulation could increase Rac1 activity. Antigenic stimulation led to activation of ERK, but not jun N-terminal kinase (JNK) (Fig. 5A). The inhibition of ERK led to decreased secretion of β -hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 5B). The data indicate that activation of ERK is responsible, at least partially, for the secretion of β -hexosaminidase from antigen-stimulated cells. We found that inhibition of ERK did not calcium influx in antigen-stimulated RBL2H3 cells (Fig. 5C). This suggests that ERK functions downstream of calcium influx in allergic inflammation. We then determined whether the peptides were able to modulate phosphorylation of ERK in antigen-stimulated RBL2H3 cells. Indeed, all of the peptides inhibited activation of ERK in antigen-stimulated RBL2H3 cells (Fig. 5D). The degree of inhibition of ERK by peptides is not completely correlated with that of inhibition of secretion of β -hexosaminidase. For example, peptide PKY showed strong inhibitory effect on phosphorylation of ERK (Fig. 5D). However, its effect on secretion of β -hexosaminidase is relatively weaker than peptide, such as GFW. This suggests that other factors may be also responsible for the regulation of secretion of β -hexosaminidase. In this study, we identified anti-allergic peptides through biopanning. These peptides showed inhibitory effects on β -hexosaminidase secretion, phosphorylation of ERK, calcium influx, and reactive oxygen species production in antigen-stimulated RBL2H3 cells. These peptides also inhibit release of histamine (Table 3), calcium influx (Fig. 3B) in lung mast cells challenged with ovalbumin. We found that these peptides inhibit phosphorylation of Lyn (Fig. 6A), PKC α (Fig. 6B), and δ (Fig. 6C). Peptides inhibited interaction between IgE and Fc ϵ RI (Fig. 6), suggesting that these peptides inhibit receptor cross-linking. It is plausible that peptides bind to Fc ϵ RI to prevent interaction between IgE antibody and Fc ϵ RI. We also found that peptides inhibit calcium influx and histamine release in ovalbumin-stimulated guinea pig lung mast cells (Fig. 3, Table 3). Anti-ovalbumin antibody is well-known to bind to

FcεRI. It is therefore possible that these peptides bind to FcεRI in guinea pig lung mast cells. It would be necessary to examine whether these peptides bind to FcεRI by labeling. Peptides inhibited interaction between FcεRI and PKCδ (Fig. 6D), suggesting that PKCδ is at least partially responsible for the activation of FcεRI. We are currently in the process of designing modified versions of peptides to examine their anti-allergic effects using *in vivo* allergy animal models employing 2, 4-dinitro-1-fluorobenzene (DNFB). By employing these modified peptides, we expect to observe enhanced anti-allergic effects. In summary, we identified anti-allergic peptides through biopanning, their effects on various signaling molecules that mediate allergic inflammation. It would be necessary to further identify targets of these peptides in allergic reaction. These peptides merit further investigation for potential use as therapeutic allergy vaccines.

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