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A Novel Insight to the Functional Role of Stathmin 1 in IgE-Mediated Activation of RBL-2H3 Cells

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ABSTRACT

IgE-mediated cell signaling, induced by cross-linking of high affinity receptor for IgE (FcεRI) in the presence of antigen (Ag), is a well known mechanism described for mast cell activation in allergy and hypersensitivity reactions, which induces a spectrum of cellular responses such as secretion and up-regulation of cell surface FcεRI. Although for several years IgE binding to FcεRI was considered to be a passive sensitization process, the outcomes of several recent studies have revealed a variety of different cellular responses to IgE binding compared to IgE plus Antigen binding.

The present study applied a functional proteomics-based approach to investigate mast cell signaling events and provided new insights to FcεRI-mediated cell signaling in RBL-2H3.1 cells, and may point to the activation of alternative signaling pathways in response to IgE or IgE plus Ag. Comparative analysis by 2-D PAGE of RBL cells activated with IgE plus Ag for three and four hours compared to non-activated cells was followed by mass spectrometric protein identification and provided evidence for the induction of Stathmin 1 (STMN1) gene expression in response to IgE plus Ag activation.

Complementary SDS-PAGE analysis showed a distinct up-regulation of STMN1 induction in response to challenge with IgE plus Ag compared to sensitization with IgE only. Phosphoproteomics analysis gave evidence for significant increase at phosphorylation of STMN1 on ser16 after 1min, though a slight rise at 5 min, and on ser38 after 1 and 5min sensitization with IgE and a similar result was observed for 1min IgE plus Ag-activation.

IgE plus Ag-activation was also found to induce the phosphorylation of ser38 to a greater extent than sensitization with IgE. In contrast, IgE alone was more effective than IgE plus Ag at inducing phosphorylation of ser16. Collectively this study provides further insights into the role of stathmin 1 in FcεRI-mediated activation of cells of mast cell lineage and might shed light on the diverse response of these cells to IgE or IgE plus Ag.

Keywords: Fc epsilon RI; Mast Cell; RBL-2H3; Stathmin

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INTRODUCTION

Cell signaling generated by cross-linking of high affinity receptor for IgE (FcεRI) in the presence of antigen/allergen in mast cells (MCs) is a well known phenomenon in allergy, asthma and anaphylaxis, and usually results in molecular and cellular responses in the forms of increased intracellular Ca²⁺ mobility, exocytosis and cytokine release.¹⁻³ Allergen is mostly described as a linker agent of IgE-sensitized FcεRI receptors and usually considered as an inductive agent of FcεRI-signaling, but several studies have also provided evidence for cellular responses being brought about by IgE receptor occupancy in the absence of cognate antigen including increased cell survival in the absence of degranulation,^{4,5} up-regulation of FcεRI,⁶⁻⁸ increases in histamine content of secretory granules⁹ and release,¹⁰ leukotriene release, receptor internalization, stimulation of DNA synthesis,¹⁰ increased responses to substance P and compound 48/80,¹¹ increase in F-actin content¹² and membrane ruffling,¹³ mast cell adhesion to fibronectin,¹⁴ migration and increase in IL-3-induced MC proliferation.¹⁵ Although, IgE and IgE plus Ag are thought to induce cellular responses via the same shared receptor (FcεRI), the diverse cellular responses given rise to them may be the outcome of regulatory effects of crosslinker proteins of branched pathways such as stathmin.

Stathmin-1 (STMN1), also known as oncoprotein 18 (Op18), pp17, pp19, metastasin and LAP18, is a ubiquitously conserved phosphoprotein between all vertebrates. Stathmin is well known as a microtubule destabilizing protein.^{16,17} STMN1 contains four phosphorylation sites on serine residues (Ser16, Ser25, Ser38 and Ser63), and these multiple phosphorylation sites suggest a regulatory role for it in regulation of various molecular reactions.

STMN1 shows binding activity to soluble free tubulin subunits with high affinity and this binding prevents molecular interaction of tubulins thereby inhibiting microtubule formation.¹⁸ Additionally, stathmin is involved in microtubule disassembly with direct catastrophic reaction on microtubules chains.¹⁹

This current study aimed to obtain information regarding the diverse aspects of STMN1 molecular changes in cell signaling pathways in high secretory variant of rat basophilic leukemia cell line (RBL-2H3.1) in response to IgE receptor occupancy only compared to receptor activation by IgE plus Antigen.

MATERIALS AND METHODS

Cell Culture

High secretory variant of RBL-2H3 cell line (RBL-2H3.1) was cultured in DMEM (Sigma), supplemented with 10% (v/v) FCS and 1% (v/v) Penicillin/streptomycin solution (Sigma) at 37°C and 5% CO₂. Cell growth continued, until reached more than 80% confluency.

Cell Cycle Synchronization and Cell Stimulation through FcεRI

Cell cycle synchronization was carried out by FCS deprivation methods as described earlier for human fibroblastic cells.²⁰ Synchronized RBL-2H3.1 cells were used for following process as below. Expressed high affinity receptors for IgE (FcεRI) were sensitized with mouse anti-DNP IgE at final concentration of 0.5μg/ml and for appropriate time courses. For short time activation, sensitized cells were washed at 37°C with DPBS and activation was carried out with DNP-HSA diluted in 37°C DPBS, and for longer time stimulations, DNP-HSA was diluted in cell culture media. Activation carried out in 37°C at presence of 5% CO₂.

Cell lysis and Sample Preparation for 2-D

Cell monolayers were washed with cold PBS for 4-5 times. Cell lysis was made in RIPA buffer containing 65mM DTT, protease inhibitor (Roche Complete cocktail) and phosphatase inhibitor (Sigma) in a concentration recommended by providers. Cell lysate was used for loading on SDS gel for 1-D electrophoresis or subjected to protein precipitation and salt removal with TCA/acetone (10% TCA in acetone) solution and then re-suspended in 2-D sample buffer containing 6M Urea, 2M Theurea, 4% CHAPS, 2% (v/v) IPG buffer, 65mM DTT and 0.001% (w/v) Bromphenol Blue (BPB). The samples provided in 2-D sample buffer were used for 2-D PAGE as below.

2-D Gel Electrophoresis

DryStrip rehydration was carried out during overnight loading of 500μg of resuspended protein in 2-D sample on 13cm IPG strips (pH 3-10 or 7-4). IEF was carried out by applying electric current at 150v for 1h, 300v for 1h and then 3500v for 5.30h at 12°C. Focused strips were used for protein isolation on SDS gel for second dimension.

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Before electrophoresis, the strips were incubated with equilibration buffer A (6M Urea, 50mM Tris-HCl pH: 8.8, 30% glycerol, 2% SDS, 100mM DTT and 0.001% BPB) for 15min and then incubated in buffer B (consist of all components of buffer A, but containing 270mM iodoacetamide instead of DTT) at the same condition as above. Strips were placed on the top of 12% SDS gel and overlaid with 1% melting agarose gel. Electrophoresis was run in tris-glycine buffer and proteins were visualized with colloidal coomassie staining or mass spectrometry compatible silver stain (ProteoSilver™ Plus, Sigma).

Image Analysis

2-D maps were visually inspected for comparative analysis. Gray stain value of selective proteins were measured by ImageJ software (downloaded from; <http://rsbweb.nih.gov/ij/download.html>) on 2-D map of proteins from activated cells and non-activated cells.

In-Gel Trypsin Digestion

Protein spots were picked up from Coomassie or mass spectrometry compatible silver stained gels and dried in vacuum centrifuge and subjected to trypsin digestion according to the instruction provided by Sigma.

MALDI-TOF Mass Spectrometry

Trypsin-digested protein was co-crystallized with Cyano-4-hydroxy-cinnamic acid (Sigma) as matrix at a concentration of 10mg/ml in 50% acetonitrile/0.05% TFA and loaded onto the target plate for matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using an ABI Voyager-DE STR instrument operated in positive ion reflector mode for protein identification by peptide mass fingerprinting method. Mass spectrometer was calibrated by commercially available calibration standards (ABI) and on average 100 spectra were acquired for protein identification.

Database Search and Data Analysis

Protein identification was performed by searching the MSDB database using the Mascot search engine with typically 100 ppm mass accuracy, trypsin as digesting enzyme, with 1 missed cleavage allowed and specifying carbamidomethylation of cysteine and oxidation of methionine. Protein identification was validated by checking the estimate PI and MW on 2-D

PAGE with those of recorded and calculated in ExPASy PI/MW computing tool.

1-D Gel Electrophoresis and Western Blot

In each case same quantity of proteins for sample and control were separated on 12% acrylamide SDS gel and transferred onto PVDF membrane. After transfer, the membrane was blocked with 5% skimmed milk and incubated with anti-stathmin 1 (abcam) for expression analysis or anti-stathmin (ser16) and anti-stathmin (ser38) antibodies (Cell Signalling) for phosphorylation analysis were used as primary antibodies and appropriate HRP-conjugated antibodies used as secondary antibody. Protein visualization was carried out on photographic film with Enhanced Chemiluminescence (ECL) method (Amersham Biosciences).

RESULTS

Comparative analysis and following densitometric analysis of protein above 17kD with ImageJ led to the detection of increased expression of this protein in cell lysates of RBLs 4h after receptor activation (Figure 1) and further mass spectrometric analysis identified the protein as STMN1 (Figure 2).

For additional confirmation, cell cycle synchronization was attempted with FCS-deprivation method (20) and equal quantities of total proteins from cell lysis of 3 and 4h IgE-sensitized and IgE plus Ag-activated cells along with sample from resting cells were analyzed by SDS gel electrophoresis and Western blotting using anti-stathmin antibody (Figure 3). The results confirmed induced expression of STMN1 in 3 and 4h IgE plus Ag-activated cells compared to resting cells. However, this is not observed when cells are sensitized with IgE alone for the same period (Figure 3). As preliminary experiments indicated differential expression of STMN1 in response to IgE plus Ag compared to IgE, further experiments were designed to evaluate molecular variation of STMN1 in these situations. For this, two phosphoserine residues of ser16 and ser38 were studied from total of four phosphoserines reported for STMN1 (Ser16, 25, 38 and 63). Anti-phosphoserine16 and anti-phosphoserine38 and Western blot analysis on sample provided from 1 and 5min IgE-sensitized or IgE plus Ag-activated cells allied to evaluate the differential phosphorylation response of STMN1 (Figure 4).

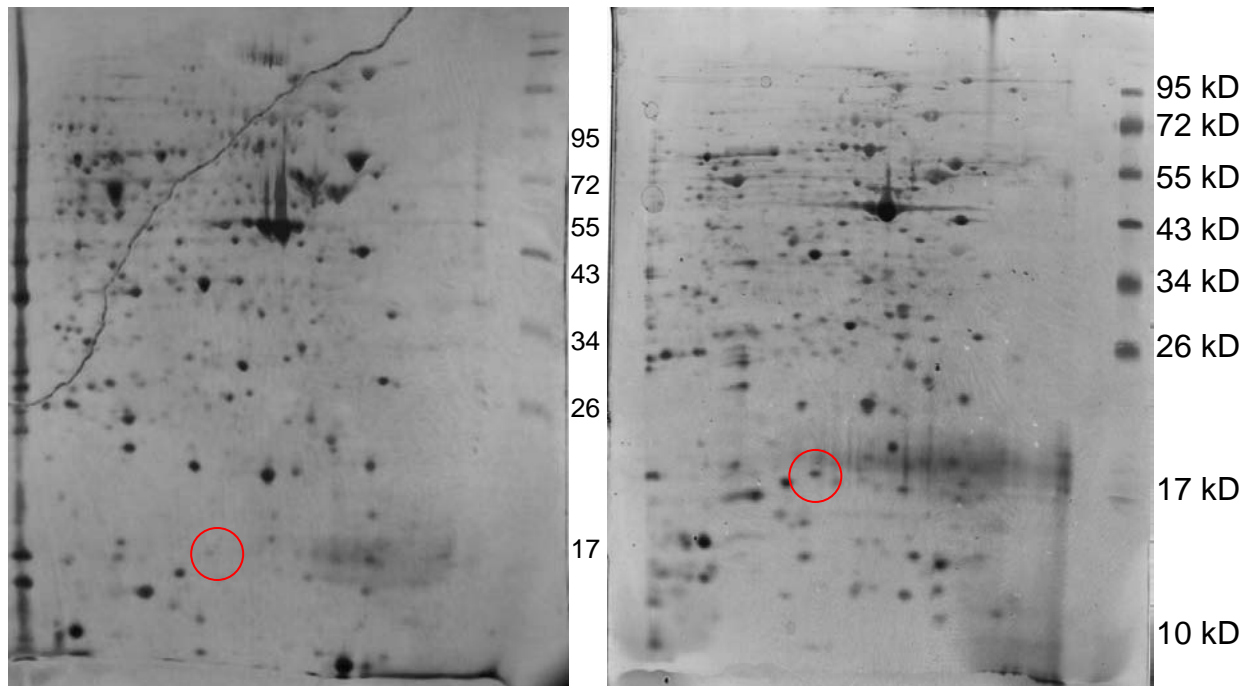


Figure 1. 2-D PAGE of proteins from cell lysate obtained from non-activated RBL-2H3 cells (left) and activated cells with IgE plus Antigen for 4 hours (right). 'Spot' circled was identified as stathmin 1.

The outcome of this study confirmed significant rise at phosphorylation of Ser16 at the time point of 1min after cell treatment by IgE and IgE plus Antigen compared to control (Cells without IgE or IgE plus Ag treatment). While, this is in higher extend in response to IgE rather than IgE plus Ag. Repeated experiment at the same condition but at 5min shows decreased level of Ser16 phosphorylation compared to 1min, though IgE response is still slightly higher compared to control. Compared to Ser16, Ser38 follows a regular pattern of phosphorylation. On the other hand, Ser38 phosphorylation increases from 1 to 5 min in both inductive condition and it is significantly higher in response to IgE plus Ag rather than IgE alone.

DISCUSSION

The results of this study introduce stathmin 1 as an inducible molecule in response to FcεRI-mediated activation of mast cells. These results also confirm differential expression and post-translational modification of STMN1 in the form of phosphorylation which may point to regulatory role of this molecule at discrimination of cellular responses to IgE or IgE plus Antigen.

Stathmin is mainly known as a protein involved in regulation of microtubule dynamics and from there it is involved in various forms of cellular functions such as cell proliferation, mitosis, intracellular mobility of sub-cellular compartments and apoptosis. Up-regulation of stathmin was initially described in highly proliferative cells such as cancerous cells,^{18,21} which could point to the pivotal role of stathmin at regulation of cell growth and mitosis.

This idea was further supported by observations that confirm the exposure of normal lymphocytes to mitogenic stimuli is able to drive the cells toward the over-expression of stathmin,²¹ but anti-proliferative agents like 4-hydroxynonenal can cause down-regulation of stathmin.²² Therefore, up-regulation of stathmin in IgE plus Ag-activated cells and not IgE-sensitized cells might be due to the activation of the MAP kinase pathway turned on by IgE plus Ag. This notion is further supported by previous observations which demonstrate an increased number of mast cells in allergic disease.²³⁻²⁵ Furthermore, increased expression of stathmin at presence of IgE plus Ag and not in the presence of IgE alone may occur as a result of IgE plus Ag to induce exocytosis which is known as a microtubule related phenomenon.

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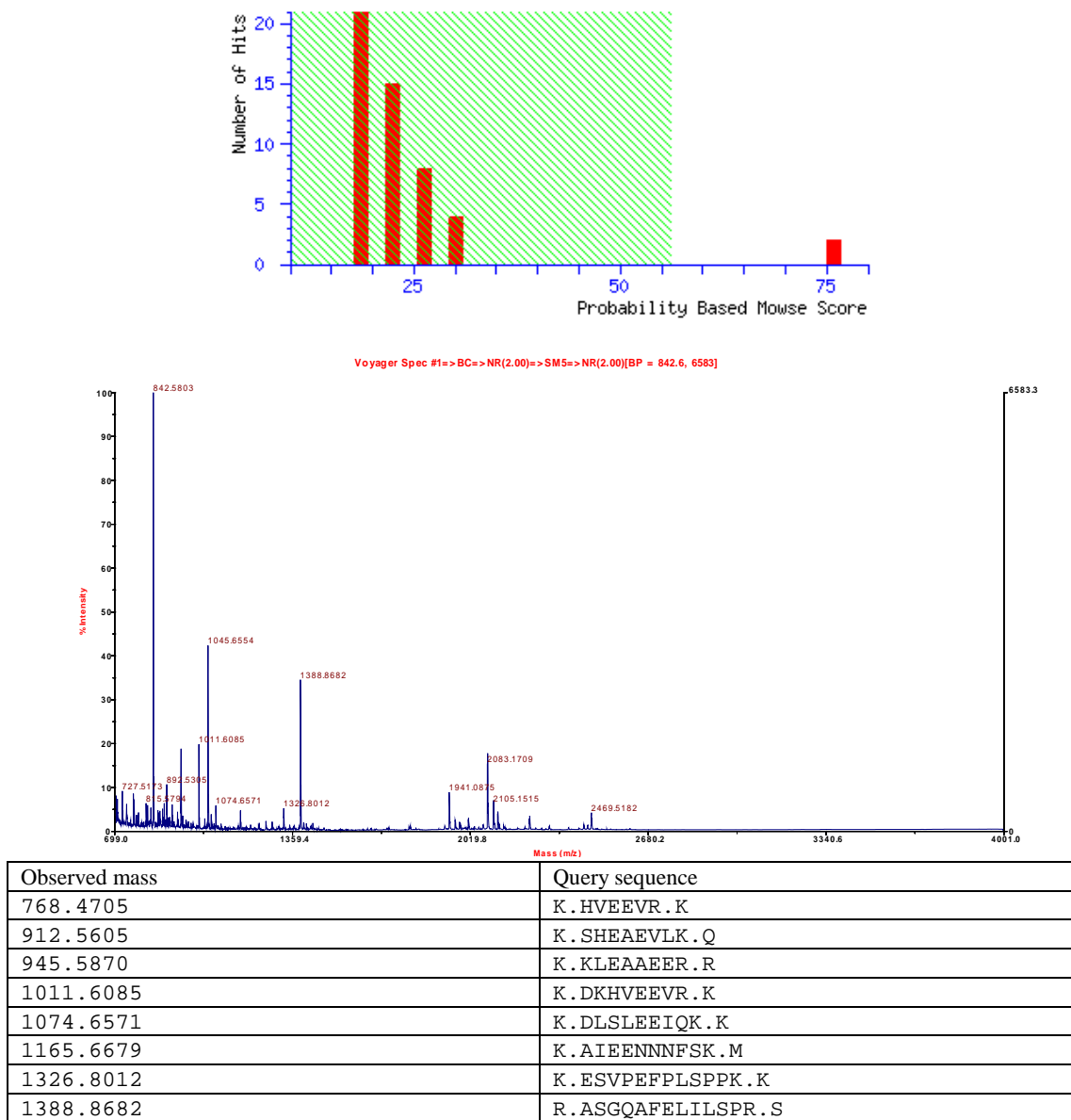


Figure 2. Mass spectrometric identification of stathmin from trypsin-digested protein excised from silver-stained 2-D gel. The picture shows probability based Mowse score diagram (Up) and mass spectrum of reporter peptide ions (Down) with m/z values of protein identified as stathmin 1. From total of 32 m/z value identified with MALDI-TOF, 8 of them uniquely belong to stathmin 1, which are listed in the table.

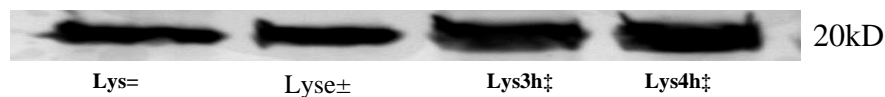


Figure 3. Expression analysis of stathmin 1 in response to IgE and IgE plus Ag. Western blot analysis was carried out with anti-stathmin1 as primary antibody and HRP-conjugated anti-rabbit antibody as secondary antibody, followed by protein visualization with enhanced chemiluminescence (ECL) revealing increased expression of stathmin 1 following cell challenge with IgE plus Ag.

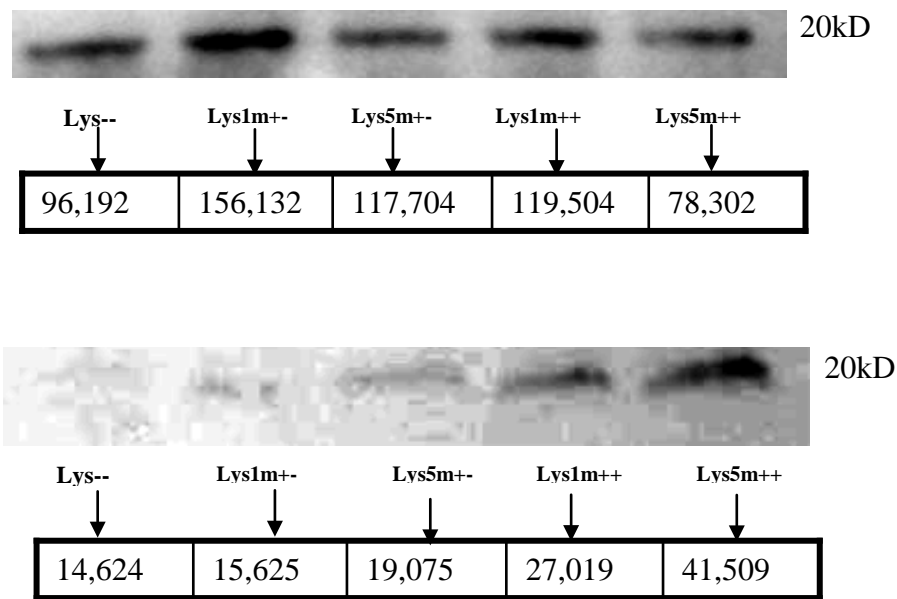


Figure 4. Western blot analysis of stathmin phosphorylation on serine16 and serine38 indicating a change in phosphorylation of stathmin 1 on serine16 (above) and serine38 (below) in the sample provided from cells in resting state (Lyse=), sensitized cells with IgE for 1 and 5min (Lyse1m± and Lyse5m±) and sensitized cells with IgE for 16h and then activated with (DNP-HSA) for 1 and 5min (Lyse 1m‡ and Lyse 5m‡). The numbers blow each lane are representative of “Integrated density” of grey stain calculated by ImageJ software at the same area about 1300 pixels for each corresponding band. Each band is representative of phosphostathmin from 30µg of total protein from cell lysate.

In contrast, monomeric IgE is unlikely to induce cell degranulation.⁵ Stathmin might be required to prepare the cells for next round of secretion and regulation of secretory granule generation from Golgi. A role of stathmin in cell secretion is further supported by experiments carried out by Strey et al.²⁶ who show that Golgi fragmentation occurs at increased concentrations of stathmin. Furthermore previous studies on RBL-2H3 cells show that Colchicine, a microtubule inhibitor, is able to inhibit intracellular mobility of secretory granules and this supports the role of microtubule, as a main substrate of stathmin, in cell secretion.²⁷ A role of stathmin for regulation of hormone secretion in rodent pituitary and insulinoma cell lines has been reported previously²⁸ and the contribution of stathmin at regulation of cell secretion has been emphasized by studies which correlate activation of Rho GTPase family and stathmin phosphorylation.^{29,30}

The role of stathmin to function at gene expression level and its biological function is tightly regulated by protein phosphorylation on four serine residue (ser16, ser25, ser38 and ser63). Phosphorylation decreases the

affinity of stathmin for tubulin, and in presence of phosphostathmin, tubulins tend towards microtubule formation.

Differential phosphorylation on ser16 and ser38 of STMN1 at presence of IgE and IgE plus Ag may serve to further discriminate cellular responses of mast cells to IgE or IgE plus Ag at molecular level. They may also support the hypothesis that IgE and IgE plus Ag is involved in diverse forms of downstream signaling or in the induction of same signaling pathway by different signal strength. Identifying specific kinase involved in phosphorylation of stathmin after FcεRI activation may further assist our understanding of the role of stathmin in mast cell signaling.

Exocytosis, the major form of mast cell response to FcεRI activation, is a cytoskeleton dependent event.^{27,31,32} Therefore, increased phosphorylation of ser16 in IgE-sensitized cells and IgE plus Ag-activated cells compared to resting cells might be explained by the role of microtubules formation toward the cell secretion (in the form of exocytosis and cytokine release). However, phosphorylation of ser16 at receptor occupancy by IgE and in response to IgE plus Ag is

interesting in several ways: Firstly, IgE induces the phosphorylation of this residue more than IgE+Ag. Secondly, IgE-induced phosphorylation is seemingly more long lasting when compared to IgE plus Ag-induced phosphorylation.

Although, it might be argued that aggregation of IgE might explain the potency of IgE to induce cell signaling in the absence of antigen, we never observed any mediator release (β -hex) in cells sensitized with IgE only when compared to unsensitized cells. Therefore, exocytosis which occurs in response to IgE plus Ag is not induced by IgE. However, the response to IgE to induce ser16 phosphorylation of stathmin may induce other forms of cell responses such as induction of cytokine synthesis release rather than induced exocytosis. This idea may further emphasize by comparative study of cytokine release at presence of IgE and IgE plus Ag⁵ which confirm the increased potency of IgE in relation to cytokine metabolism. However, as microtubule destabilizing activity of stathmin is regulated by three other phosphoserine residues (ser25, ser38, ser63), as well as ser 16, phosphorylation of these residues must also be taken into account when discussing the cellular function of stathmin.

The current study also shows induced phosphorylation of ser38 in response to IgE and IgE plus Ag. In this case, IgE plus Ag give rise to a more potent signal than IgE alone. This might be explained by higher potency of IgE plus Ag activation toward exocytosis compared to IgE sensitization, or by considering CDKs and MAPKs as major kinases involved in phosphorylation of ser38³³⁻³⁵ and these observations may further relate to other cellular responses in the form of induced gene expression, cell proliferation and mitosis.

Collectively, our observations reveal differential induction of STMN1 in presence of IgE and IgE plus Ag and these findings may form the basis for further investigations that may shed light on the central regulatory role of this molecule at cellular responses to IgE or IgE plus Ag.

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