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# Compartmentalization of the Type I Fcε receptor and MAFA on mast cell membranes

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**Abstract**

The Mast cell Function-associated Antigen (MAFA) is a membrane glycoprotein on rat mast cells (RBL-2H3) expressed at a ratio of ~1:30 with respect to the Type I Fcε receptor (FcεRI). Despite this stoichiometry, clustering MAFA by its specific mAb G63 substantially inhibits secretion of both granular and de novo synthesized mediators induced upon FcεRI aggregation. Since the FcεRIs apparently signal from within raft micro-environments, we investigated possible co-localization of MAFA within these membrane compartments containing aggregated FcεRI. We used cholera toxin B subunit (CTB) to cluster the raft component ganglioside GM1 and studied the effects of this perturbation on rotation of FcεRI and MAFA by time-resolved phosphorescence anisotropy of erythrosin-conjugated probes. CTB treatment would be expected to substantially inhibit rotation of raft-associated molecules. Experimentally, CTB has no effect on rotational parameters such as the long-time anisotropy ($r_\infty$) of unperturbed FcεRI or MAFA. However, on cells where FcεRI-IgE has previously been clustered by antigen ($DNP_{14}$-BSA), CTB treatment increases the FcεRI-IgE's $r_\infty$ by 0.010 and MAFA's by 0.014. Similarly, CTB treatment of cells where MAFA had been clustered by mAb G63 increases MAFA's $r_\infty$ by 0.010 but leaves FcεRI's unaffected. Evaluation of raft localization of FcεRI and MAFA using sucrose gradient ultracentrifugation of Triton X-100 treated membrane fragments demonstrates that a significant fraction of MAFA molecules sediments with rafts when FcεRI is clustered by antigen or when MAFA itself is clustered by mAb G63. The large excess of FcεRI over MAFA explains why clustering MAFA does not substantively affect FcεRI dynamics. Moreover, in single-particle tracking studies of individual FcεRI-IgE or MAFA molecules, these proteins, upon clustering by antigen, move into small membrane compartments of reduced, but similar, dimensions. This provides additional indication of constitutive interactions between FcεRI and MAFA. Taken together, these results of distinct methodologies suggest that MAFA functions within raft microdomains of the RBL-2H3 cell membrane and thus in close proximity to the FcεRI which themselves signal from within the raft environment.


*Keywords:* FcεRI; MAFA; 2H3; Plasma membrane rafts; Signaling; Single-particle tracking

## 1. Introduction

The Type I Fcε receptors expressed on mast cells and basophils (FcεRI) are specific for the respective Fcε domains of IgE molecules and bind them with a stoichiometry of 1:1. Although binding by itself does not the initiate secretory response of these cells, clustering of the FcεRI via the bound IgEs which serve as antigen-specific, divalent adaptors provide the signal leading to the above cellular response [1]. This response involves secretion of both granule-stored mediators

*Abbreviations:* 2H3, rat mucosal-type mast cells of the 2H3 cell line; BSS, balanced salt solution; CTB, cholera toxin B subunit; *D*, diffusion coefficient; DMEM, Dulbecco's Modified Eagle's Medium; DNP, 2,4-dinitrophenyl; Er, erythrosin isothiocyanate; FcεRI, Type I Fcε receptor; FPR (or FRAP), fluorescence photobleaching recovery; GPI, glycosylphosphatidyl-inositol; ITIM, immuno-receptor tyrosine-based inhibitory motif; M, fractional mobility; mAb, monoclonal antibody; MAFA, mast cell function-associated antigen; MEM, minimal essential medium; PTK, protein tyrosine kinase.

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such as histamine as well as the de novo synthesized ones [2] utilizing a signaling network that includes recruitment and activation of specific protein tyrosine kinases (PTK) [3], a transient increase in tyrosine phosphorylation of several cellular proteins [4,5] and free intracellular calcium ion concentrations [6] and activation of PLCγ and phosphatidyl inositide hydrolysis [7].

Several membrane proteins, including a membrane glycoprotein termed the Mast cell Function-associated Antigen (MAFA), have been found to suppress the FcεRI-mediated secretory response [8–12] upon being clustered by the specific mAb G63. Although there is only approximately one MAFA expressed for every thirty FcεRI, clustering MAFA was shown to inhibit by up to 80% the secretory response of the rat mucosal-type mast cells of the RBL-2H3 line to subsequent FcεRI clustering [13]. MAFA apparently delivers negative signals overriding those of the aggregated FcεRI upstream of PLCγ activation by suppressing both phosphatidylinositide phosphate hydrolysis and transient intracellular calcium elevation [13,14] and these inhibitory effects are not due to MAFA interference with IgE-FcεRI interactions [13]. Pecht et al. have shown that MAFA clustering induces phosphorylation of the tyrosine residue of its immuno-receptor tyrosine-based inhibitory motif (ITIM) by the PTK *lyn* [15] . This induces recruitment to this ITIM of both SHIP, presumably causing reduction of PIP3 levels [15], and SHP2, lowering *syk* activity [16].

Previous studies suggest functionally significant interactions between FcεRI and MAFA within the cell membrane. Fluorescence resonance energy transfer measurements demonstrate proximity of FcεRI to MAFA binding mAb G63 [17]. Time-resolved phosphorescence anisotropy measurements of rotational dynamics of FcεRI and MAFA [18] suggest that these molecules are constitutively associated with one another, at least to some extent, irrespective of MAFA's aggregation state. Thus, it is important to determine whether these interactions occur at arbitrary sites in the plasma membrane or in well-defined membrane compartments. Membrane microdomains include so-called rafts which, because of their high cholesterol and sphingolipid content, float in sucrose gradients. Rafts are enriched, not only with sphingolipids and cholesterol [19], but also with glycosylphosphatidyl-inositol (GPI)-anchored proteins [20] and, in some cells, comprise a substantial fraction of the plasma membrane [21,22]. These microdomains can contain membrane proteins necessary for cell signaling [23,24] and have been reported to diffuse laterally as intact entities within the plasma membrane [25]. Baird's group has shown that unperturbed, non-aggregated FcεRI are dispersed within the plasma membrane but, upon aggregation, are translocated into lipid rafts [26]. To independently examine the effects of FcεRI clustering on the compartmentalization of MAFA or FcεRI, we have now combined several methods, time-resolved phosphorescence anisotropy measurements of protein rotation, density gradient ultracentrifugation and single-particle tracking of individual protein molecules on viable cells in order to evaluate the characteristics of membrane compartments accessed by MAFA and FcεRI.

## 2. Materials and methods

### 2.1. Cells and culture

Rat mucosal-type mast cells of the RBL-2H3 line were kindly provided by Dr. Reuben Siraganian of the National Institutes of Health. Cells were grown and characterized as described [18] in Minimum Essential Medium Eagle with Earle's salts (VWR), containing HEPES buffer and non-essential amino acids (Sigma-Aldrich, St. Louis, MO) and supplemented with 10% fetal bovine serum (Gemini Bio Products, Woodland, CA). Cells were grown to approximately 90% confluence in BD Falcon non-treated, plug-seal tissue culture flasks (VWR), which they prefer, and then seeded into Petri dishes for high-throughput cell culture. For raft experiments, 20 dishes were usually grown to provide the necessary 25 million cells per sample. Cells were harvested from culture using a 5 mM EDTA in PBS for 10 min at 4 °C and then washed down using Hank's Balanced Salt Solution.

### 2.2. Antibodies, proteins and conjugates

mAb G63 ($IgG_1$) was purified from hybridoma culture supernatants by chromatography [18]. Monoclonal 2-4-dinitrophenyl (DNP)-specific A2 mouse IgE was purified from ascitic fluid by binding to DNP-Sepharose and elution with DNP-glycine [13]. FcεRI alpha chain-specific antibody was obtained from Upstate Cell Signaling Solutions, Lake Placid, NY. $DNP_{14}$-BSA, derivatized with an average of 14 DNP-groups per molecule, was prepared as described earlier [27]. Antibodies were derivatized with erythrosin isothiocyanate (Er, Molecular Probes, Eugene, OR) using a modification [18] of methods described by Johnson and Holborow [28]. Prior to use, all dye-derivatized proteins were centrifuged at 130,000×*g* for 10 min in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA) to remove any protein aggregates formed during storage.

### 2.3. Time-resolved phosphorescence anisotropy measurements

Time-resolved phosphorescence anisotropy (TPA) experiments were performed using methods previously described [29,30] as adapted for MAFA [18]. Cells were labeled either

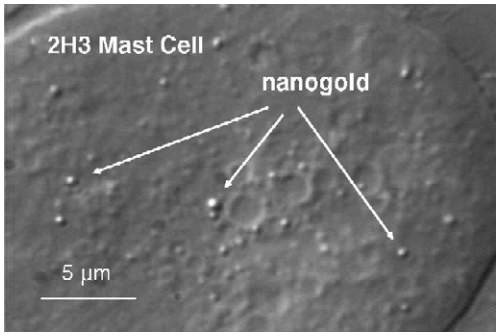


Fig. 1. Photograph of 2H3 cell showing IgE-nanogold particles bound to individual Type I Fcε receptors.