

# Regulation of mast cells' secretory response by co-clustering the Type 1 Fc $\epsilon$ receptor with the mast cell function-associated antigen

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The mast cell function-associated antigen (MAFA) is a type II membrane glycoprotein first identified on rat mast cells and basophils. Clustering MAFA inhibits these cells' secretory response to the type 1 Fc $\epsilon$  receptor (Fc $\epsilon$ RI) stimulus. To quantitatively characterize this inhibition and its dependence on MAFA-Fc $\epsilon$ RI co-clustering, we investigated the secretory response of rat mucosal-type mast cells of the RBL 2H3 line carrying an IgE class, 2,4 dinitrophenyl (DNP) specific monoclonal antibody to DNP-conjugated Fab and F(ab')<sub>2</sub> fragments of (1) mouse IgG, and (2) of the MAFA-specific, monoclonal antibody G63. The first reagent clusters Fc $\epsilon$ RI-IgE complexes into oligomers by reacting with the DNP residues. The DNP conjugated G63 Fab and F(ab')<sub>2</sub> fragments, additionally aggregate MAFA and form Fc $\epsilon$ RI-IgE-MAFA co-clusters. All experiments using these ligands were performed in the absence or presence of an excess of intact mAb G63, which clusters MAFA molecules. Empirical Hill functions were used to relate the secretory response of mast cells to the equilibrium concentrations of Fc $\epsilon$ RI-IgE or MAFA clusters and co-clusters calculated as function of the employed ligands concentrations. This analysis of the experimental results indicates that co-clustered MAFA molecules have a markedly higher inhibitory capacity than MAFA-clusters alone. The molecular basis of the enhanced inhibition observed upon co-clustering MAFA with the Fc $\epsilon$ RI is most probably the increased concentration of the inhibitory cell components in the immediate proximity of the activation coupling elements.

Received 26/12/04

Accepted 21/2/05

[DOI 10.1002/eji.200425964]

## Key words:

Mast cells · Type I Fc $\epsilon$ -receptor  
· Signal transduction  
· Regulation

## Introduction

Response of immune system cells to stimuli provided by their respective antigen receptors is a tightly regulated process. A family of receptors endowed with the capacity

of inhibiting the activation processes triggered by the antigen receptors has been characterized during recent years [1, 2, 3]. The inhibitory receptors carry in their cytosolic tails one or more sequence motifs generally referred to as immunoreceptor tyrosine-based inhibition motif (ITIM). These inhibitory receptors include amongst other the CTLA-4 and CD22 expressed by T and B cells, respectively, murine Fc $\gamma$ RIIB on B and mast cells or the killer cell inhibitory receptors (KIR) on natural killer (NK) cells [1, 2, 3]. These receptors, upon being co-clustered with the activating receptors undergo phosphorylation of their ITIM tyrosine residues and recruit SH2 domain containing phosphatases, which interfere with the activating receptors' stimulus-response coupling cascade [1, 3, 4, 5]. The mast cells

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**Abbreviations:** **RBL-2H3:** Rat basophilic leukemia cells, subline 2H3 · **MAFA:** Mast cell function-associated antigen · **DNP:** Dinitrophenyl · **ITIM:** Immunoreceptor tyrosine-based inhibition motif · **Fc $\epsilon$ RI:** Type I Fc $\epsilon$  receptor · **Fc $\gamma$ RIIB:** Type II Fc $\gamma$  receptor on B cells

function-associated antigen (MAFA), another inhibitory receptor has a C-type lectin domain in its extracellular part and, like the other members of the above family, its intracellular domain contains an ITIM sequence [6]. MAFA was discovered on rat mast cells by means of a specific monoclonal antibody (G63), that binds it with considerable affinity and induces its clustering [6, 7]. Clustering was found to be required and sufficient for inhibiting the secretory response to the type 1 receptor for IgE (FcεRI) [7–9]. This is one important feature, which distinguishes MAFA as an inhibitory receptor from the majority of the other that require their co-clustering with the activating receptors in order to induce inhibition [1–4].

Binding studies revealed that rat mucosal-type mast cells of the RBL-2H3 line carry an average of  $\text{ca. } 1 \times 10^4$ – $2 \times 10^4$  MAFA copies per cell on their surface [7], *i.e.* less than tenfold that of FcεRI ( $3 \times 10^5$ – $5 \times 10^5$ ) [10, 11]. Therefore the question arose how this comparatively small number of MAFA molecules is capable of inhibiting the cells' response to the FcεRI stimulus and was rationalized by the assumption that the MAFA recruited enzymes have the capacity of amplifying the inhibitory signal. Moreover, fluorescence microscopy studies suggested that MAFA is at least partly co-localized with aggregated FcεRI [12]. This was further supported by measurements of the fluorescence resonance energy transfer between MAFA bound mAb G63 and FcεRI bound IgE on the RBL-2H3 cells, indicating that at least some MAFA molecules and FcεRI are indeed in close proximity even on unperturbed cells [13]. Further evidence for a possible association of these two membrane components emerged from recent emission anisotropy studies; Song et al. [14] reported phosphorescence anisotropy measurements of MAFA-bound Fab fragments of mAb G63, performed in the presence and absence of FcεRI aggregation, and found that clustering of the latter reduces the rotational relaxation time of the MAFA-G63-Fab complex. Taken together, all these results suggest that at least a partial degree of FcεRI-MAFA association exists on the surface of unperturbed RBL-2H3 cells.

The above observations combined with the fact that the absolute majority of the ITIM containing receptors require co-clustering with the activating receptors for exerting their inhibitory function raises the questions: (1) does also FcεRI-MAFA association negatively regulate the cells' response? (2) If so, can one correlate this association with the inhibitory action? To answer these questions we have now investigated the inhibition of the RBL-2H3 cells' secretory response as a function of co-clustering MAFA with the FcεRI-IgE complexes. This was done using bi-specific reagents with different valences, which can react simultaneously with both these membrane components. The inhibition caused by

co-clustering MAFA with the FcεRI was then compared with that caused by clustering MAFA by intact mAb G63. The resulting data were analyzed in terms of a computational model, which describes both, cell secretion and its inhibition as a function of the equilibrium distribution clusters and co-clusters of FcεRI-IgE-MAFA on the cells' surface.

## Results

### Theory

The following clustering agents were prepared and employed in the present study: (1)  $\text{F(ab')}_2$  and Fab fragments of normal mouse polyclonal IgG conjugated with a different number of DNP groups (notation:  $\text{m(Fab')}_2\text{DNP}_n$ , and  $\text{mFabDNP}_n$  respectively;  $n$ : average number of DNP per Fab or  $\text{F(ab')}_2$  fragment). These reagents react with cell-bound, DNP-specific monoclonal IgE and cluster them into oligomers of different sizes, depending on the number of attached DNP groups. (2) The MAFA-specific mAb G63 and its  $\text{F(ab')}_2$  and Fab fragments. The latter two were also derivatized with DNP (notation:  $\text{G63F(ab')}_2\text{DNP}_n$  and  $\text{G63FabDNP}_n$ , respectively).

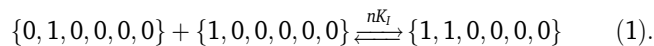
For the manifold of all these reagents we use in the following the term 'ligand'. To describe the multiple interactions between the ligands and their respective receptors; FcεRI-IgE and MAFA, the following terminology will be used throughout the paper. FcεRI-IgE oligomer formation by its interaction with the hapten (DNP) carrying  $\text{F(ab')}_2$  fragments of mouse polyclonal (nonspecific) IgG and the oligomerization of MAFA by mAb G63 or its  $\text{F(ab')}_2$  fragments is termed ligand induced receptor clustering. The induction of MAFA association with FcεRI-IgE-oligomers by hapten derivatized mAb G63 is termed co-clustering. The term 'association' is generally used for all types of receptor-ligand aggregation.

A quantitative comparison of the inhibitory capacity of MAFA clustering and co-clustering requires knowledge of different thermodynamic parameters of the process, *e.g.* concentrations of the different reacting species. Hence, a theoretical simulation based on the below outlined model has also been performed aiming at calculating the dose response of RBL-2H3 cells to the above listed reagents. This involves calculation of the equilibrium concentrations of all FcεRI-IgE and MAFA containing clusters as a function of the total ligand concentration. To efficiently account for the entire manifold of clusters produced upon reacting mast cells carrying a hapten (DNP)-specific mAb with the above mentioned reagents we utilize the representations  $\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}$  and  $\{l_g, m_g\}_m$ .  $\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}$  represents a complex with  $r$  FcεRI-

RI-IgE-,  $l$  DNP-conjugated ligands,  $m_{fm}$  MAFA-monomers and  $m_{fd}$  MAFA dimers co-clustered with a given Fc $\epsilon$ RI-IgE aggregate by means of monovalent G63-Fab-DNP<sub>n</sub> and divalent G63F(ab')<sub>2</sub>DNP<sub>n</sub>, and  $m_p$  MAFA monomers pre-aggregated with the Fc $\epsilon$ RI-IgE ( $m_p$ ).  $n_c=0,1$  denotes open and closed ring aggregates, respectively. Characteristic aggregates are illustrated in Fig. 1. An open aggregate (Fig. 1d, e, g) has either one or two free antigen binding sites on the IgE molecules. In a closed aggregate, the ligand has reacted with all binding sites of the IgE to form a ring-like structure (Fig. 1f). The second representation  $\{l_g, m_g\}_m$  reflects the status of those MAFA molecules which are not co-clustered with the Fc $\epsilon$ RI, yet are subject to clustering by intact G63 or its

F(ab')<sub>2</sub> fragments [F(ab')<sub>2</sub>G63].  $l_g=0$ ,  $m_g=0$  denotes the absence of these agents and, thus, of clustering.  $l_g=1$ ,  $m_g=1$  denote binding of one Fab-binding site of G63 to the MAFA and  $l_g=1$ ,  $m_g=2$  denotes two MAFA molecules clustered through G63 [intact or F(ab')<sub>2</sub>].

For the purpose of illustration, some important and representative reactions considered in our model are listed in the following. For instance, the binding of mF(ab')<sub>2</sub>DNP<sub>n</sub> and G63F(ab')<sub>2</sub>DNP<sub>n</sub> to cell bound IgE is written as:



The aggregate  $\{1, 1, 0, 0, 0, 0\}$  is depicted in Fig. 1c. For the sake of simplicity we assume that all DNP groups

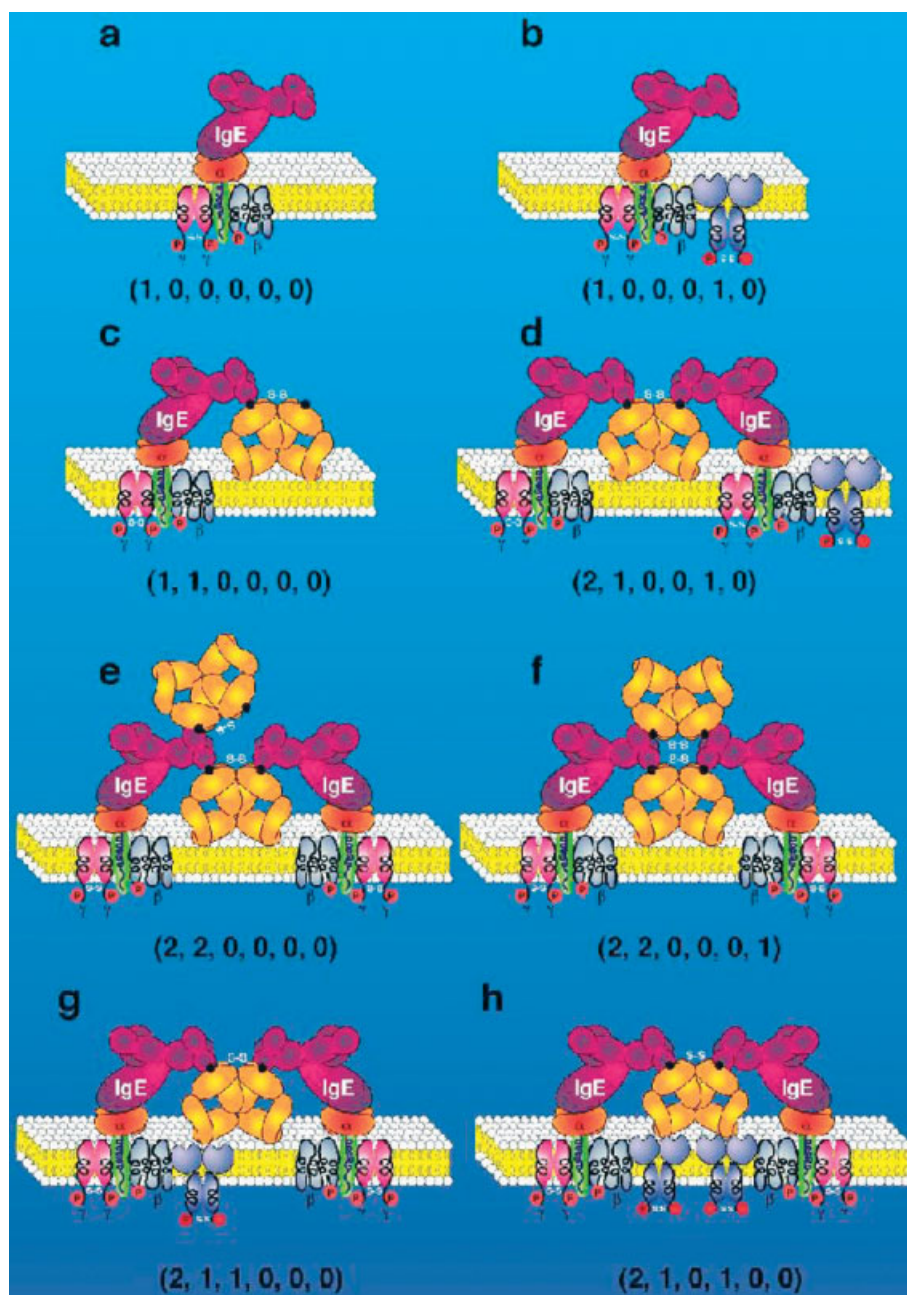
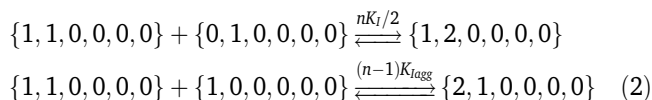
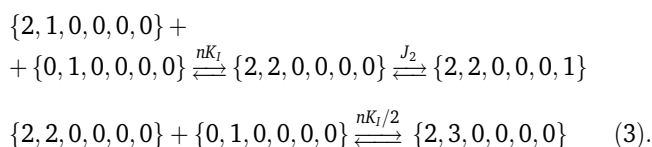


**Fig. 1.** A scheme illustrating several of the possible A2-IgE-Fc $\epsilon$ RI-MAFA aggregates produced by the binding of DNP-derivatized F(ab')<sub>2</sub> fragments of either mouse IgG or of the MAFA-specific mAb G63 to the antigen combining sites of a DNP-specific IgE and/or MAFA epitopes. The different aggregates are characterized by the vector like representations  $\{r, l, m_{fm}, m_{fd}, m_p, n_c\}$ , that represent a complex with  $r$ -Fc $\epsilon$ RI- IgE receptors,  $l$  DNP-conjugated ligands,  $m_{fm}$  MAFA-monomers and  $m_{fd}$  MAFA dimers co-clustered within a given Fc $\epsilon$ RI-IgE aggregate by means of the divalent G63F(ab')<sub>2</sub>DNP<sub>n</sub> ligands and MAFA pre-aggregated with the Fc $\epsilon$ RI ( $m_p$ ).  $n_c=0, 1$  denotes open and closed ring aggregates, respectively.

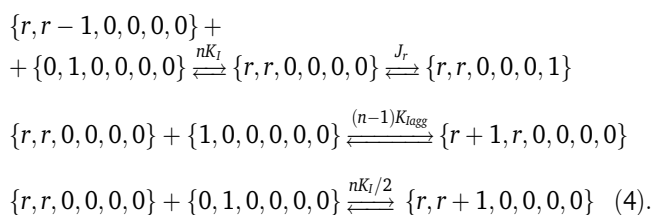
are bound with the same binding affinity  $K_L$ . In fact,  $nK_L$  is an apparent, average affinity. In the next step, the FcεRI-IgE complex can react either with another ligand, or with another FcεRI-IgE to yield a dimer:



where  $K_{Iagg}$  is the equilibrium constant for aggregation on the cell surface per DNP-binding site. The factor  $(n-1)$  reflects the fact that one DNP-binding site has been consumed by the first binding reaction. The dimer can again react with another ligand to yield the following clusters:

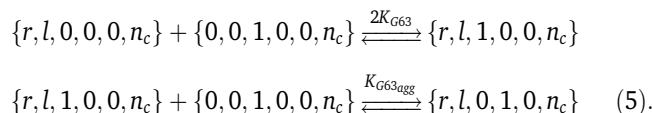


The species  $\{2, 2, 0, 0, 0, 0\}$  and  $\{2, 2, 0, 0, 0, 1\}$  are illustrated in Fig. 1e and f. The latter is a closed ring dimer which is produced by the reaction of a divalent DNP-ligand with IgE-antibodies in solution [15].  $J_2$  is the respective equilibrium constant for the closed ring formation.  $\{2, 2, 0, 0, 0, 0\}$  and  $\{2, 3, 0, 0, 0, 0\}$  are nuclei for higher order aggregation, which involves the binding of free epitopes of their ligands to free binding sites of IgE bound to the FcεRI. The manifold of reactions can generally be described by the general reaction scheme:



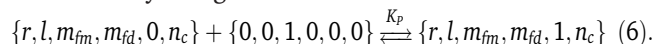
The notation reflects the fact that clusters of  $r$ -th order containing  $r$  receptors and may contain  $l=r-1$ ,  $r$ , or  $r+1$  ligands. The first reaction accounts for the binding of a ligand to one of the free IgE antigen-combining sites. Subsequently, the thus formed species can react with another FcεRI-IgE to form a cluster of  $(r+1)$ -th order (second reaction). Alternatively, it can bind a ligand to its free antigen-combining site (third reaction) or form a closed ring (last step of first reaction). We neglect reactions involving the binding of more than two DNP to IgE antigen combining sites because we consider them as unlikely for steric reasons.

Now the co-clustering of MAFA by G63F(ab')<sub>2</sub>DNP<sub>n</sub> has to be taken into consideration: this process adds MAFA to the FcεRI-IgE aggregates (Fig. 1g and h). The general reaction scheme can then be written as:

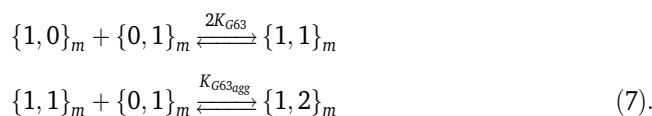


$K_{G63}$  is the association constant of G63 Fab to its MAFA epitope,  $K_{G63agg}$  is the equilibrium constant for MAFA cluster formation on the cell surface [7, 16]. For the number of ligands  $l$ , only the values  $r-1$ ,  $r$ , and  $r+1$  are possible. MAFA has been found to exist in the RBL-2H3 membranes as both, monomers and disulfide-linked homodimers [6]. Since the binding isotherms for the G63-MAFA reaction measured by Ortega Soto and Pecht [7] do not indicate any positive or negative cooperativity, we can ignore this complication, since all binding sites can be considered as independent.

Finally, as described elsewhere [16] and above, evidence from different experimental approaches suggests that a fraction of the MAFA molecules are associated with the FcεRI even in the absence of external clustering agents (Fig. 1b and d). This is described by the general reaction:



In some experiments we added intact mAb G63 in addition to mF(ab')<sub>2</sub>DNP<sub>n</sub> or G63-F(ab')<sub>2</sub>-DNP<sub>n</sub> in order to examine the impact of independent MAFA clustering on the secretory response. In this case we have to add the following to the reaction scheme:



The dose response to the clustering of FcεRI-IgE by the above DNP conjugated F(ab')<sub>2</sub> is treated by empirical Hill functions as described in earlier publications [17, 18]. This yields:

$$\begin{aligned} R(l_T) = \sum_r \sum_{l=r-1}^{r+1} \sum_{m_p=0}^r \sum_{n_c=0}^1 \left[ \tilde{R}_{l,r,m_p,n_c} \cdot \frac{\left( \frac{[r,l,0,0,m_p,n_c]}{\Gamma_{l,r,m_p,n_c}} \right)^{n_a}}{1 + \left( \frac{[r,l,0,0,m_p,n_c]}{\Gamma_{l,r,m_p,n_c}} \right)^{n_a}} \cdot \right. \\ \left. \cdot \left( 1 + \left( \frac{[r,l,0,0,m_p,n_c]}{\eta_{l,r,m_p,n_c}} \right)^{n_d} \right)^{-1} \right] \quad (8) \end{aligned}$$

where  $R_{l,r,m_p,n_c}$  is the maximal secretion induced by the cluster  $\{r, l, 0, 0, m_p, n_c\}$ ,  $\Gamma_{l,r,m_p,n_c}$  and  $\eta_{l,r,m_p,n_c}$  are its activation and inhibition constants in units of M and  $n_a$  and  $n_d$  are the Hill coefficients of the activation and inhibition function, respectively. Eq. (8) accounts for the experimentally established fact that a large fraction of FcεRI aggregates causes a deactivation of cells [18]. Apparently, eq. (8) disregards contributions from FcεRI-clusters with one or more co-clustered MAFA. Hence it is

based on the assumption that co-clustering completely eliminates the capacity of an FcεRI cluster to induce secretion.

MAFA clustering by mAb G63 has earlier been shown to cause partial inhibition of RBL-2H3 cells' secretory response [11]. This is described by an empirical hyperbolic function of the concentration of MAFA-dimers {1,2}:

$$D_{M_{free}}(l_T, l_{G63}) = \left[ 1 + \left( \frac{[1,2]}{\kappa_{M_{free}}} \right)^{n_{inhib1}} \right]^{-1} \quad (9)$$

where  $\kappa_{M_{free}}$  is a constant reflecting the average efficiency of the inhibition of the secretory response to FcεRI-IgE clusters with and without MAFA-FcεRI pre-aggregation. It also accounts for the additional inhibition which can be caused by the weak interaction of MAFA bound intact mAb G63 antibodies with Fcγ receptors on the RBL-2H3 cells' surface.

As it will be shown below, an additional inhibitory signal is created by MAFA co-clustered to FcεRI. This is described by another hyperbolic function:

$$D_{coagg}(l_T, l_{G63}) = \sum_r \sum_{l=r-1}^{r+1} \sum_{m_f=0}^l \sum_{m_{fd}=0}^l \sum_{m_p=0}^r \sum_{n_c=1}^0 \left[ 1 + \left( \frac{[r, l, m_f, m_{fd}, m_p, n_c]}{\kappa_{coagg}} \right)^{n_{inh2}} \right]^{-1} \quad (10)$$

where  $\kappa_{coagg}$  denotes the efficiency of inhibition by MAFA co-clustering. It is zero for  $m_{fm}=m_{fd}=0$ .

To obtain the final dose response function, we have to multiply eqs. (8), (9) and (10) yielding the final expression:

$$R'(l_T, l_{G63,T}) = R(l_T) \cdot D_{M_{free}}(l_T, l_{G63,T}) \cdot D_{coagg}(l_T) \quad (11).$$

Application of eqs. (8)–(11) requires calculating the concentration of all species produced by the above reactions. The respective formalism is described in the appendix.

### MAFA-FcεRI co-clustering enhances the inhibition of the secretory response

Based on preliminary experiments evaluating the optimal response to the different reagents, the cells were usually first reacted with a constant concentration ( $10^{-10}$  M) of the monoclonal DNP specific A2-IgE. The different clustering and co-clustering reagents have been described above.

Fig. 2 presents results of a typical set of experiments done in this study. This was repeated at least four times with every one of the outlined protocols; each done in triplicates or quadruplicates. Results shown in Fig. 2a illustrate the secretory dose response of RBL-2H3 cells reacted with  $10^{-10}$  M IgE, to the addition of

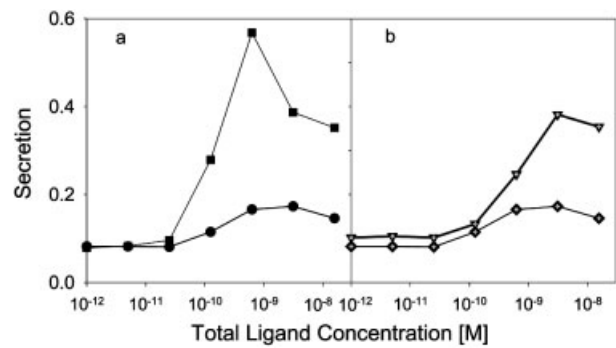


**Fig. 2.** A typical illustration of the impact which co-clustering the FcεRI and MAFA has on the secretory response: RBL-2H3 cells ( $1 \times 10^5/100 \mu\text{l}/\text{well}$ ) were plated in 96-well plates. Cells suspended in medium containing the DNP-specific A2IgE (typically  $0.1 \mu\text{l}/\text{ml}$  of ascites fluid i.e. ca.  $1 \times 10^{-10}$  M), were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. Then,  $50 \mu\text{l}$  of the respective secretion-inducing reagent was added, (or  $100 \mu\text{l}$  for experiments where no competing mAb G63 were added), and the cells were further incubated at  $37^\circ\text{C}$  for 30 min. The release was determined as described in Materials and methods. The dependence of RBL 2H3 cells' secretion (expressed as fraction of the cell's total content) on the concentration of the following clustering and co-clustering reagents is shown. (a) F(ab')<sub>2</sub> fragments of mAb G63 (circles), and of mouse IgG (squares), both derivatized with an average of 2.9 or 3.4 DNP molecules, respectively. (b) The effect on secretion of co-clustering without (diamonds) and with the addition of excess intact mAb G63 (triangles). The cells were primed with the DNP specific mAb A2I.

mF(ab')<sub>2</sub>DNP<sub>3.4</sub> or G63F(ab')<sub>2</sub>DNP<sub>2.9</sub>. The marked decrease in secretion induced by G63F(ab')<sub>2</sub>DNP<sub>2.9</sub> compared with that caused by clustering the FcεRI-IgE only, clearly establishes the enhanced inhibition by the co-clustered MAFA. Moreover, we observe that addition of intact mAb G63 reduced rather than increased that inhibition (Fig. 2b). These results suggest that separate MAFA clusters are considerably less effective than those co-clustered with the FcεRI in inhibiting the secretion process. As detailed above, a quantitative comparison of the inhibitory capacity of clustering and co-clustering MAFA requires consideration of its dependence on the concentrations of the different reacting species. Hence, simulation based on the above outlined model has been performed.

In the following we quantitatively analyze several dose-response curves, which were obtained with ligands carrying a different number of DNP groups. To this end we simulated dose response curves expected under the respective experimental conditions. We used binding constants obtained in earlier studies [7, 15, 16] and determined the other parameters stepwise starting with the simplest reaction (i.e. with mFabDNP<sub>2</sub> as ligand) and ending with the most complicated one (i.e. with G63F(ab')<sub>2</sub>DNP<sub>10</sub> and G63 as ligands). This procedure

minimizes correlations between parameters and ascertains maximal self-consistency. It should be emphasized that our simulations aim at achieving internal consistency using a minimal number of free parameters rather than in obtaining the best fit to individual dose-response data sets.

### Secretory response to mFabDNP<sub>2</sub> and G63FabDNP<sub>1.7</sub>

In view of the above illustrated enhancement of the inhibition efficacy of co-clustered FcεRI and MAFA, we proceeded with a detailed quantitative analysis of the secretory response to the mFabDNP<sub>2</sub> and G63FabDNP<sub>1.7</sub>. Both ligands can cluster FcεRI-IgE into dimers while the latter can additionally co-cluster MAFA. As shown in Fig. 3, secretion is induced by mFabDNP<sub>2</sub> when its concentration is higher than 10<sup>-10</sup> M. The secretion induced by G63FabDNP<sub>1.7</sub> is significantly lower, particularly at a concentration range of 10<sup>-7</sup>–10<sup>-9</sup> M. Thus, the difference between the dose response to mFabDNP<sub>2</sub> and to G63FabDNP<sub>1.7</sub> can be related to FcεRI clusters which contain no or one co-clustered MAFA. Thus, we find that already the co-clustering of a single MAFA molecule is capable of inhibiting the secretion induced by the respective FcεRI-IgE aggregates.

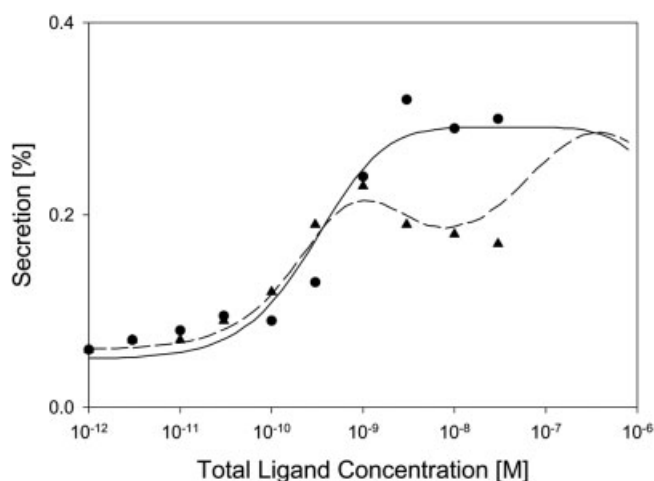


**Fig. 3.** Concentration dependence of the secretory response (expressed as fraction of the cell's total content) of RBL-2H3 cells primed with mAb A2 IgE (1.0×10<sup>-10</sup> M) to mF(ab)DNP<sub>2</sub> (circles) and G63F(ab)DNP<sub>1.7</sub> (triangles). Cells suspended in medium containing the DNP-specific A2IgE (typically 0.1 μl/ml of ascites fluid i.e. ca. 1×10<sup>-10</sup> M), were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. Then, 50 μl of the respective secretion inducing reagent was added, and the cells were further incubated at 37°C for 30 min. The release was determined as described in Materials and methods. The solid and dashed lines are the result of the fitting procedure outlined in the text.

### Analysis of the dose response to mFabDNP<sub>2</sub> and G63FabDNP<sub>1.7</sub>

We first analyzed the dose response to mFabDNP<sub>2</sub> as depicted in Fig. 3. Initially we used the equilibrium constant  $K_I=10^7\text{M}^{-1}$  of DNP binding to the A2 IgE obtained in earlier binding studies [15]. It turned out, however, that a satisfactory simulation of the data requires a somewhat higher value (4×10<sup>7</sup> M; probably reflecting avidity of the interactions). To model FcεRI-IgE clustering we assumed  $K_{Iagg} \geq K_I$ , because it occurs in the two-dimensions of the cells' surface, where the relatively high FcεRI-IgE density creates an effective reactant concentration that is higher than its apparent molar concentration [18, 19]. Schweitzer-Stenner et al. [18] obtained  $K_{Iagg}/K_I$  ratios between 4.5×10<sup>3</sup> and 50 for the FcεRI-clustering by monoclonal antibodies. For the present experiments, one expects a lower enhancement factor because less than 10% of the FcεRIs were occupied by IgE under the employed experimental conditions ([IgE-FcεRI] = 10<sup>-10</sup> M). We therefore assumed a  $K_{Iagg}/K_I$  ratio of 10 for our simulations. This should be considered as a lower limit. For the sake of simplicity we further assumed that all open IgE-FcεRI dimers ( $n_C=0$ ) formed upon reaction with the above reagents have the same secretion inducing capacity. Closed rings ( $n_C=1$ ), however, were assumed to be incapable of inducing secretion, in accordance with our own observations [20] and those of Baird and colleagues [21].

As indicated by the subscript, the activation and inhibition parameters,  $\Gamma_{l,r,m_p,0}$ ,  $\eta_{l,r,m_p,0}$  and the maximal secretion  $\tilde{R}_{l,r,m_p,0}$  depend on the state of FcεRI-aggregation  $\{r, l, m_{f_m}=0, m_{f_d}=0, m_p, n_C=0\}$ . However, it is practically impossible to discriminate between these parameters on the basis of our experimental data, because the coexisting aggregation states exhibit a very similar dependence on the total ligand concentration. Hence,  $\Gamma_{eff}, \eta_{eff}$  and  $\tilde{R} = \tilde{R}_{l,r,m_p,n_C=0}$  were used as apparent parameters for the respective activation, inhibition and saturation representing all FcεRI clusters. These and  $n_a$ ,  $n_d$  were varied in order to obtain the best simulation of the experimental data, which is illustrated by the solid line in Fig. 3. Next, we calculated the dose response to G63FabDNP<sub>2</sub> assuming that the observed inhibition is solely due to the complete abrogation of the FcεRI clusters stimulus upon co-clustering MAFA. For binding of a G63 to MAFA we used  $K_{IG63}=10^8\text{M}^{-1}$  [11]. However, this calculation yielded only a negligibly small reduction of the secretory response over the entire ligand concentration range used in these experiments. From this it follows, that direct abrogation cannot explain the higher inhibitory efficiency of co-clustered MAFA. Therefore, we employed eq. (10) to simulate the inhibition by co-clustered MAFA, which reflects a

**Table 1.** Parameters used for and derived from the analysis of the secretory dose response of RBL-2H3 cells primed with IgE to mF(ab')<sub>2</sub>DNP<sub>n</sub>, G63F(ab')<sub>2</sub>DNP<sub>n</sub>, n=2, 10, mF(ab)DNP<sub>2</sub> and G63F(ab)DNP<sub>1.7</sub>

| Parameter                              | mF(ab') <sub>2</sub> DNP <sub>2</sub><br>G63F(ab') <sub>2</sub> DNP <sub>2</sub><br>[G63]=10 <sup>-7</sup> M | mF(ab') <sub>2</sub> DNP <sub>10</sub><br>G63F(ab') <sub>2</sub> DNP <sub>10</sub><br>[G63]=10 <sup>-7</sup> M | mF(ab') <sub>2</sub> DNP <sub>2</sub><br>G63F(ab') <sub>2</sub> DNP <sub>10</sub><br>[G63]=3·10 <sup>-8</sup> M | mFabDNP <sub>2</sub><br>G63FabDNP <sub>1.7</sub> |
|----------------------------------------|--------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------------|
| K <sub>I</sub> [M <sup>-1</sup> ]      | 4×10 <sup>7</sup>                                                                                            | 4×10 <sup>7</sup>                                                                                              | 4×10 <sup>7</sup>                                                                                               | 4×10 <sup>7</sup>                                |
| K <sub>Iagg</sub> [M <sup>-1</sup> ]   | 4×10 <sup>8</sup>                                                                                            | 4×10 <sup>8</sup>                                                                                              | 4×10 <sup>8</sup>                                                                                               | 4×10 <sup>8</sup>                                |
| K <sub>p</sub> [M <sup>-1</sup> ]      | 5×10 <sup>9</sup>                                                                                            | 5×10 <sup>9</sup>                                                                                              | 5×10 <sup>9</sup>                                                                                               | 5×10 <sup>9</sup>                                |
| K <sub>G63</sub> [M <sup>-1</sup> ]    | 10 <sup>8</sup>                                                                                              | 10 <sup>8</sup>                                                                                                | 10 <sup>8</sup>                                                                                                 | 10 <sup>8</sup>                                  |
| K <sub>G63agg</sub> [M <sup>-1</sup> ] | 5×10 <sup>8</sup>                                                                                            | 5×10 <sup>8</sup>                                                                                              | 5×10 <sup>8</sup>                                                                                               | 10 <sup>9</sup>                                  |
| Γ [M]                                  | 3×10 <sup>-13</sup>                                                                                          | 3×10 <sup>-13</sup>                                                                                            | 10 <sup>-14</sup>                                                                                               | 3×10 <sup>-13</sup>                              |
| η [M]                                  | 2×10 <sup>-13</sup>                                                                                          | 2×10 <sup>-13</sup>                                                                                            | 2×10 <sup>-13</sup>                                                                                             | 2×10 <sup>-13</sup>                              |
| n <sub>a</sub>                         | 1.0                                                                                                          | 0.4                                                                                                            | 1.0                                                                                                             | 1.0                                              |
| n <sub>d</sub>                         | 0.1                                                                                                          | 0.1                                                                                                            | 0.5                                                                                                             | 0.1                                              |
| $\tilde{R}$                            | 0.75                                                                                                         | 0.5                                                                                                            | 0.30                                                                                                            | 0.35                                             |
| κ <sub>Mfree</sub> [M]                 | 1×10 <sup>-12</sup>                                                                                          | 1×10 <sup>-12</sup>                                                                                            | 1×10 <sup>-12</sup>                                                                                             | –                                                |
| κ <sub>coagg</sub> [M]                 | 4×10 <sup>-14</sup>                                                                                          | 2×10 <sup>-15</sup>                                                                                            | 4×10 <sup>-14</sup>                                                                                             | 4×10 <sup>-14</sup>                              |
| n <sub>inh</sub>                       | 1.0                                                                                                          | 1.0                                                                                                            | 0.5                                                                                                             | 1.0                                              |

statistical character of the underlying process. This yielded the dashed line in Fig. 3. The  $\kappa_{coagg}$  and  $n_{inh2}$  were varied to achieve an optimal reproduction of the experimental data. The respective parameter values for (Table 1)  $\Gamma$ ,  $\eta$ ,  $\kappa_{coagg}$ ,  $n_a$ ,  $n_d$  and  $n_{inh}$ , were then used for the analysis of the more complex reaction taking place with mF(ab')<sub>2</sub>DNP<sub>n</sub> and G63F(ab')<sub>2</sub>DNP<sub>n</sub> as described below.

### Secretory response to mF(ab')<sub>2</sub>DNP<sub>n</sub> and G63F(ab')<sub>2</sub>DNP<sub>n</sub>

Fig. 4 shows the dose response of RBL-2H3 cells reacted with A2IgE to the addition of mF(ab')<sub>2</sub>DNP<sub>n</sub> or G63F(ab')<sub>2</sub>DNP<sub>n</sub>, where n=2 (Fig. 4a) or 10 (Fig. 4b) in the absence and presence of intact G63 (10<sup>-7</sup> M). As expected, the higher DNP-conjugation shifts all dose response curves to lower ligand concentrations, reflecting the increased avidity of the IgE-FcεRI for these ligands. The addition of G63 caused only a very small inhibition of the secretory response to mF(ab')<sub>2</sub>DNP<sub>10</sub>, while its effect is nearly negligible for n=2. The reaction with G63F(ab')<sub>2</sub>DNP<sub>n</sub>, however, induced a much lower level of secretion, illustrating again that MAFA co-clustering causes a more pronounced inhibition. Interestingly, adding an excess of intact mAb G63 prior to the reaction with G63F(ab')<sub>2</sub>DNP<sub>n</sub> did not cause further decline of the secretory response. In contrast, for n=2, at high ligand concentrations (>10<sup>-9</sup> M), secretion is even increased upon addition of mAb G63, while for n=10, it is much higher at ligand concentrations above 10<sup>-10</sup> M (cf. also Fig. 2).

### Analysis of the dose response to mF(ab')<sub>2</sub>DNP<sub>n</sub> and G63F(ab')<sub>2</sub>DNP<sub>n</sub>

For simulation of the cellular response to FcεRI clustering and its inhibition by MAFA exhibited in Fig. 4, we assumed that FcεRI-IgE aggregates larger than trimers can be neglected because of their comparatively low concentrations. In fact, our calculations show that trimer concentrations are already more than an order of magnitude lower than those of the respective dimers. We utilized again apparent activation and inhibition constants  $\Gamma_{eff}$  and  $\eta_{eff}$ , which represent a weighted average of different activation and inhibitory capacities of all types of open ring dimers and trimers. Moreover, we assumed that closed ring oligomers are not capable of inducing secretion [21].

For the simulation of the secretory response to mF(ab')<sub>2</sub>DNP<sub>2</sub> (Fig. 4A) we used again  $K_I=4\times 10^7$  M<sup>-1</sup> and  $K_{agg}=10^8$  M<sup>-1</sup>. The molar concentrations of FcεRI-IgE- complexes were calculated to be 10<sup>-10</sup> M.  $\Gamma_{eff}$ ,  $\eta_{eff}$ ,  $n_a$ ,  $n_d$  and  $\tilde{R}$  were used as variable parameters. This yielded the black line in Fig. 4A. The parameters used for this simulation was then used to calculate the secretory response to mF(ab')<sub>2</sub>DNP<sub>2</sub> (red data points in Fig. 4A) and G63F(ab')<sub>2</sub>DNP<sub>2</sub> in the presence of 10<sup>-7</sup> M intact G63 (green data points) and to G63F(ab')<sub>2</sub>DNP<sub>2</sub> in the absence of intact G63 (blue data points). Moreover, we employed the values for  $K_{IG63}$ ,  $K_{IG63agg}$ ,  $\kappa_{coagg}$ ,  $n_a$ ,  $n_d$  and  $n_{inh}$  as obtained from the simulation of the data shown in Fig. 3. Thus, only  $\kappa_{Mfree}$  and  $\tilde{R}$  remained as adjustable parameters. This yielded satisfactory simulations of the experimental data (solid lines in Fig. 3). For fine-tuning

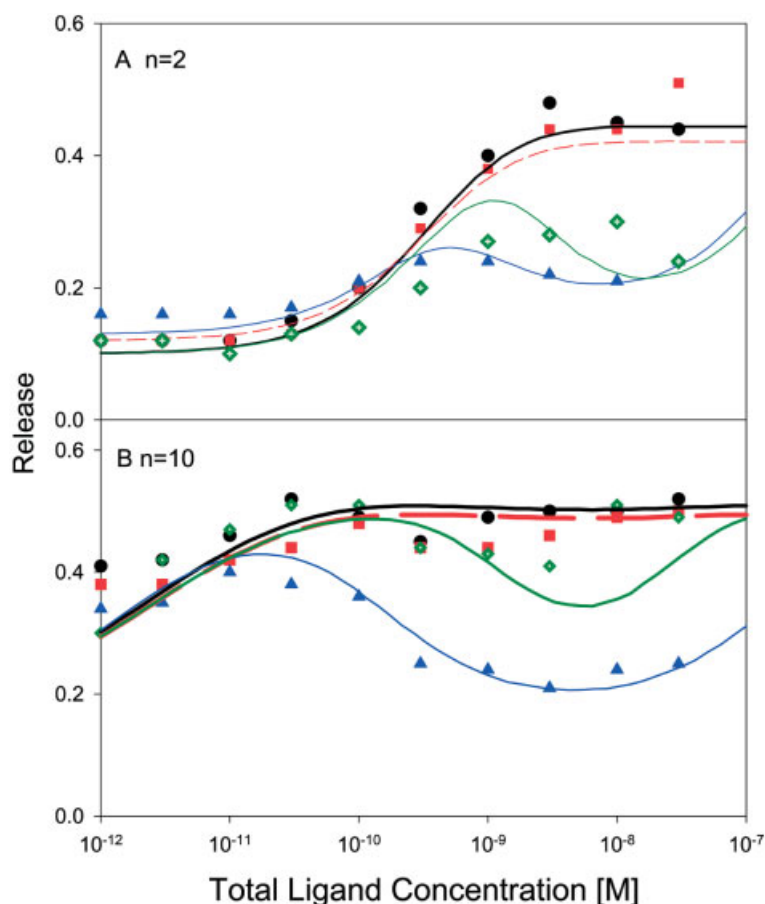


**Fig. 4.** Concentration dependence of the secretory response (expressed as fraction of the cell's total content) of RBL-2H3 cells primed with the mAb A2 IgE ( $10^{-10}$  M) to mF(ab')<sub>2</sub>DNP<sub>n</sub> in the absence (black circles) and presence (red squares) of  $10^{-7}$  M intact mAb G63, and to G63F(ab')<sub>2</sub>DNP<sub>n</sub> in the absence (blue triangles) and presence (green diamonds) of  $10^{-7}$  M intact mAb G63. The average number  $n$  of DNP derivatization is indicated in the figure. Cells suspended in medium containing the DNP-specific A2IgE (typically 0.1  $\mu$ l/ml of ascites fluid i.e. ca.  $1 \times 10^{-10}$  M), were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. Then, 50  $\mu$ l of the respective secretion inducing reagent was added, (or 100  $\mu$ l for experiments where no competing mAb G63 were added), and the cells were further incubated at 37°C for 30 min. The release was determined as described in Materials and methods. The solid lines are the result of the fitting procedure outlined in the text. The dashed blue lines result from a calculation for G63F(ab')<sub>2</sub>DNP assuming that the inhibition solely results from the complete suppression of the release of co-clustered FcεRI-aggregates.

we assumed a somewhat lower value for  $K_{IG63agg}$  ( $5 \times 10^8$  M instead of  $10^9$  M). The parameters used for this calculation are listed in Table 1.

To further check the validity of this analysis we used the same model to calculate the dose response to mF(ab')<sub>2</sub>DNP<sub>10</sub> and G63F(ab')<sub>2</sub>DNP<sub>10</sub> in the presence and absence of  $10^{-7}$  M mAb G63 (Fig. 4B). A final adjustment solely required a modification of  $n_a$  and  $\kappa_{coagg}$ . For the former we obtained a value of 0.4, which most likely reflects some heterogeneity of the binding affinities of the DNP haptens carried on the ligands. For  $\kappa_{coagg}$  we obtained a slightly lower value ( $2 \times 10^{-15}$  M), indicating a higher inhibitory capacity of the co-clustered MAFA. The solid lines in Fig. 4B illustrate the results of the latter simulations. The fact that all results of the experiments shown in Fig. 4 can be simulated using the same parameters (with the exception of  $n_a$  and  $\kappa_{coagg}$ ) strongly corroborates the suitability of the employed model. Again it was impossible to reproduce the dose response to G63F(ab')<sub>2</sub>DNP<sub>n</sub> ( $n=2, 10$ ) by assigning the inhibition capacity of MAFA to the suppression of the response, exclusively to co-clustered FcεRI (dashed blue lines in Fig. 4). Rather, our results suggest that the co-clustered MAFA molecules inhibit secretion like free MAFA-clusters, yet much more efficiently. This is revealed by the values obtained for

$\kappa_{Mfree}$  and  $\kappa_{coagg}$  (Table 1), which are two-to-three orders of magnitudes larger for co-clustered than for free MAFA clusters: the co-clustered MAFA concentration required for an optimal inhibition is  $4 \times 10^{-2}$  ( $n=2$ ) and  $2 \times 10^{-3}$  ( $n=10$ ) lower than that of MAFA clustered separately by mAb G63. In other words, co-clustering MAFA with the FcεRI dramatically amplifies the inhibitory efficiency. The employed intact mAb G63 concentration (i.e.  $10^{-7}$  M) is not the optimal one for inhibition since the concentration of MAFA dimers produced by mAb G63 reaches a maximum at around  $[l_{G63T}] = 5 \times 10^{-9}$  M, which, as expected is close to  $1/(2 \cdot K_{IntG63})$  [22]. Consequently, one expects that a lower G63 concentration (about  $10^{-8}$  M) should be more effective. Indeed, another data set obtained by reacting IgE primed RBL-2H3 cells with mF(ab')<sub>2</sub>DNP<sub>10</sub> without and with  $3 \times 10^{-8}$  M G63 (Fig. 5) shows that the G63 induced a statistically significant inhibition of secretion. We first used the parameters derived from analysis of the data presented in Fig. 4 to simulate the dose-response curves presented in Fig. 5. To optimize the agreement with the experimental data,  $n_d=0.5$  was eventually used instead of 0.1. This yielded the solid lines in Fig. 5. The agreement with the experimental data is satisfactory and strongly supports the validity of the employed model.

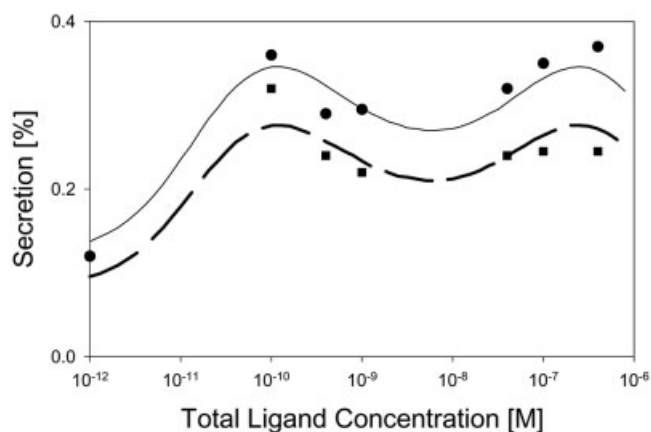


**Fig. 5.** Concentration dependence of the secretory response (expressed as fraction of the cell's total content) of RBL-2H3 cells primed with the mAb A2IgE to mF(ab')<sub>2</sub>DNP<sub>10</sub> in the absence (circles) and presence (squares) of  $3 \times 10^{-8}$  M intact mAb G63. Cells suspended in medium containing the DNP-specific A2IgE ca.  $1 \times 10^{-10}$  M, were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. Then, 50  $\mu$ l of the secretion inducing reagent was added, (or 100  $\mu$ l for experiments where no competing mAb G63 were added), and the cells were further incubated at 37°C for 30 min. The release was determined as described in Materials and methods. The solid and dashed lines are the result of the fitting procedure outlined in the text.

A final check for the applicability of the employed model of cellular response to Fc $\epsilon$ RI clustering and its inhibition by MAFA has been carried out by analyzing the effect of a monovalent competitor of Fc $\epsilon$ RI-IgE clustering, the hapten DNP-caproic acid (DNP-cap) on the dose response to G63F(ab')<sub>2</sub>DNP<sub>2,9</sub> in the absence and presence of  $10^{-7}$  M G63 (Fig. 6). The secretion observed in the absence of the monovalent hapten could be qualitatively reproduced by using the parameters obtained from all the previous simulations; with the sole exception of the Hill coefficients  $n_a$  and  $n_{inhib2}$  that had to be modified ( $n_a=1.5$  instead of 1.0,  $n_{inhib2}=2.0$  instead of 1.0). Some discrepancies between the simulations and the respective experimental results appear at high ligand concentrations. This may be a result of our neglect of the fact that using a representative, average value for the distribution of the DNP conjugation ratios of the employed mF(ab')<sub>2</sub>DNP<sub>n</sub> ligands is less appropriate for intermediate levels of DNP-conjugation (the relative standard deviation  $\Delta n / \langle n \rangle$  of the average conjugation ratio decreases with  $n$ ). Since our model does not consider the binding of monovalent hapten, we could not quantitatively reproduce the respective dose response data obtained in the presence of excess DNP-cap. However, since this excess hapten competes for the antigen-binding sites and thus disrupt Fc $\epsilon$ RI-IgE clusters, we simulated the free haptens' influence on the dose

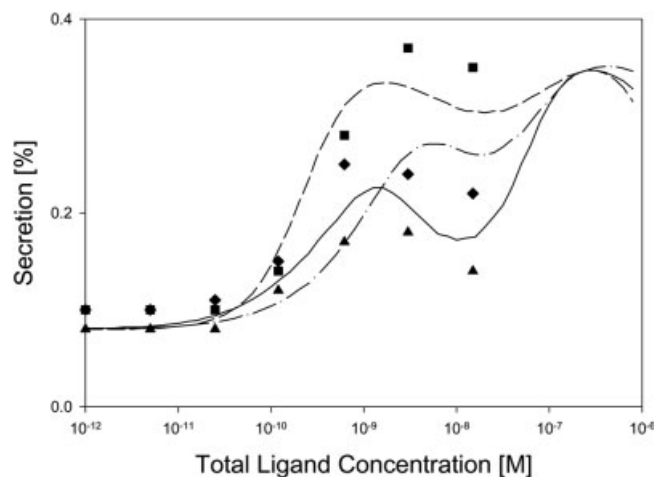


**Fig. 6.** Concentration dependence of the secretory response (expressed as fraction of the cell's total content) of RBL-2H3 cells primed with the mAb A2gE to G63F(ab')<sub>2</sub>DNP<sub>2,9</sub> in the absence (triangles) and presence (squares) of  $10^{-7}$  M intact mAb G63 and, additionally in the presence of  $10^{-7}$  M DNPcap (diamonds). Cells suspended in medium containing the DNP-specific A2IgE ca.  $1 \times 10^{-10}$  M, were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. Then, 50  $\mu$ l of the respective secretion inducing reagent was added, (or 100  $\mu$ l for experiments where no competing mAb G63 were added), and the cells were further incubated at 37°C for 30 min. The release was determined as described in Materials and methods. The solid, dashed and dashed-dotted lines are the result from the fitting to the data points labeled by triangles, squares and diamonds, respectively.

response by employing lower values for the hapten binding constant  $K_I$  ( $10^{-7}$  instead of  $4 \times 10^{-7}$  M). As shown in Fig. 6, this qualitatively reproduces the enhancement of the secretion at high ligand concentration, even though it does not perfectly fit the data. This enhancement seems to be counterintuitive, since one would expect that a decrease in Fc $\epsilon$ RI-IgE oligomer concentration would decrease rather than increase the secretion. However, in our case, occupation of the antigen binding sites by monovalent haptens increases the clustering of separate MAFA molecules at the expense of the co-clustered ones and as a consequence the overall inhibition is reduced. Therefore, this reproduction of the enhancement provides additional support for the validity of the employed model.

Finally, it should be emphasized again that practically all the parameters used in the present analysis should be considered as effective ones.  $K_I$  represents the average affinity of the DNP binding sites of an ensemble of DNP-conjugated ligands. The same holds for  $K_{Iagg}$  where the employed value (i.e.  $K_I=10 K_{Iagg}$ ) might be an underestimate, but it was not used as a free parameter because of its correlation with the activation and inhibition parameters  $\Gamma_{eff}$  and  $\eta_{eff}$ . The parameters

related to G63 binding and its influence on secretion ( $K_{G63}$ ,  $K_{G63agg}$ ,  $\kappa_{Mfree}$ ,  $\kappa_{coagg}$ ) are significantly more reliable because  $K_{G63}$  and  $K_{G63agg}$  were directly determined in earlier studies [7, 16].

## Discussion

In order to achieve a deeper understanding of the mutual functional relation between the Fc $\epsilon$ RI and MAFA as expressed in mast cells' secretory response, we have measured and analyzed its dose dependence on a set of reagents which are capable of producing homo- and/or hetero clusters of Fc $\epsilon$ RI-IgE complexes and MAFA. Clustering of Fc $\epsilon$ RI primed with the monoclonal DNP specific A2IgE was achieved by using DNP-conjugated Fab and F(ab')<sub>2</sub> fragments derived from either polyclonal mouse IgG or from the mAb G63. While the former causes Fc $\epsilon$ RI-IgE-aggregation mainly into dimers and trimers, the latter additionally causes the co-clustering of MAFA. Three different (average) levels of DNP conjugation were prepared and used, namely  $n=2$ , 2.9 and 10 (cf. Fig. 3–5). Moreover, we have also measured the secretory dose response to these reagents in the presence of excess concentrations of intact mAb G63 so as to examine its effect on the inhibitory capacity of separately clustered and Fc $\epsilon$ RI-IgE-co-clustered MAFA-molecules. The dose response to the DNP conjugated G63-Fab-fragments were used to determine the inhibition capacity of MAFA molecules co-clustered with the Fc $\epsilon$ RI-IgE. The model employed to analyze all these data only invokes the mass-action law in order to calculate the equilibrium concentrations of the variety of involved species. Dose- response data obtained to the different reagents [i.e. mF(ab')<sub>2</sub>DNP<sub>n</sub>; G63F(ab')<sub>2</sub>DNP<sub>n</sub>,  $n=2$ , 2.9, 10; mFabDNP<sub>2</sub>, G63FabDNP<sub>1.7</sub>], all in the absence or the presence of excess intact mAb G63 were simulated well with a practically identical set of fitting parameters. Our analysis rules out the possibility that the stronger inhibition exerted by co-clustered MAFA is solely due to a direct blockade of the stimulus produced by the co clustered Fc $\epsilon$ RI. It revealed instead that co-clustering causes a markedly amplified inhibitory action which, as indicated by the respective constants  $\kappa_{Mfree}$  and  $\kappa_{coagg}$  (Table 1), is about two orders of magnitude more efficient than that caused by the clustering MAFA molecules separately.

MAFA belongs to the family of ITIM containing inhibitory receptors, which suppress the response to activation induced by ITAM containing receptors on the respective different cell types. Xu et al. [23, 24] identified two phosphatases, the SH2 domain containing protein tyrosine phosphatase, SHP2, and the one specific for hydrolyzing 5' inositol phosphates, SHIP, as mediating the inhibitory action of MAFA. Both enzymes

were shown to be recruited to the phosphorylated tyrosine of MAFA ITIM. This phosphorylation is induced upon MAFA clustering by the protein tyrosine kinase Lyn whose SH3 domain is assumed to bind to the ITIM adjacent PAAP motif of MAFA. The recruited SHP-2 was shown to interfere with most proximal events of Fc $\epsilon$ RI activation by reducing the tyrosine phosphorylation of two important components of the Fc $\epsilon$ RI coupling cascade: Syk and Lat. The MAFA recruited SHIP exerts its activity by hydrolysis and the subsequent decline in the cell membranes' phosphatidyl inositides trisphosphates. Activities of both enzymes eventually cause the suppression of the cells' secretory response, but the latter has been proposed to be dominant [23, 24].

It is noteworthy that the inhibitory activity of practically all the currently known ITIM containing receptors requires their co-clustering with the relevant ITAM containing activating receptors, while MAFA is an exception. However, the here presented experimental findings and their analysis clearly show that the Fc $\epsilon$ RI-IgE co-clustered MAFA molecules are much more effective inhibitors than MAFA molecules clustered separately. This observation can be understood by taking into account that co-clustering increases the number of MAFA molecules in the proximity of the activating, ITAM containing Fc $\epsilon$ RI-IgE clusters. This, in turn, is also increasing the concentration of inhibitory enzymes recruited to the proximity of the activating Fc $\epsilon$ RI in the cells' plasma membrane [25]. One of the involved enzymes is the protein tyrosine kinase Lyn, known to phosphorylate both, the canonical tyrosines of the Fc $\epsilon$ RI- $\beta$  and  $\gamma$  subunits ITAM as well as the one of the MAFA ITIM [23, 24]. Hence, it could be that Fc $\epsilon$ RI co-clustered MAFA encounters a higher local concentration of Lyn than separately clustered MAFA. This would amplify the inhibitory process by involving Lyn in further phosphorylation of the MAFA ITIM and the subsequent recruitment and activation of SHIP. Since earlier results indicate a steady state association of a fraction of MAFA molecules with the Fc $\epsilon$ RI, even in the absence of any clustering reagents, it is likely that separate MAFA clusters are also exerting their inhibition by virtue of this basal Fc $\epsilon$ RI association. The present results raise the intriguing question whether or not such co-clustering can also be promoted by other, still unknown membrane components on the mast cells or by the still unknown natural MAFA ligand. Such a ligand may provide both MAFA clustering as well as its potential enhanced association with the Fc $\epsilon$ RI and thereby cause a higher inhibitory action.

## Materials and methods

### Reagents

All chemical reagents used were of analytical grade, and unless otherwise stated, were purchased from Sigma (St. Louis, MO). DNP<sub>11</sub>-BSA (BSA fraction V), conjugated with an average of 11 2,4-dinitrobenzene molecules, was produced in our laboratory by reaction with 2,4-dinitro-benzensulfonic acid. The hybridoma clone A2 producing the DNP specific monoclonal IgE [26], was originally obtained from Dr. V. T. Oi (Stanford University, CA). The IgE molecules were purified by affinity chromatography on DNP-Sepharose and eluted with 0.1 M DNP-Glycine (pH 7.4), dialyzed extensively, and finally equilibrated with 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, pH 5.7, and passed through Dowex X8 Cl column (0.5×4 cm). The Fab and F(ab')<sub>2</sub> fragments of polyclonal mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). mAb G63 (IgG1 subclass) was produced and isolated in our laboratory [7]. G63-F(ab')<sub>2</sub> was produced from the intact antibody by ficin digestion using the modified technique of Mariani et al. [27] and the Fab fragments by a slight modification of the method described by Herzenberg and Oi [28] by papain (1% w/w) digestion.

### Conjugation of DNP to m-Fab, m-F(ab')<sub>2</sub>, G63-Fab and G63-F(ab')<sub>2</sub>

This conjugation was carried out in the following two-step reaction: (a) Preparation of DNP-ε amino caproate-N-hydroxy succinimide (NHSI). DNP ε amino caproic acid, DCC and NHSI were reacted in the ratio 1.1:1:1 in dry DMF. First, DNP-ε-amino caproic acid was mixed with NHSI ester, and subsequently allowed to cool on ice. The DCC was also cooled on ice and added to the mixture. The test tube was sealed and stirred on ice for 1 h, and the reaction allowed to proceed for additional 2 h or overnight at room temperature. (b) Conjugation of the above proteins with DNP: in order to derivatize the proteins with a different average number of DNP groups, the synthesis was performed with the appropriate ratios of the DNP-ε amino caproate-NHSI to protein molecule (i.e. between 4 and 100 activated DNP groups per protein). The calculated volumes of DNP-ε amino caproate-NHSI containing solution were added to that of the respective protein in 0.1 M NaHCO<sub>3</sub>, pH 8.1. The reaction was allowed to proceed overnight while stirring at room temperature and subsequently the sample was centrifuged to sediment slight precipitate. The transparent solution was loaded on a 4.5-ml G-25 column, in a 5-ml pipette, for gel filtration. Optical densities of the products were determined at 280 nm and 366 nm and used to calculate the DNP/protein ratios. Thus, the following derivatives were prepared: m-Fab<sub>1</sub>-DNP<sub>n</sub> with n=1.7, m-F(ab')<sub>2</sub>-DNP<sub>n</sub> with n=2.1 and 10.4, G63-Fab-DNP<sub>n</sub> with n=2; G63-F(ab')<sub>2</sub>-DNP<sub>n</sub> where n=2.1; 2.4; 10.4.

### Cells

Rat mucosal mast cells of the RBL-2H3 line were kindly provided by Dr. R. P. Siraganian (NIH, Bethesda ME), and grown as monolayer cultures in DMEM (Bio-Lab, Beit Haemek,

Israel), supplemented with 10% fetal calf serum (FCS, Gibco-BRL, USA), 4 mM L-glutamine, and combined antibiotics (Bio-Lab, Jerusalem, Israel), in a humidified atmosphere with 7% CO<sub>2</sub>, at 37°C. Cells from confluent cultures were detached and harvested by incubation with 10 mM EDTA in DMEM, for 10 min, and suspended in fresh DMEM medium. Cells were maintained during the experiments in Tyrod's buffer: 130 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM Glucose, 20 mM Hepes buffer pH 7.4, and BSA 0.1 mg/ml.

### Secretory response assay

RBL-2H3 cells (1×10<sup>5</sup>/100 μl/well), suspended in medium containing the DNP-specific A2IgE (typically 0.1 μl/ml of ascites fluid i.e. ca. 1×10<sup>-10</sup> M), were plated in 96-well plates. Cells were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrod's buffer. For competition experiments, 50 μl of mAb G63 (1×10<sup>-7</sup> M) was added, and the plates were incubated at room temperature for 15 min. Then, 50 μl of secretion inducing reagent was added, (or 100 μl for experiments where no competing mAb G63 were added), and the cells were further incubated at 37°C for 30 min. 20 μl of the supernatants were transferred to a new 96-well plate and reacted with 50 μl of substrate solution (p-nitrophenyl-N-acetyl-β-D-glucose amine 1.3 mg/ml, in 0.1 M citrate, pH 4.5). The mixture was incubated at 37°C, for 1 h, and the reaction terminated and developed by addition of 150 μl 'stop' solution (0.2 M glycine, pH 10.7). The intensity of the yellow color formed at this pH, due to the nitrophenol produced by the enzymatic hydrolysis of the substrate, is proportional to the concentration of the secreted enzyme. The optical density of the color intensity was measured at 405 nm in an ELISA plate reader. All these assays were repeated at least four times and their results are expressed as net percent of the cells' total enzyme activity contents ±5–10%.

### Appendix

The concentration of all species involved in the reactions described by eqs. (1)–(6) can be calculated by utilizing the following general equation:

$$[\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}] = J_r^{n_c} (n \cdot K_l)^{r-1} \cdot \left( \frac{n^a \cdot (n-1)^\beta \cdot K_l^{l-r+1}}{2^\beta} \right) \cdot ((n-1) \cdot K_{I_{agg}})^{r-1} \cdot K_p^{m_p} \cdot K_{G63}^{m_{f_m}} \cdot K_{G63agg}^{m_{f_d}-1} [r_f]^r \cdot [l_f]^l \cdot [m_f]^{(m_{f_m}+m_{f_d}+m_p)} \quad (A1)$$

The exponents  $a$  and  $\beta$  are defined as follows:

$$a = \begin{cases} 1 & \text{for } l = r \\ 0 & \text{for } l = r-1, r+1 \end{cases} \quad \beta = \begin{cases} 1 & \text{for } l = r+1 \\ 0 & \text{for } l = r-1, r \end{cases} \quad (A2).$$

In order to facilitate the understanding of this relatively condensed formalism, we shall now briefly discuss its individual factors.  $J_r^{n_c}$  accounts for the closed ring formation at all levels of clustering. A ligand induced cluster of  $r$ -th order (e.g.  $r=2$  for dimers,  $r=3$  for trimers, etc.) requires: (1) the binding of  $r-1$  ligands to different FcεRI-IgE to form  $r-1$  FcεRI-

IgE-ligand complexes; (2) the clustering of these reaction products by the binding of one of its free DNP-groups to the free Fab-combining site on another FcεRI-IgE-ligand complex; and finally (3) the interaction of a free DNP of the thus produced cluster with one of the two Fab-combining sites of a FcεRI-IgE complex is accounted for by the factors  $(n \times K_I)^{r-1}$  (step 1) and  $[(n-1) \times K_{Iagg}]^{r-1}$  (step 2 and 3), respectively. The factor  $\left(\frac{n^a \cdot (n-1)^\beta \cdot K_I^{l-r+1}}{2^\beta}\right)$  describes the binding of ligands to the free binding sites of monomers, dimers, trimers, etc. (Fig. 1a, b, d, g, h). For the ligand induced clusters with two free Fab-combining sites  $l=r-1$ , thus  $a=\beta=l-r+1=0$  so that  $\left(\frac{n^a \cdot (n-1)^\beta \cdot K_I^{l-r+1}}{2^\beta}\right)$ . For the binding of the first ligand  $l=r$  and  $a=1, \beta=0$ , so that one obtains  $nK_I$ . The binding of the second ligand yields a complex with  $l=r+1$ , so that  $a=0$  and  $\beta=1$  which yields  $(n-1)K_I^2/2$ . The product term  $K_p^{m_p} \cdot K_{G63}^{m_{f_m}} \cdot K_{G63agg}^{m_{f_d}-1} [r_f]^r \cdot [l_f]^l \cdot [m_f]^{(m_{f_m}+m_{f_d}+m_p)}$  reflects MAFA-FcεRI-IgE pre-aggregation and the co-clustering of one and two MAFA molecules by DNP-conjugated F(ab')<sub>2</sub>G63.

The direct clustering of free MAFA by G63 or F(ab')<sub>2</sub>G63 is accounted for by the equation:

$$[\{l_g, m_g\}_m] = 2 \cdot K_{G63agg}^{m_g-1} \cdot K_{G63agg} [\{1, 0\}_m \cdot \{0, 1\}_m^{m_g-1}] \quad (A3).$$

Eqs. (A1) and (A3) express the clusters' concentrations in term of the free ligand, FcεRI-IgE and MAFA concentrations. In order to obtain these quantities from the known total concentrations of these reagents ( $l_T, r_T, m_T, l_{G63,T}$ ) we utilized the law of mass conservation expressed by the following nonlinear equations:

$$\begin{aligned} l_T &= \sum_r \sum_{l=r-1}^{r+1} \sum_{m_{f_m}=0}^l \sum_{m_{f_d}=0}^l \sum_{m_p=0}^r \sum_{n_c=0}^1 (l \cdot [\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}]) \\ r_T &= \sum_r \sum_{l=r-1}^{r+1} \sum_{m_{f_m}=0}^l \sum_{m_{f_d}=0}^l \sum_{m_p=0}^r \sum_{n_c=0}^1 (r \cdot [\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}]) \\ m_T &= \sum_r \sum_{l=r-1}^{r+1} \sum_{m_{f_m}=0}^l \sum_{m_{f_d}=0}^l \sum_{m_p=0}^r \sum_{n_c=0}^1 ((m_{f_m} + 2 \cdot m_{f_d} + m_p) \cdot [\{r, l, m_{f_m}, m_{f_d}, m_p, n_c\}]) + \sum_{m_g=1}^2 m_g \cdot [\{1, m_g\}_m] \\ l_{G63,T} &= [\{1, 1\} + \{1, 2\} + \{1, 0\}] \quad (A4). \end{aligned}$$

We have solved these equations by a numerical, iterative procedure so as to obtain the respective free ligand concentrations, which were then used to calculate the concentrations of the different complexes and clusters by means of eqs. (A1) and (A3).

The FcεRI-IgE and MAFA concentrations and aggregation constants in eqs. (A1), (A3) and (A4) should, in principle be expressed in units of molecules/Å<sup>2</sup> and Å<sup>2</sup>/molecules, respectively. In order to express all concentrations in these equations in units of molarity, the surface densities have to be multiplied and the aggregation constants have to be divided by the factor [7, 29]:

$$\xi = \frac{c_{cell} A}{L} \quad (A5)$$

where  $c_{cell}$  is the number of cells per unit volume, A denotes the average surface area of the cell and L is the Avogadro's number. However, this conversion does not account for the fact that the effective concentration of the reactants involved in receptor clustering and co-clustering is larger than indicated by the (average three-dimensional) molar concentrations used for our calculations. As shown by Grasberger et al. [19] and Schweitzer-Stenner et al. [18] this can be compensated by allowing  $K_{Iagg} > K_I$  and  $K_{IG63} > K_{G63}$ , which would be physically incorrect if the respective reactions occurred in three dimensions.

**Acknowledgements:** Research reported in this paper was supported by a grant number 661/00 from The Israel Science Foundation to I.P. Part of the research of R.S.S. reported in this paper was carried out at the University of Puerto Rico, Río Piedras Campus, which was supported in part from a NIH-COBRE II grant for the Center for Research in Protein Structure, Function and Dynamics (P20 RR16439-01).

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