

Differential targeting of secretory lysosomes and recycling endosomes in mast cells revealed by patterned antigen arrays

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Summary

Polarized response towards a contact interface is a common theme in intercellular signaling. To visualize spatial regulation of stimulated secretion within a contact region, we exposed IgE-sensitized rat basophilic leukemia (RBL) mast cells to a surface that was patterned on the μm scale with hapten-containing lipid bilayers to activate cell surface IgE-receptor complexes. We find that, within 10 minutes of stimulation, fusion of individual secretory lysosomes is targeted towards the cell-substrate interface, but is spatially segregated from the patterned bilayers and receptor signaling complexes. By contrast, stimulated outward

trafficking of recycling endosomes is preferentially targeted towards the patterned bilayers. High spatial resolution of both antigen presentation in these arrays and detection of exocytotic events provides direct evidence for the heterogeneity of polarized responses.

Supplementary material available online at
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Key words: Differential targeting, IgE receptors, Polarized response, Recycling endosome, Secretory lysosome

Introduction

Considerable evidence supports a polarized response following cell-cell contact in the immune system, as exemplified by reorientation of the T-cell microtubule organizing center and enrichment of granular contents or membrane towards its interface with an antigen-presenting cell (Kupfer and Dennert, 1984; Kupfer et al., 1991; Linsley et al., 1996; Egen and Allison, 2002; Kuhn and Poenie, 2002; Clark et al., 2003; Bossi and Griffiths, 2005). One of the postulated functions of the immunological synapse, the ordered μm -scale structure formed at this cell-cell interface is targeted secretion (Stinchcombe et al., 2001). Specific targeting also occurs when antigens are presented on beads (Lawson et al., 1978; Tapper et al., 2002), indicating that intracellular machineries of effector cells alone can achieve this localization. In addition to secretion of soluble mediators, constitutive and stimulated trafficking occurs in cells to maintain or modulate plasma membrane composition. Localized targeting of membrane components, often carried out through recycling endosomes, operates in parallel with intracellular sorting mechanisms to cause surface expression that is laterally heterogeneous (Bajno et al., 2000; Ehrlich et al., 2002; Mamdouh et al., 2003; Bonello et al., 2004; Das et al., 2004). However, it remains unknown whether directed secretion and recycling occur locally at the sites of initial receptor clustering or are simply polarized towards cell-cell or cell-bead interfaces.

In investigations of cell-cell interactions, supported lipid bilayers have proven useful for presenting mobile ligands that engage receptors on the apposing cell surface as well as for providing a planar substrate favorable for optical imaging (Grakoui et al., 1999). In earlier studies we developed a novel

and biocompatible fabrication method for patterning supported lipid bilayers that incorporate hapten-labeled lipids (hereafter referred to as hapten-containing patterned bilayers) for specific binding of IgE, both in solution and when bound to cell surface receptors (Fc ϵ RI) on RBL mast cells (Orth et al., 2003; Wu et al., 2004). By restricting hapten presentation to μm -sized patches for controlled engagement of cellular receptors, it is possible to distinguish a receptor-targeted response from a response that is polarized generally towards the contacting surface. Stimulated exocytosis in mast cells, like T cells or other secretory cells of hematopoietic lineage, uses dual-function secretory lysosomes instead of more specialized secretory granules, such as those of neuroendocrine cells (Blott and Griffiths, 2002).

In the present study, we have examined secretory lysosome fusion with the plasma membrane at the single-vesicle level in spatial relationship to the initial stimulus. We find that this secretion is directed towards the substrate where the stimuli are located, but not targeted to the sites of receptor clustering. By contrast, stimulated outward trafficking of cholera toxin subunit B (CTB), which is bound to ganglioside GM1 and localized to recycling endosomes in mast cells (Naal et al., 2003), is preferentially targeted towards the localized regions of the initial stimulus. Overall, our results show that activated mast cells use a spatially regulated network to respond to external stimuli.

Results

Steady-state degranulation visualized by an annexin V binding assay

We first used an annexin V binding assay to examine RBL cell

activation by hapten-containing patterned bilayers. Annexin V binds to phosphatidylserine with high affinity in the presence of physiological concentrations of Ca^{2+} . In resting cells, phosphatidylserine is localized to the cytosolic face of the plasma membrane but becomes exposed to the external medium by flipping across the membrane as a result of mast cell activation; this then causes exogenous, soluble annexin V to bind to the cell surface (Demo et al., 1999). The amount of annexin V binding has been found to correlate quantitatively with secretory granule release, making it useful as a readout for exocytosis in individual mast cells, although the exact molecular mechanisms relating granule release and phosphatidylserine exposure are not clear. This process is different from phosphatidylserine exposure during apoptosis, because phosphatidylserine exposure in activated mast cells is much faster and is reversed within hours after stimulation.

RBL cells that had been sensitized with anti-DNP IgE were incubated with patterned bilayers, with or without haptenated (DNP-cap-DPPE) lipid. In the presence of this ligand, fluorescently labeled IgE becomes highly concentrated at the patterned features (Orth et al., 2003; Wu et al., 2004) (supplementary material Fig. S1). After 20 minutes and 40 minutes incubation, 94% and 96% of cells contacting pattern

show clustered IgE (J. L. Díaz-Pérez, A. Torres and B.A.B., unpublished data). As shown in Fig. 1A,B, fluorescence images reveal clear differences in stimulated exocytosis as indicated by binding of labeled annexin V. In the absence of the DNP ligand, the total number of pixels that are brighter than the background is consistently less than 10% of that in the presence of this ligand for similar cell densities. On the same chip containing haptenated lipids, clear differences in annexin V binding are also seen between the patterned and unpatterned surfaces (Fig. 1C). Comparing two regions with the same total area and cell density (and using the same threshold as background) the number of pixels that are brighter than background in the region without lipid bilayer is consistently about 10% of that in the region covered with haptenated lipid bilayer, indicating that stimulation is confined to cells in contact with ligand-containing patterns. Cells that appear bright in the absence of haptenated lipid typically show continuous instead of punctate annexin V labeling and probably represent a small population of damaged cells, possibly undergoing apoptosis. These results confirm that annexin V binding is selective for activated, degranulating cells. Closer inspection of individual cells on the μm -sized patterns of lipids containing the DNP-ligands reveals that the annexin V label does not localize to these regions of clustered receptors where the initial stimulus occurs (Fig. 1D). This result is similar to that observed with IgE receptors crosslinked by soluble antigen (supplementary material Fig. S2).

In contrast to annexin V, upon stimulation, phosphorylated tyrosine and Lyn kinase strongly localize with IgE at the patterned bilayers on the time scale of cell activation (Wu et al., 2004). Possible explanations for the absence of annexin V localization to these patterned bilayers are that the initial phosphatidylserine exposure sites bear no spatial relationship to the sites of stimulation or exocytosis, or the spatial correlation is lost due to lateral redistribution of phosphatidylserine after exposure and/or annexin V binding.

Although this annexin V binding assay does not satisfactorily answer the question of spatial localization of exocytosis, it addresses two important aspects of RBL cell activation by hapten-containing patterned bilayers. First, it provides a cell-by-cell assessment of stimulated degranulation that is statistically robust: exposure of inner leaflet lipid phosphatidylserine as consequence of signaling occurs in essentially all cells interacting with the patterned surfaces. Second, these results indicate that secondary responses do not occur with bystander cells that are not directly associated with the patterns. The activated response is shown to be specific, as cells interacting with haptenated lipids are at least ten times more responsive than cells adhering to unmodified surfaces. Thus, we can interpret the spatial regulation of exocytosis in our dynamic measurements of single vesicle fusion as described below.

Spatial segregation of secretion from initial stimulus

We established new probes that would allow us to visualize secretory granules directly. In a preliminary evaluation with confocal microscopy, we found that cellular granules labeled with a variety of markers (CD63-EGFP, EGFP-VAMP7, LysoTracker Red) are randomly distributed with respect to patterned bilayers under conditions of cell stimulation. However, interpretation of these images is complicated by a

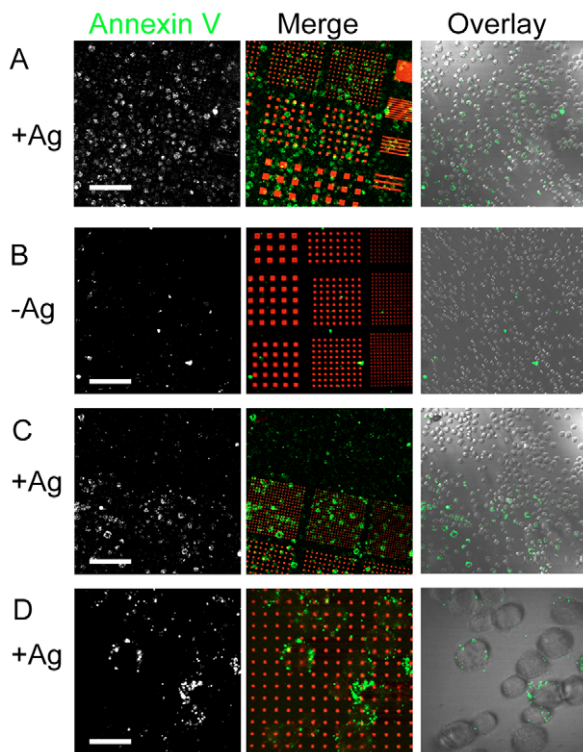


Fig. 1. Stimulated exocytosis detected by annexin V binding. (A,B) Substantial differences are observed for exocytosis stimulated by patterned bilayers (A) with and (B) without lipid ligand DNP-cap-DPPE ('Ag'). (C,D) The Alexa Fluor-488-annexin V label (green) is localized to cells on patterned regions (red) compared with cells (reflected light overlay) off patterns when only half of the chip is covered by haptenated lipids (C), but it is not localized to the defined regions of stimulus at the subcellular level (D). Cells were incubated with the patterns for 30 minutes at 37°C. Bars, 100 μm (A-C), 20 μm (D).

high background of granules in the cytoplasm and also by the possibility of an intrinsic bias towards inactive granules because transiently releasing granules may be missed. Therefore, we focused on the dynamic fusion events of individual granules to address the spatial regulation of the releasable pool of secretory granules. For a marker of the secretory granules we used CD63-EGFP, also known as lysosome-associated membrane protein 3 (Lamp3), a tetraspanin membrane protein containing a lysosome-targeting domain (Levy and Shoham, 2005). In general, CD63 labels secretory lysosomes that cycle between the endocytic pathway and the plasma membrane (Blott and Griffiths, 2002; Nishikata et al., 1992; Kobayashi et al., 2000). With low surface expression in resting cells, CD63 is reported to translocate to the plasma membrane after activation in a wide variety of cell types (Jaiswal et al., 2002) and to play an important role in intracellular trafficking, recycling of plasma membrane components, integrin-dependent post-adhesion functions, and T-cell co-stimulation (Duffield et al., 2003; Pfistershammer et al., 2004; Kraft et al., 2005). In RBL cells, CD63 has been shown to colocalize with SNARE proteins syntaxin 3 and VAMP7, which are involved in fusion of secretory granules with plasma membranes (Puri et al., 2003).

Fig. 2 and Movie 1 (supplementary material) provide a dynamic view of stimulated exocytosis by two-color confocal fluorescence imaging of a live cell stimulated globally with soluble antigen. RBL mast cells are transfected with CD63-EGFP and loaded with LysoTracker Red, which is taken up spontaneously into acidic secretory granules (supplementary material Fig. S3). The fusion process is characterized by rapid loss of granular contents, and more gradual merging of granule and plasma membranes, indicated by the profile analysis of the respective markers: LysoTracker Red and CD63-EGFP. This imaging assay based on confocal fluorescence microscopy is consistently reliable for visualizing secretion that occurs at the equatorial surface of RBL mast cells when stimulus is delivered globally (three positive experiments out of three trials, 32 single-fusion events observed). However, in multiple trials ($n=10$) to visualize CD63-EGFP- or LysoTracker-Red-labeled vesicle fusion in RBL mast cells stimulated by supported bilayers, we observed numerous fusion events occurring at the surface and none away from the surface. This is consistent with previous reports on peritoneal mast cells that the degranulating response is polarized towards the stimulating surface (Lawson et al., 1978).

We employed total internal reflection fluorescence microscopy (TIRFM) to look preferentially at cellular structures near the cell-surface interface. Because cell adhesion

and surface receptor crosslinking leading to exocytosis take place simultaneously in these experiments, it is challenging to image exocytosis during the initial minutes of cell-surface contact. To circumvent this problem, we used a standard approach to decouple the initial adhesion and exocytosis by withholding Ca^{2+} during the initial settling of the cells onto the patterned bilayers. After cells became adherent and visible by TIRFM, exocytosis was initiated by replenishing Ca^{2+} . In parallel degranulation assays using soluble antigen and a 20-minute delay in Ca^{2+} addition, no significant difference was observed in the total amount of secretion measured by release of β -hexosaminidase when compared with no delay (data not shown).

With TIRFM the exponentially increasing excitation field as vesicles approach the interface enables us to capture single fusion events of RBL mast cells stimulated by hapten-containing patterned bilayers (Fig. 3A, supplementary material Movie 2). For a given exocytosis event, the fluorescence signal of a single granule, labeled by CD63-EGFP, undergoes a two-stage change as quantified by (1) the granule peak intensity and, (2) the width of the bright spot within the set area (Fig. 3B). In the first stage, peak intensity increases while the width of the spot remains essentially the same, suggesting that the granule approaches the surface but remains intact. In the second stage, an increase of spot width indicates that vesicle fusion with the plasma membrane has begun. At the same time, peak intensity decreases. With these parameters we can distinguish vesicle fusion from vesicle motion away from the surface within the cell (Schmoranz et al., 2000).

In three experiments of this type on different dates, we observed approximately 5% of the fluorescent secretory lysosomes in the excitation volume to undergo fusion with the plasma membrane at the ventral cell surface imaged. This apparent low activity, compared with the release of ~70% of stimulated secretory lysosomes that is detected by confocal fluorescence microscopy (Williams and Webb, 2000) (our observations), is due in part to the differential visualization capacity of different imaging conditions and is consistent with TIRFM measurements of exocytosis in other cell types (Allersma et al., 2006). All the observed fusion events occurred only after Ca^{2+} replenishment and within 15 minutes of this addition. When presented with DNP-ligand in the patterned bilayers, we observed that essentially all of the fusion events occurred away from the receptors that were clustered by these patterned bilayers. Fig. 3C provides a summary image of the exocytosis sites observed in a single cell, and Fig. 3D is a summary of distance distributions from the nearest patterned bilayer over three experiments (35 fusion events). This spatial

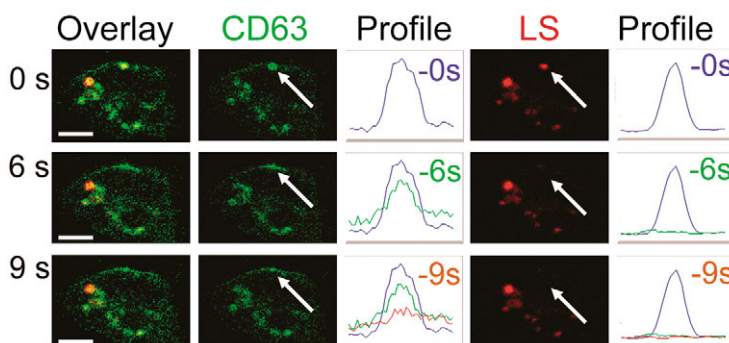


Fig. 2. Secretory lysosome labeled by transmembrane protein CD63-EGFP undergoes antigen-stimulated exocytosis as observed by confocal fluorescence microscopy. Vesicular secretion is characterized by the instantaneous loss of LysoTracker Red (LS) fluorescence (red) and flattening of the CD63-EGFP-labeled granular membrane (green) with the plasma membrane. The profile analysis shows a more gradual spreading of membrane bound fluorescence compared with release of LysoTracker Red. Blue, green, orange lines correspond to 0 seconds, 6 seconds and 9 seconds, respectively. Bars, 10 μm .

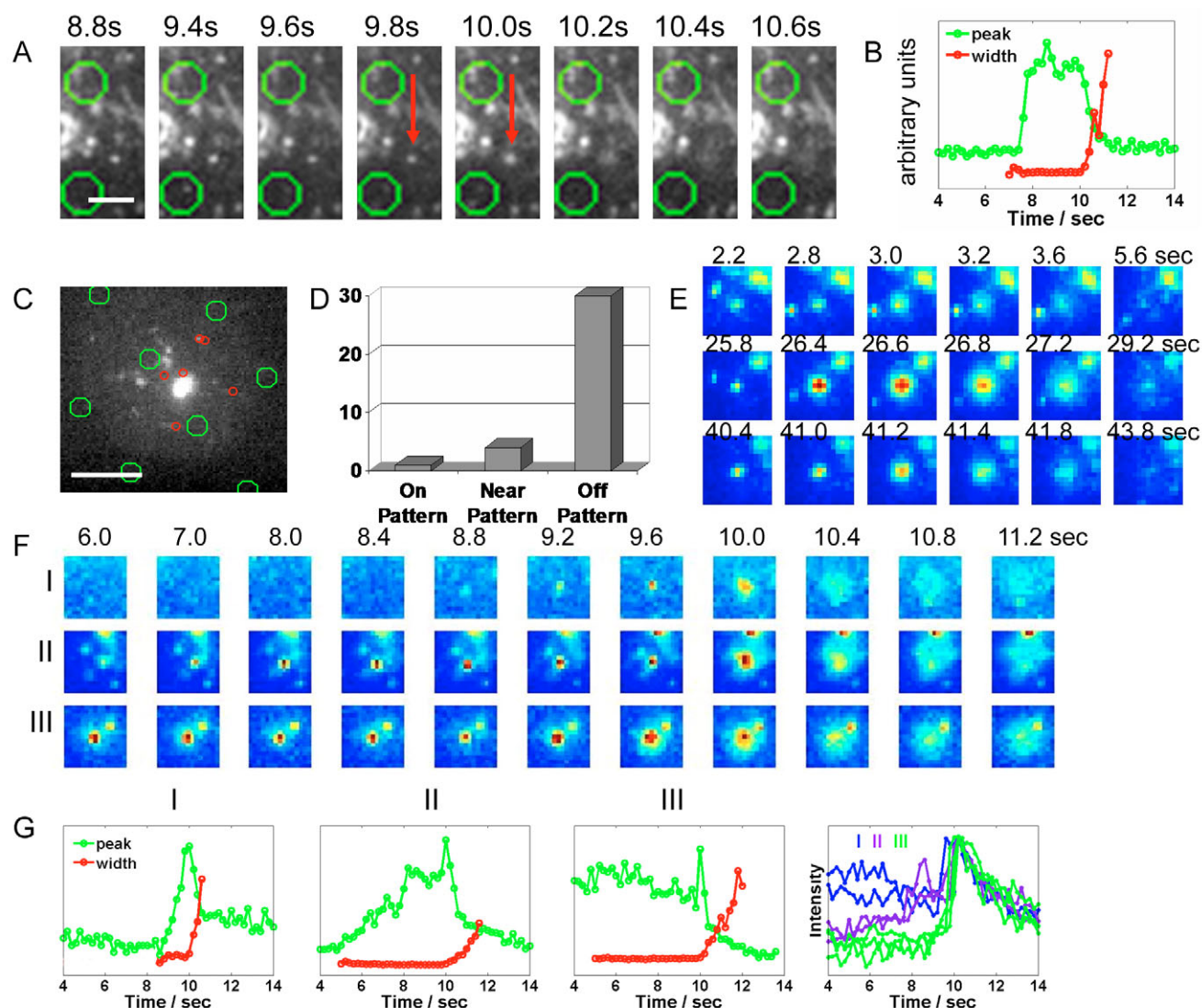


Fig. 3. TIRFM reveals that exocytosis occurs away from the initial stimulus. (A,B) Representative single exocytosis event (A) in which the fluorescence signal of a single secretory granule labeled by CD63-EGFP (arrows) undergoes a two-stage change, quantified in B by granule peak intensity (green) and width of the bright spot (red) that is fitted by a 2D Gaussian distribution. (C) Summary of visualized exocytosis sites (red circles) from a single representative cell. Boundaries of patterned bilayers in A and C are shown in green. (D) Frequency of exocytosis events on pattern, near pattern and off pattern; the distance from fusion site to the nearest pattern bilayer is less than $0.6\ \mu\text{m}$, between $0.6\ \mu\text{m}$ and $1.2\ \mu\text{m}$, and larger than $1.2\ \mu\text{m}$, respectively. The radius of the pattern feature size is $0.6\ \mu\text{m}$, and the distance between closest features is $4.8\ \mu\text{m}$. (E) Three exocytotic events that happened within 40 seconds and took place at exactly the same location; all three events are full fusion. (F) Example fusion events representing three different scenarios before the final full fusion: (I) fusion event shortly after the appearance of the granule, (II) granule is close to membrane but moves in x - y direction before fusion, (III) granule is docked at fusion site for some time before fusion. (G) Quantification of the events I, II and III in terms of peak intensity (green) and width (red). The fourth panel shows normalized average intensity of a 7×7 pixel area centered at peak for several representative cases of transient (I, blue), intermediate (II, purple) or long-docking step (III, green) prior to fusion. Time is indicated in (A,B). (F,G) is referenced by setting the initial fusion frame as 10 seconds. Bars, $2\ \mu\text{m}$ (A), $5\ \mu\text{m}$ (C). Image size of E and G is $2.5\ \mu\text{m}$.

distribution does not appear to have any detectable dependence on the time of fusion (supplementary material Fig. S4).

Interestingly, we observed that 21% of the released vesicles shared a fusion site with one or more other vesicles undergoing exocytosis from the same cell. Fig. 3E (supplementary material Movie 3) shows an example of three exocytotic events occurring sequentially at the same location within a period of 40 seconds. All three events are full fusions of three different granules as indicated by spreading of the granular membrane

marker, making it highly unlikely that a single granule is responsible for the repeated events observed. In fact, 'kiss and run' transient opening of a fusion pore does not result in exchange of granular membrane and is not detected under these conditions. Our results suggest that there are preferred sites at the plasma membrane where exocytosis occurs, and these sites are spatially distinct from sites of receptor activation.

The dynamic traces of granular motion prior to fusion are rather diverse (Fig. 3F). In 55% of 36 exocytotic events

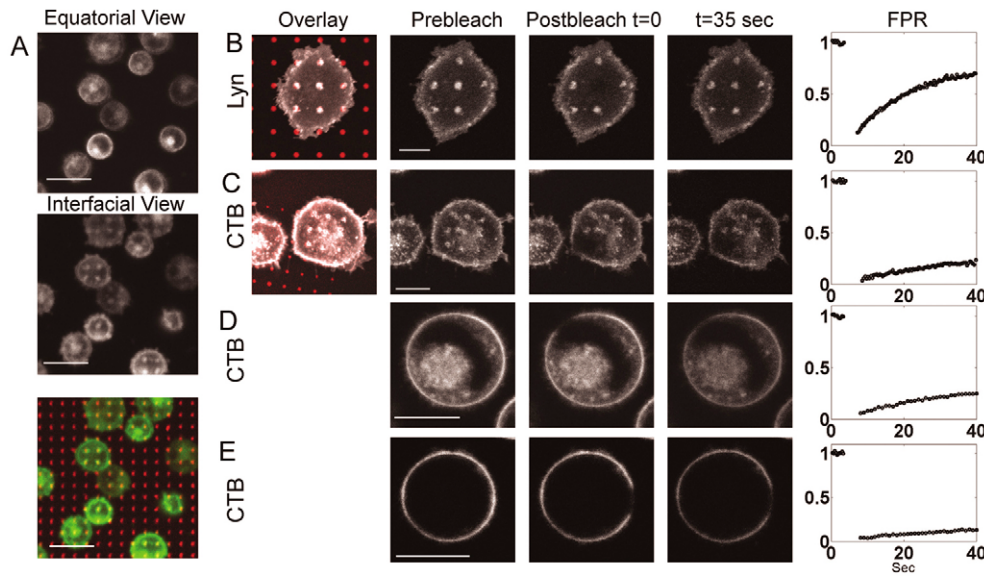


Fig. 4. Recycling endosomes labeled by Alexa Fluor-488 CTB-GM1 are targeted locally to the site of initial stimulus. (A) Confocal fluorescence images illustrate the intracellular pool of CTB-GM1 (equatorial view) and the enrichment of CTB-GM1 over the patterned bilayer (interfacial view). (B,C) Differential mobility of (B) Lyn-EGFP and (C) CTB-GM1 in patterns measured by FPR. (D,E) Slow mobility of surface CTB-GM1 measured by FPR in unstimulated cell (D) with or (E) without the internal perinuclear pool fluorescently labeled to assess contribution of outward trafficking to the fluorescence recovery on the plasma membrane. Bars, 25 μm (A), 10 μm (B-E).

observed, fusion occurs just after the granule appears. In these cases the total visible time from granule appearance to flattening out into the plasma membrane is typically less than 4 seconds, suggesting that the granule has been directly transported in the z direction towards its fusion site. In 39% of exocytotic events, the granules move around in x - y plane before fusion occurs. 21% of fusion events occur with granules that appear to be docked at their fusion sites, remaining immobile for longer than 8 seconds before fusion. Fig. 3G shows quantification of intensity dynamics for sample fusion events where a docking step is evident in some cases (II and III) but not in others (I).

Localized targeting of recycling endosomes

We find that stimulated exocytosis of CTB bound to ganglioside GM1 is targeted differently from CD63. As shown previously, CTB-GM1 is internalized by RBL cells and colocalizes with a perinuclear pool of recycling endosomes containing transferrin receptors (Naal et al., 2003). CTB-GM1 has an apparently higher plasma membrane distribution than transferrin receptors at steady-state, possibly due to slow internalization of CTB-GM1 (supplementary material Fig. S5). The CTB-GM1-labeled pool of perinuclear endosomes also strongly colocalizes with EGFP-Rab11 (supplementary material Fig. S6), consistent with their assignment as recycling endosomes (Sonnichsen et al., 2000). When directly compared in unstimulated cells, CD63-EGFP in secretory granules is distributed throughout the cytoplasm, whereas CTB-GM1 has a distinctive perinuclear localization (supplementary material Fig. S7). The perinuclear pool of internalized CTB-GM1 is spatially distinct from the Golgi complex in these cells (Naal et al., 2003), but its ultrastructural identity has not yet been defined. Our previous work showed that IgE-receptor-mediated signaling causes rapid, sustained net outward trafficking of intracellular CTB-GM1 (Naal et al., 2003). Using the patterned DNP ligands we found that, in contrast to CD63-EGFP secretory granules, stimulated trafficking of CTB-GM1 is targeted to the clustered IgE-receptors, taking place within 5 minutes at 37°C (Fig. 4A). This apparently localized exocytosis is not due to rapid lateral redistribution with the clustered IgE-receptors of labeled CTB

originating on the plasma membrane: we previously showed that CTB does not concentrate at the patterned bilayers under these conditions if only plasma membrane GM1 is labeled (before or after incubation of the cells with the substrates) (Wu et al., 2004). The rapid kinetics of stimulated targeting suggest that the apparent enrichment is not likely to be due to differential endocytosis rates for CTB at or away from the patterned bilayers, because internalization of CTB-GM1 is very slow (Naal et al., 2003). The accumulation of CTB-GM1 at the patterned DNP ligands does not occur at 4°C, as expected for a process that depends on intracellular trafficking (data not shown).

Because of the high background of the CTB-GM1 at the plasma membrane, we have not yet been able to visualize individual fusion events by TIRFM. To test whether CTB-GM1 labeled endosomes that are delivered to the plasma membrane undergo fusion at those targeted locations, we used an antibody specific for FITC conjugated to CTB. When added during stimulation of live cells, Alexa Fluor-488 labeled antibody became coincident with the patterned bilayers (supplementary material Fig. S8). The striped distribution of this labeled antibody coincident with the line patterns of hapten-containing lipid bilayers used in this experiment demonstrates accessibility of the newly trafficked FITC-CTB at the outer leaflet of the plasma membrane. Control experiments showed that concentration of labeled anti-FITC at the patterned bilayers depends on pre-internalization of FITC-CTB into recycling endosomes. Taken together, these results provide strong evidence that newly trafficked CTB-GM1 is delivered preferentially to sites of stimulation at the patterned bilayers.

We assessed why newly delivered CTB remains concentrated at the target site without diffusing into other parts of the plasma membrane. Quantitative measurements of fluorescence photobleaching recovery (FPR) show that fluorescent CTB at the patterned bilayers diffuses very slowly (Fig. 4C). By contrast, we observed substantially faster recovery due to diffusion of two other probes that are anchored to the inner leaflet of the plasma membrane and colocalize with clustered IgE receptors: Lyn-EGFP (Fig. 4B) and PM-EGFP (Wu et al., 2004), an analogue containing the N-terminal

segment of Lyn that is dually acylated with palmitate and myristate. This slow mobility of CTB also occurs in the absence of the patterned bilayer, either in the presence or absence of a fluorescently labeled internal pool (Fig. 4D,E). In the presence of the internal pool of fluorescently labeled CTB (Fig. 4D), the recovery rate is increased relative to that in the absence of the labeled internal pool (Fig. 4E), suggesting that net outward trafficking contributes to photobleaching recovery. Furthermore, these results indicate that the slow mobility of the bound CTB cannot be ascribed simply to an increased surface level of CTB-GM1 as a result of stimulated outward trafficking. Slow lateral diffusion of CTB-GM1 has been observed in other cell types (Bacia et al., 2002; Kenworthy et al., 2004), and its physical basis remains to be determined, although it has been suggested to be pH dependent (McCann et al., 1997; Pelkmans et al., 2004). In any case, this slow lateral mobility causes freshly delivered CTB-GM1 to accumulate at a targeting site that corresponds to the clustered IgE receptors and initial stimulus.

Discussion

Spatial regulation of stimulated cellular exocytosis plays an important physiological role in how a cell responds effectively and directionally to environmental signals. Previous studies showed that mast cells (Lawson et al., 1978; Galli et al., 2005) as well as neutrophils, helper T cells and cytotoxic T cells (Kupfer and Dennert, 1984; Kupfer et al., 1991; Clark et al., 2003; Stinchcombe et al., 2001; Tapper et al., 2002; Bossi and Griffiths, 1999) have a polarized response when stimulated by large functionalized beads, antigen-presenting cells or target cells. However, the contact areas in these cases represent a substantial fraction of the cell surface, and heterogeneity within the contact region is usually not examined. In one study of immunological synapses formed between cytotoxic T lymphocytes and target cells, secretory granules were observed by immunofluorescence to be enriched in the synapse but spatially segregated from a signaling domain (Stinchcombe et al., 2001). Our studies on RBL mast cells, combining surface-patterned ligands and dynamic visualization of individual vesicles with TIRFM, directly demonstrate spatial segregation within the cell-surface contact and differential targeting for secretory lysosomes (labeled with CD63-EGFP) compared with recycling endosomes (labeled with CTB-GM1).

We found that stimulated degranulation of secretory lysosomes in RBL mast cells occurs away from the patterned ligands and, correspondingly, away from the clustered IgE receptors. The question regarding the mechanism for directing these exocytotic events remains open, and we expect that structural rearrangements associated with the cytoskeleton play a significant role. We observed that more than half of fusing secretory lysosomes appeared briefly (less than 4 seconds) in the evanescent field (less than 100 nm away from the surface) prior to fusion, indicating they are transported from inside of cell in response to the stimulus. Long distance movements of CD63 labeled vesicles are microtubule dependent, as they are abolished in the presence of the microtubule polymerization inhibitor colchicine (supplementary material Movie 4) (Nishida et al., 2005; Smith et al., 2003). Microtubule-dependent targeting of intracellular vesicles towards focal adhesions has been visualized and implicated in local regulation of trafficking in endothelial cells (Ezratty et al.,

2005). In addition, the tetraspanin CD63 is known to associate directly with integrins (Mannion et al., 1996). Tetraspanin-enriched microdomains that contain tetraspanin-integrin complexes are emerging as a possibility for spatial regulation that is distinct from standard definitions of lipid rafts based largely on lipid properties (Hemler, 2005; Tarrant et al., 2003). In recent experiments, we have observed segregation of EGFP- $\alpha 5$ integrin away from the patterned bilayers (A. Torres, D. Holowka and B. Baird, unpublished results). Although limited, current evidence is consistent with the view that the exocytotic location we observe is regulated with spatial cues coming from integrin-mediated adhesion complexes. Further experiments will be necessary to test this hypothesis.

In contrast to CD63-EGFP, the patterned bilayers revealed that outward trafficking of CTB-GM1, which also occurs within minutes after stimulation, is targeted towards the regions of receptor clustering and initial signaling complexes. Differential targeting can be achieved by specific intracellular trafficking routes and machinery and/or different kinetics for internalization and recycling to the surface. The observation that mast cells maintain constitutive internalization and outward trafficking of membrane components such as GM1, and also maintain potential for stimulated activity (Naal et al., 2003), might be relevant to the capacity of these cells to respond rapidly to antigen through modulation of plasma membrane composition. Similarly, T cell receptors (Das et al., 2004), LAT (Bonello et al., 2004) and Lck (Ehrlich et al., 2002) have been observed to associate with recycling endosomes in T cells, and these intracellular pools appear to translocate towards the surface of stimulation within minutes of activation. Thus, localized targeting could be a general mechanism for delivering new membrane to specific sites of activation that in turn maintains particular environments for targeting of other signaling pathways.

We find that a set of early signaling molecules, including the inner leaflet protein Lyn-EGFP (Wu et al., 2004), and the transmembrane protein LAT-EGFP (Senaratne et al., 2006), are recruited to clustered receptors. By contrast, key components that are important for secretion both quantitatively and possibly spatially, such as phosphatidylinositol (4,5)-bisphosphate (as indicated by PLC δ PH-EGFP) (Wu et al., 2004) and SNAP23 (data not shown) do not preferentially localize to the hapten-containing patterned bilayers. It is notable that trafficking of CTB-labeled recycling endosomes to the plasma membrane does not depend on protein kinase C activity (Naal et al., 2003), whereas secretory lysosomal exocytosis depends strongly on this enzyme (Blank and Rivera, 2004; Wolfe et al., 1996). In addition, stimulated outward trafficking of CTB-GM1 does not depend on microtubule polymerization (N. L. Smith and D.H., unpublished results), whereas colchicine inhibits transport and release of secretory lysosomes in these cells (supplementary material Movie 4 and data not shown) (Nishida et al., 2005). Therefore, it is possible that downstream signaling associated with microtubule rearrangements play some role in this differential spatial targeting.

In summary, we show that μ m-sized patterned antigens provide a defined spatial reference to visualize and quantify intracellular vesicle trafficking and targeting to the plasma membrane. We find the response is generally polarized to the surface bearing the stimulus. However, distinctly heterogeneous targeting occurs at higher spatial resolution, and

this could reflect a general mechanism for cells to maintain an overall efficient response as well as to allow specialization of different types of exocytotic events. In future studies we expect that this capacity to resolve both the stimulus and the response will facilitate elucidation of the molecular mechanisms involved in these targeting processes.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[6-[(2,4-dinitrophenyl) amino] hexanoyl] (DNP-cap-DPPE), were purchased from Avanti Polar Lipids. DiIC₁₈, Alexa Fluor-488-annexin V, Alexa Fluor-488-cholera toxin subunit B (CTB) and LysoTracker Red were purchased from Molecular Probes. 5-Hydroxytryptamine and colchicine were purchased from Sigma Chemical Co. The CD63-EGFP, EGFP-VAMP7, and EGFP-Rab11 constructs were gifts from Juan Bonifacio (NIH, Bethesda, MA), Paul Roche (NIH, Bethesda, MA), and Ruth Collins (Cornell University, Ithaca, NY), respectively.

Patterned bilayer formation

Silicon wafers (4 inches) with a thermally grown oxide layer (Silicon Qwest, Santa Clara, CA) and 100-mm glass wafers (Precision Glass & Optics, Santa Ana, CA) were used for substrates. Wafers were thoroughly cleaned in Nanostrip (Cyantek Corp., Fremont, CA) to remove surface contaminants and with lipid patterned bilayers as described (Wu et al., 2004).

Cell preparations

RBL-2H3 cells were maintained as described previously (Wu et al., 2004). Transient transfections with CD63-EGFP and other constructs were carried out using Lipofectamine (Invitrogen, Carlsbad, CA). In all experiments, cells were sensitized with anti-DNP IgE at 0.5 µg/ml overnight or at 1 µg/ml for 1 hour at 37°C just prior to the experiments. In single-vesicle dynamics experiments (both confocal and TIRFM), cells were incubated with 250 µM of 5-hydroxytryptamine overnight (Williams and Webb, 2000). When used, granule probe LysoTracker Red was loaded at 3 µM for 2 minutes prior to stimulation, then cells were stimulated by adding 1 ml of DNP-BSA (100 ng/ml). For indicated experiments cells were incubated with colchicine (20 µM) for 1 hour at 37°C prior to stimulation to inhibit microtubule polymerization.

For experiments using patterned bilayers, cells were washed twice and resuspended at 1×10^6 cells per ml in buffered saline solution (BSS; 135 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES pH 7.4, containing 1 mg/ml BSA). Labeled cells were incubated with patterned bilayers for specific periods of time and imaged live in BSS.

For the annexin-V-binding assay, cells were incubated with the patterned bilayers for 30 minutes at 37°C, then washed in cold PBS twice and incubated with Alexa Fluor-488-annexin V (1:40 dilution) at room temperature for 15 minutes in BSS. For CTB trafficking experiments, Alexa Fluor-488-CTB (5 µg/ml) was added to cell aliquots for 1 hour at 37°C just prior to the experiment, then cells were washed twice and resuspended in BSS at 1×10^6 cells per ml. After incubation with the patterned bilayers for 5 minutes at 37°C, cells were imaged by confocal fluorescence microscopy.

For exocytosis visualized with CD63-EGFP and TIRFM, Ca²⁺-free BSS was initially used during cell adherence at 37°C. After cells became attached and optimized in the view of evanescent field (about 15 minutes), 2× Ca²⁺ BSS was added to the solution to initiate exocytosis.

Confocal fluorescence microscopy

Imaging of all samples was carried out at room temperature. A BioRad confocal head stage and an Olympus AX 70 inverted microscope were used for confocal microscopy imaging. DiIC₁₈ was observed with a 585-nm LP filter, and Alexa Fluor-488 was observed with a 522/35-nm filter.

Confocal-based fluorescence photobleaching recovery (FPR)

A Leica Spectral Confocal Microscope System was used to measure FPR. In these experiments, a circular region of interest (ROI) was photobleached using full laser power for ten iterations (0.34 seconds per iteration) and monitored for fluorescence recovery using low-intensity laser excitation for 35 seconds (100 frames). Photobleaching of internal CTB to optically remove outward trafficking (Fig. 4E) is accomplished by bleaching the internal pool at full laser power for 34 seconds. Image analysis was carried out with Matlab (Mathworks, Natick, MA). Background fluorescence was subtracted frame by frame using average fluorescence intensity of a blank region. Photobleaching during image acquisition was corrected by calculating the ratio between the average intensity of the bleached region and that of a nonbleached region.

Total internal reflection fluorescence microscopy (TIRFM)

An inverted Olympus IX71 microscope was modified for through-the-objective

evanescent field illumination (Axelrod, 2001). Krypton-argon laser beams were focused off-axis onto the back focal plane of a 60×, 1.45 NA Olympus TIRFM objective to achieve total internal reflection at the interface between the glass substrate and the solution or cell. 525/30 and 600LP filter sets were used for EGFP and DiIC₁₈ emission, respectively. Fluorescence images were recorded by an EMCCD camera (Andor iXon, Model#DV887) at 200-millisecond intervals. An image of the patterned lipid bilayer was obtained before and after each movie to determine locations of receptor clustering. All TIRFM experiments were carried out at 37°C with temperature controlled by an objective heater (Biopetech, Butler, PA) and stage incubator (Nevtek, Williamsville, VA). The zero time point was recorded as the addition of 2× Ca²⁺ BSS.

Image analysis

All image analysis was carried out in Matlab. For the annexin-V-binding assay, an automatic threshold routine was used, and numbers of positive pixels were compared for samples with or without haptenated lipids. In the case of partial bilayer coverage, two areas with equal size and cell density were selected manually, one containing lipid bilayer only and one containing unpatterned surface only. The ratio between numbers of positive pixels in two areas was calculated for quantification.

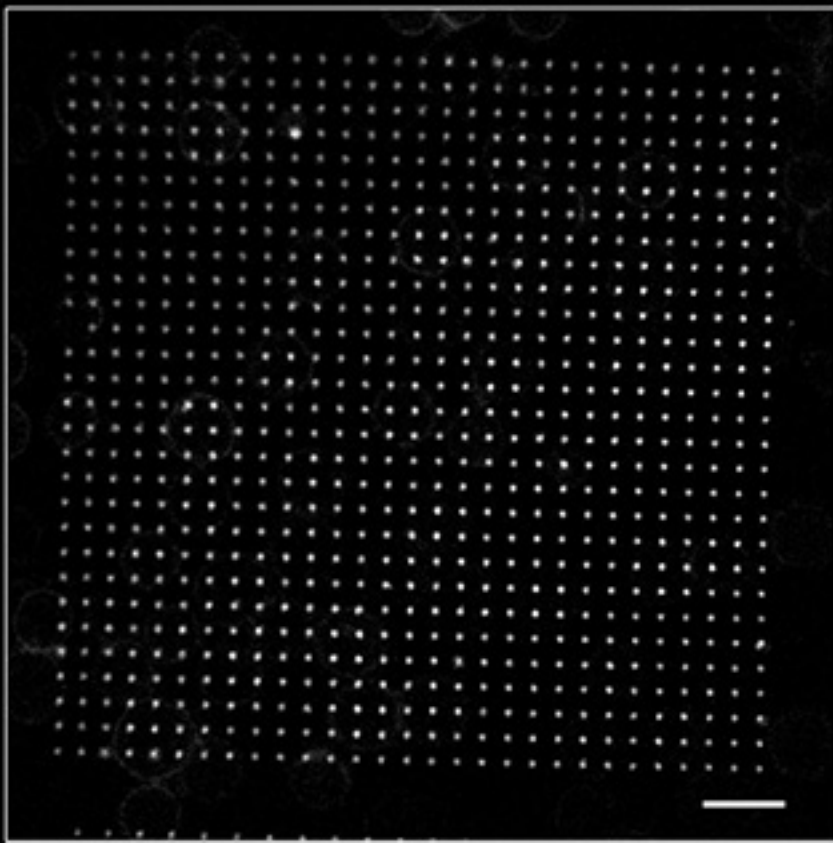
For exocytosis dynamics, pattern locations were obtained from the red channel by using a threshold. Boundaries of the pattern were overlaid with each movie. Fusion events were first identified visually. A 7×7 pixel area centered with the brightest pixel was used to quantify peak intensity and average intensity. The intensity over this area was fitted by a 2D Gaussian profile with peak, center coordinates and width (standard deviation) as free parameters. A 15×15 pixel area was used for presentation in the figures.

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IgE

Annexin

