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Enzymatic generation of ceramide induces membrane restructuring: Correlated AFM and fluorescence imaging of supported bilayers

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ABSTRACT

The effect of enzymatic generation of ceramide on phase separated bilayers with a mixture of co-existing fluid and liquid-ordered phases has been examined using a combination of atomic force microscopy (AFM) and fluorescence imaging. Supported lipid bilayers prepared from a DOPC/sphingomyelin/cholesterol mixture were imaged prior to, during and after incubation with sphingomyelinase by total internal reflection fluorescence (TIRF) microscopy. Enzyme treatment resulted in the growth of large dye-excluded regions. The growth kinetics for these patches are consistent with activity of a variable number of enzyme molecules in different regions of the bilayer. Correlated AFM and fluorescence imaging shows that some of the large dye-excluded patches form around the original liquid-ordered domains, which become heterogeneous in height with many raised ceramide-rich regions around their periphery. However, some of the dye-excluded patches correspond to areas of the bilayer where the initial domains have largely or partially disappeared. The dye-excluded patches observed by fluorescence are shown to be areas of increased adhesion in lateral deflection AFM images and are postulated to form by incorporation of both cholesterol and ceramide in the original fluid phase and to vary in composition throughout the bilayer. This is evident from the observation that the dye-excluded areas are all detected as areas of increased friction, but do not always show a distinct height difference in topographic images. These results highlight the utility of a multi-modal imaging approach for understanding the complex membrane restructuring that occurs upon enzymatic generation of ceramide.

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1. Introduction

Ceramide is an important signaling molecule that mediates a range of cellular processes, including apoptosis, cell cycle arrest and bacterial pathogenesis (Bollinger et al., 2005; Cremesti et al., 2002; Grassme et al., 2003). Although the concentration of ceramide is low in resting cells, it increases rapidly in response to various stresses and can reach levels as high as 10% of the total membrane lipid of apoptotic cells (Hannun, 1996). The increased concentration of ceramide is primarily due to enzymatic hydrolysis of sphingomyelin (SM) by Sphingomyelinases (SMase). Since SM is one of the main constituents of membrane raft domains (Pike, 2006; Simons and Ikonen, 1997; Simons and Vaz, 2004), the generation of ceramide has a marked effect on membrane compartmentalization. In particular, a number of studies have proposed that ceramide production leads to coalescence of small rafts to give larger membrane platforms, as summarized in recent reviews (Bollinger et al., 2005; Cremesti et al., 2002). These ceramide-enriched

membrane platforms appear to provide a mechanism for organizing signaling molecules to facilitate and amplify signaling via specific membrane receptors. It has been hypothesized that the transformation of small inactive rafts to larger signaling platforms explains the function of ceramide in cellular activation by many receptors, in stress stimulation and in developmental processes (Bollinger et al., 2005; Zhang et al., 2009).

Ceramides are hydrophobic lipids with high melting temperatures and a strong propensity for hydrogen bonding, both of which contribute to their large impact on the physical properties of membranes (Goni and Alonso, 2006, 2009). These effects include increases in molecular order, promotion of phase separation and domain formation, enhanced membrane permeabilization and transbilayer lipid flip-flop, and formation of non-lamellar phases. The importance of ceramide in modulating membrane physical properties and biological function has prompted a number of studies of ceramide incorporation in model membranes. Several recent investigations have examined the effects of ceramide incorporation on coexisting ordered and fluid domains in phase separated binary and ternary lipid mixtures that are used to model membrane rafts (Castro et al., 2007; Fidorra et al., 2006; Silva et al., 2007; Sot et al.,

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2006, 2008). This work has demonstrated that ceramide leads to increased membrane heterogeneity and that its effects depend on the membrane composition. Ceramide has also been shown to displace cholesterol from raft domains in model and cellular membranes (Ali et al., 2006; Chiantia et al., 2006; Megha and London, 2004; Megha et al., 2006; Yu et al., 2005b). Several recent studies have concluded that the ability of ceramide to modulate the properties of liquid-ordered domains and form ceramide-enriched gel domains is affected by the chain length and that short-chain ceramides (C_{12}) are not good models for studies of the biological activity of ceramide (Chiantia et al., 2007; Megha et al., 2007).

Enzymatic generation of ceramide in model membranes has also been examined and is no less complex than the direct incorporation in lipid mixtures. For example, treating phosphatidylcholine/SM vesicles with SMase produced ceramide patches that coalesced into larger domains and then formed vesicles (Holopainen et al., 2000; Nurminen et al., 2002). Several other studies have provided evidence for ceramide-enriched domains in monolayers (Fanani et al., 2002; Hartel et al., 2005), in fluid phospholipid vesicles (Taniguchi et al., 2006) and in giant vesicles prepared from SM/ceramide or ceramide/POPC/cholesterol mixtures (Fidorra et al., 2006; Sot et al., 2006). These studies in monolayers and vesicles indicate that enzymatic generation of ceramide leads to more complex changes in membrane morphology than does direct ceramide incorporation in the same lipid membranes. Of particular relevance to the influence of ceramide on membrane raft domains are several recent studies that have compared direct and enzymatic generation of ceramide in vesicles and supported bilayers prepared from ternary lipid mixtures with coexisting ordered and fluid phases (Chiantia et al., 2006, 2008; Ira and Johnston, 2006, 2008; Lopez-Mentero et al., 2007; Silva et al., 2007; Trajkovic et al., 2008). Atomic force microscopy (AFM) and fluorescence microscopy have shown that both direct and enzymatic incorporation of C_{16} or C_{18} ceramide causes formation of a new ceramide-rich ordered phase for various ternary lipid mixtures. However, enzymatic generation of ceramide in supported bilayers also leads to a pronounced bilayer restructuring that has some analogies to the proposed coalescence of rafts in cellular membranes (Ira and Johnston, 2006, 2008). Work from our and other laboratories has illustrated the utility of AFM for studying the nanoscale details of this ceramide-induced membrane restructuring in supported lipid bilayers (Chiantia et al., 2006; Ira and Johnston, 2006, 2008; Lopez-Mentero et al., 2007). In addition to producing ceramide-enriched islands within individual ordered domains, enzyme treatment leads to clustering of individual domains, formation of areas of fluid phase that are devoid of domains and membrane defects. The extent of membrane restructuring varies with the initial size and composition of the ordered domains and is consistent with effects due to ceramide-induced cholesterol expulsion from the domains (Ira and Johnston, 2006, 2008).

AFM is particularly useful for probing membrane morphology at high spatial resolution in aqueous solution (Connell and Smith, 2006; Johnston, 2007), but provides limited information on chemical composition for samples with multiple components and has limitations for following the time course of enzyme activity over a range of time and length scales. Therefore, we have examined the enzymatic generation of ceramide using a combination of total internal reflection fluorescence (TIRF) microscopy and correlated AFM and fluorescence microscopy. The TIRF studies allow one to monitor effects of ceramide on both domains and fluid phase and to separate effects of initial enzyme activity from subsequent lipid reorganization. Correlated AFM/fluorescence provides a detailed understanding of the complex effects of enzyme activity upon both the domains and surrounding fluid membrane.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), egg sphingomyelin (ESM), ganglioside GM1 and cholesterol were obtained from Avanti Polar Lipids and were used as received. Sphingomyelinase (SMase) isolated from *Staphylococcus aureus* (*S. aureus*) was from Sigma-Aldrich. Texas Red[®] 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE) and Cholera toxin subunit B (Ctx-B) Alexa 647 conjugate were from Invitrogen. All aqueous solutions were prepared with 18.3 MΩ cm Milli-Q water. SMase buffer: 125 mM NaCl, 10 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, pH 7.4. Ctx-B buffer: 20 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaN₃, pH 7.4.

2.2. Preparation of bilayers

Small unilamellar vesicles were prepared as previously described (Ira and Johnston, 2006). Briefly, chloroform solutions of phospholipids and fluorescently labeled lipids were mixed in the appropriate ratios and the lipid films obtained after drying the solvent were hydrated in water and vortexed to obtain multilamellar vesicles. The sample was then sonicated in a water bath sonicator to clarity to form SUVs with a final lipid concentration of 1 mM. Vesicles were used immediately for bilayer preparation or stored at 4 °C for up to a week prior to use.

Vesicle solution (150 μl) and 300 μl CaCl₂ (15 mM) were added to freshly cleaved mica (60–70 μm thick for fluorescence imaging) clamped in a liquid cell. After incubation at 45 °C for 90 min, bilayers were cooled to RT and then rinsed extensively with water to remove unattached vesicles before imaging. The presence of occasional defects allowed us to measure the bilayer thickness, confirming the presence of a single bilayer. Control experiments in which vesicle and bilayer preparation was carried out in the presence of antioxidants (*N*-propyl gallate and ascorbic acid) showed that bilayer morphologies for the ternary lipid mixture were similar to those in their absence.

2.3. TIRF imaging

Fluorescence images of bilayers were taken on an Olympus 1X81 total internal reflection fluorescence (TIRF) microscope equipped with a high resolution CCD camera (CoolSNAP, Photometrics, US) and a 60x/1.45 NA Plan Apochromat objective. TR-DHPE was excited at 543 nm and emission collected at 593 (±20) nm. Ctx-B Alexa 647 was excited at 633 nm with emission collected at 690 (±20) nm. For SMase treatment, the bilayers were washed with SMase buffer and imaged. The effect of enzyme on the bilayer was followed in time by imaging the same area before and after addition of buffered enzyme solution at room temperature. Control experiments indicated that irradiation of bilayers containing TR-DHPE for similar time periods to those used for the SMase treatments led to no changes in bilayer morphology, although significant photobleaching of the dye was observed. Images for the individual time course experiments are displayed on a fixed intensity scale unless otherwise noted. For the kinetic analysis, the area of individual dark patches/domains was calculated using the area function of the ImagePro software. Plots display the total dye-excluded area for a specific region of the image as a function of time. A minimum of three independent bilayers was prepared and imaged for each enzyme concentration and several areas of each sample were examined.

2.4. Atomic force microscopy

AFM images were obtained on a PicoSPM atomic force microscope (Molecular Imaging) in MAC-mode using magnetic coated

silicon tips (Type II and Type IV) with spring constants of ~ 0.5 N/m and resonance frequencies between 7 and 35 kHz in aqueous solutions. Either a $30 \times 30 \mu\text{m}^2$ or $5 \times 5 \mu\text{m}^2$ scanner was used with a scan rate between 0.7 and 1.4 Hz. All images shown are flattened raw data. Two or three independently-prepared samples were imaged for each bilayer composition, and several areas were scanned for each sample. Domain heights were measured as the difference between the condensed phase domains and the fluid phase and 20–40 height estimates were collected per image. Results of several different experiments for each composition were used to generate histograms that were fitted with a Gaussian function. The reported heights are the peak of the Gaussian distribution. For SMase treatment, the bilayers were imaged in water prior to replacing the water by buffered enzyme solution, incubated at room temperature for 2–30 min, rinsed extensively with water and re-imaged.

2.5. Correlated AFM and fluorescence imaging

Correlated images were recorded using a JPK NanoWizard®II BioAFM (JPK Instruments, Berlin, Germany) integrated with an Olympus IX81 inverted optical microscope. AFM was performed in contact mode and images were captured using DNP-S (Veeco, CA) AFM cantilever/tips with spring constants of 0.15–0.32 N/m. Images were recorded in both topographic and lateral deflection modes. The contrast in lateral deflection scans depends on the normal force applied to the sample and the scan speed. The contrast can be adjusted with the imaging parameters, but the regions of different lipid phases are constant. Fluorescence images used lamp excitation with a TRITC WF filter set (Chroma Technology) and a Cascade 512 CCD camera (Photometrics). For the correlated imaging, AFM and fluorescence images of the same area were recorded sequentially, after locating a suitable area. Fluorescence images had acquisition times of <1 s, much shorter than the ~ 10 min required to record the AFM images. Typically fluorescence images were recorded both before and after AFM images of the same area to verify that the membrane morphology had not changed dramatically during the time required for AFM imaging. In some experiments the bilayer was re-imaged after washing to remove enzyme (which requires moving the sample), and it was necessary to find the same area again in order to make a direct comparison of before enzyme, after incubation with enzyme and after washing. This was accomplished by using line defects in the mica as a marker.

3. Results

In a previous study we used AFM to examine SMase-mediated generation of ceramide in 2:2:1 DOPC/ESM/cholesterol bilayers (Ira and Johnston, 2006). This lipid mixture has the coexisting liquid-ordered and fluid phases that are typical of several ternary lipid mixtures used to model the behavior of membrane rafts in vesicles and supported membranes (London, 2005; Simons and Vaz, 2004; Veatch and Keller, 2005). SMase treatment of 2:2:1 DOPC/ESM/cholesterol bilayers led to formation of heterogeneous domains with raised ceramide-enriched islands (Ira and Johnston, 2006). In addition, the bilayers showed significant restructuring which resulted in formation of (1) clusters of domains in a new phase with height intermediate between that of the domains and fluid phase, (2) membrane defects and (3) areas of a lower DOPC-rich phase that were devoid of domains. This work raised questions on the effects of enzyme concentration and exposure time and indicated that both enzyme activity and subsequent lipid reorganization contribute to the observed changes in membrane morphology. In order to address these issues, we have examined the enzyme-mediated membrane restructuring using one and

two-color TIRF microscopy to follow the bilayer reorganization over a wider range of time and length scales and enzyme concentrations than can be readily done by AFM, and correlated AFM and fluorescence imaging to better understand the membrane restructuring.

3.1. TIRF imaging of *in situ* ceramide generation in DOPC/ESM/cholesterol bilayers

Bilayers were prepared from vesicles of a 2:2:1 DOPC/ESM/cholesterol lipid mixture containing 0.5% TR-DHPE. Vesicles were incubated in the TIRF cell at a higher temperature and longer time than in our previous AFM study of bilayers prepared from this lipid mixture, a procedure that gives slightly larger domain sizes that can be more easily visualized by fluorescence microscopy. Fig. 1A shows a typical TIRF image for such a bilayer. TR-DHPE partitions strongly into the fluid phase with the liquid-ordered domains consequently appearing as micrometer-sized dark spots in the bright fluid phase.

The effects of incubation with *S. aureus* SMase were followed by TIRF imaging of the same area of a bilayer at selected time points after enzyme addition. The images in Fig. 1B–H show the results of a typical experiment in which 1 U/ml SMase was added and the bilayer was imaged over a period of 70 min. The images from time zero to 30 min are all on the same intensity scale and indicate modest bleaching ($\sim 30\%$ decrease in intensity) of the dye due to repeated irradiation over the time course of this experiment. A control experiment for bilayers in the absence of SMase indicates a similar level of bleaching but no other changes in the overall size or arrangement of the initial domains over a period of 1 h.

Changes in the bilayer are evident as early as 1 min after addition of SMase; selected areas of the bilayer are outlined and numbered in images before and 30 min after SMase addition in order to illustrate these changes. As shown in Fig. 1B, after 1 min a relatively large dark region that excludes dye appears in area 1. This dark patch appears to form by disappearance of the dye-labeled phase between and around two pre-existing domains in image 1A and continues to grow in size by both expansion of the dye-excluded region and incorporation of adjacent dark domains (images C–E). This behavior is typical of other regions, for example those marked 2, 3, and 4; in each case the dark area around existing domains expands until individual small domains coalesce to give larger dark patches. By contrast, there are domains that show little, if any, change over the same time period, as for example the domains in regions 5, 6, and 7. Occasionally, we observed formation of new patches at later times that also expanded during the first 5 min after their appearance (data not shown). As shown in Fig. 1F–H, the dark patches that form during the first 10 min after enzyme addition continue to increase slowly in size and some merge with other dye-excluded areas to form larger dark patches. The number and size of the dye-excluded areas vary with the area imaged and between independently-prepared samples.

The evolution of the bilayer over the first 30 min is shown in Fig. 1I, which plots the total dye-excluded area for marked regions of the images, and in the expanded view of the data at early times in Fig. 1J. Note that the increase in area is due to formation of new dye-excluded regions, whereas the initial value (at time zero) reflects the total area of the original dark domains (before enzyme addition) within specific regions of the bilayer. These plots show clearly the three distinct types of behavior described above: (1) some areas show no increase in size of the initial domains up to a period of 30 min after enzyme addition; (2) some regions exhibit rapid expansion of the dye-excluded region around one or more initial domains at early times and (3) most of the dark regions that

Fig. 1. Following the SMase mediated bilayer changes in real time with TIRF microscopy. A DOPC/ESM/cholesterol (2:2:1) bilayer with 0.5% TR-DHPE was imaged before (A) and after (B–H, times indicated) injection of 1 U/ml SMase. Images A–F are on the same intensity scale (max 2700) and the maximum intensity has been reduced to 2000 for images G and H for clearer visualization of the membrane morphology. The increase in the dye-excluded area for each numbered region in images A and F is plotted as a function of time after enzyme injection in (I). The plot in (J) shows an expanded view of the data at early times. The intercept at time zero for each region corresponds to the area covered by the domains prior to enzyme addition.

expand rapidly in the first few minutes show a slower increase in the area of dye-excluded patch at later times. The rapid initial change in the amount of dye-excluded area occurs within 2–3 min, with variable slopes for the various regions analyzed. After approximately 4 min the rate of growth of the dye-excluded patches decreases dramatically and then continues to increase at a much slower level. The change in area is only plotted for 30 min since the boundaries of many of the dark patches eventually overlap to give a single larger patch.

In order to examine whether the membrane changes at later times required the presence of enzyme, a bilayer was incubated with 1 U/ml SMase for 15 min and then washed extensively with water to deactivate and remove the enzyme. The bilayer was imaged before enzyme treatment, during the SMase incubation and at several time points after washing (Supporting Information, Fig. S1). The dark patches formed by enzyme activity during the 15 min incubation were similar to those shown at early time points in Fig. 1. After the bilayer was washed to remove SMase, the sizes of individual dark patches continued to increase slowly in a qualitatively similar manner to experiments for unwashed bilayers. Consistent with this result, the size of dye-excluded regions also continued to increase slowly when EDTA was added to bilayers after a short incubation with enzyme. By contrast, bilayers that were incubated with enzyme solution plus EDTA showed no change over a period of approximately 1 h, as expected based on the known requirement for Mg^{2+} for enzymatic hydrolysis (Goni and Alonso, 2002).

3.2. Effect of SMase concentration

SMase concentrations that vary by three orders of magnitude were examined to probe the effect of enzyme concentration on ceramide generation and membrane reorganization. A time course experiment for a bilayer incubated with 0.1 U/ml SMase (Fig. 2) gave qualitatively similar results to those in Fig. 1; in both cases large dye-excluded patches appear and increase in area and there are domains that do not change significantly in size (see regions outlined in Fig. 2A and F and plots in Fig. 2I and J). However, the kinetic data in Fig. 2I and J indicate a significant lag period (~10 min) before the first detectable changes to the bilayer for the lower enzyme concentration, whereas changes were observable within the first minute for 1 U/ml SMase. Although the time lag varied from experiment to experiment, 0.1 U/ml SMase consistently led to longer time lags than 1 U/ml SMase. The initial slopes of the area vs time graphs show that each dye-excluded patch expands at a different rate.

Treatment of bilayers with 0.01 U/ml SMase from the same stock used for Fig. 2 showed no evidence for SMase-induced changes by TIRF up to 45 min after enzyme addition. In other cases, the growth of dye-excluded patches was observed after addition of 0.01 U/ml SMase, but after longer lag periods than for the higher concentrations. The variable results may reflect problems with reproducible addition of small enzyme concentrations and adsorption of enzyme to the cell walls at low SMase concentrations. Enzyme-induced changes to DOPC/ESM/cholesterol bilayers were

Fig. 2. Following the SMase mediated bilayer changes in real time with TIRF microscopy. A DOPC/ESM/cholesterol (2:2:1) bilayer with 0.5% TR-DHPE was imaged before (A) and after (B–H, times indicated) injection of 0.1 U/ml SMase. All areas are displayed on the same intensity scale. The increase in the dye-excluded area for the numbered regions in images A and F is plotted as a function of time after enzyme injection in (I), with an expanded view of data at early time in (J).

observed by AFM for 0.01 U/ml using SMase from a different bacterial source (*Bacillus cereus*) in our earlier study (Ira and Johnston, 2006). However, the threshold level of ceramide required to detect changes in membrane morphology may be different for AFM and TIRF.

3.3. Two-color TIRF microscopy to probe changes to domains and fluid phase

TIRF experiments using Texas Red-DHPE to label the fluid phase provide no information on either the identity of the new dye-excluded patches or the fate of the initial domains in these regions. For example, it is not possible to ascertain whether the dark patches represent defects or new bilayer regions that exclude TR-DHPE, nor if the initial domains disappear. Two alternate approaches were employed to address these questions, as outlined below and in the following section. First, two-color, double-label TIRF experiments were used to probe changes in both domains and fluid phase. As noted recently, it is difficult to find a dye-labeled lipid that will reliably partition into liquid-ordered domains and is compatible with excitation at >480 nm (Baumgart et al., 2007). However, GM1 has been shown to localize in the liquid-ordered phase for a variety of ternary lipid mixtures and can be easily detected by labeling with cholera toxin (Burns et al., 2005; Chiantia et al., 2008; Hammond et al., 2005; Shaw et al., 2006; Yuan et al., 2002). We prepared bilayers from DOPC/ESM/cholesterol vesicles containing 1% GM1 and used binding of Alexa 647 labeled cholera toxin subunit B (Ctx-B) to visualize the protein-labeled ganglioside by both fluorescence and AFM. Fig. 3A shows a bilayer containing 1% GM1 stained with TR-DHPE (pseudo color green) and Fig. 3B

the same area after Ctx-B binding (Alexa 647 channel, pseudo color red). Fig. 3C gives the merged image for TR-DHPE and Alexa 647 channels after Ctx-B binding. Ctx-B binds predominantly to the domains, consistent with localization of GM1 primarily in the liquid-ordered phase. No change in the bilayer was observed over a period of 1 h after addition of protein. There was no detectable fluorescence at 647 nm after incubation of a control bilayer (DOPC/ESM/cholesterol without GM1) with Ctx-B, indicating minimal non-specific protein binding.

DOPC/ESM/cholesterol bilayers labeled with both TR-DHPE and GM1/Ctx-B were incubated with SMase in order to visualize changes to both the fluid phase and the domains during enzyme-mediated bilayer restructuring. Selected time-points of a typical series of fluorescence images for a Ctx-B labeled bilayer before and during incubation with 1 U/ml SMase are shown in Fig. 3. Changes in the bilayer are evident as early as 1 min after SMase addition (Fig. 3D–F); a small dark patch forms at the lower right corner of the image and during the next 3 min numerous additional small dark patches appear (see arrows in image 3E). Between 4 and 10 min, there is little change in the bilayer with only slight increases in area of the existing dark patches and no new patches observed. The combination of large and small dark patches is typical of multiple areas and several independent samples. In all the cases the dark patches are in the TR fluorescence channel whereas the Ctx-B labeled domains are unchanged. Furthermore, most of the dark patches start adjacent to the red domains and expand outward, similar to the observations for samples where only the fluid phase is labeled (Figs. 1 and 2). Photobleaching of the relatively immobile Ctx-B–Alexa 647 made it difficult to follow the SMase reaction at longer times.

Fig. 3. TIRF images for a DOPC/ESM/cholesterol (2:2:1) bilayer with 1% GM1 and 0.5% TR-DHPE before (A, TR channel, green) and after labeling with Ctx-B–Alexa 647 conjugate (B, Ctx-B channel, red). (C) A merged image showing both TR-DHPE (red) and Ctx-B (green) channels after Ctx-B labeling. Ctx-B binds predominantly to the liquid-ordered domains that exclude TR-DHPE. (D, E, F) TIRF images for the same bilayer after treatment with SMase. The arrows in image E show several new dye-excluded areas.

The enzyme-induced changes visualized by TIRF microscopy are qualitatively similar for bilayers with and without GM1–Ctx-B. The effect of protein binding was next examined in more detail by AFM imaging of bilayers with Ctx-B labeled domains before and after incubation with SMase. Fig. 4A and B show a typical bilayer containing 1% GM1 before and after labeling with Ctx-B; the liquid-ordered domains increase in height by ~ 4 nm and become more heterogeneous after addition of Ctx-B, as shown by the histograms in Fig. 4D and E. The broad height distribution for Ctx-B bound domains reflects a non-uniform distribution of protein, consistent with observations of GM1 islands in a variety of ordered membrane phases (Vie et al., 1998; Yuan et al., 2002; Yuan and Johnston, 2001).

Fig. 4C shows the same bilayer after treatment with 1 U/ml SMase for 15 min, followed by washing. Ceramide generation resulted in formation of higher regions around the outer edges of the Ctx-B bound domains, with a broad height distribution (Fig. 4F). However, there was no evidence for rearrangement of the membrane to give new raised regions around the domains or areas of lower phase from which domains had disappeared, both of which were observed in our earlier study of bilayers without GM1–Ctx-B (Ira and Johnston, 2006). An additional control experiment was performed to test whether protein or GM1 was responsible for these differences. A bilayer containing 1% GM1 was incubated with 1 U/ml SMase for 15 min prior to washing and

Fig. 4. AFM images of a DOPC/ESM/cholesterol (2:2:1) bilayer with 0.5% TR-DHPE and 1% GM1 before (A) and after (B) labeling with Ctx-B. (C) shows the same bilayer after 15 min incubation with 1 U/ml SMase. The z-scale is 5 nm for A and 10 nm for B and C. Distribution of domain heights obtained for two independent experiments are shown as histograms: (D), untreated bilayers; (E), Ctx-B labeled bilayers; and (F), Ctx-B labeled bilayers treated with SMase. The data were fit to 1 or 2 gaussians and peak values of the Gaussian fits are indicated in Å.

imaging by AFM (Fig. S2). After enzymatic ceramide generation, the domains were heterogeneous with raised subdomains on their edges and in the interior and there were relatively large regions of bilayer that were devoid of domains, confirming that enzyme activity was not modified by inclusion of GM1 in the bilayer. Thus, the AFM data provide qualitative evidence for reduced membrane restructuring when domains are pre-labeled with protein. This observation, plus difficulties in interpreting the changes in domain heights induced by enzyme in the presence of Ctx-B, prompted us to use correlated imaging to further elucidate the nature of the membrane restructuring visualized by TIRF microscopy.

3.4. Correlated AFM and fluorescence imaging of restructured bilayers

The variability of the membrane morphology after enzymatic generation of ceramide requires that one correlate optical and AFM images for the same area during the bilayer restructuring. The results of a correlated imaging experiment using an integrated AFM-inverted optical microscope are shown in Fig. 5 and Fig. S3. Before enzyme addition, both fluorescence and AFM images show the same distinctive pattern of domains, while smaller scale AFM images recorded in height and lateral deflection modes indicate that the domains and the surrounding fluid phase are uniform (Fig. S3). Both the domain shape and size and the height difference between domains and fluid phase are similar to our previous results (Ira and Johnston, 2006).

SMase (0.3 U/ml) was added to the bilayer and fluorescence images were recorded over a period of 37 min; large dye-excluded regions similar to those observed by TIRF appeared during the first minute and slowly increased in size (data not shown). The bilayer was then washed before obtaining correlated AFM and fluorescence images of the membrane; note that a different area is imaged after enzyme treatment. The fluorescence image (Fig. 5A) shows many small dark domains that are qualitatively similar to those observed before enzyme addition, plus a number of larger dye-excluded regions. AFM images (Fig. 5B–F) of a region

within the fluorescence image confirms that the small raised domains are similar in size and shape to those in the original bilayer but are heterogeneous in height, in agreement with our earlier AFM study which had assigned the raised domains to ceramide-rich sub-domains (Ira and Johnston, 2006). The centre of the domains is 1 nm higher than the fluid phase and the edges are 2–3 nm higher than the interior of the domains (Fig. 5D and E). In addition to the domains with raised edges, the topographic images have a number of irregularly-shaped raised areas that are ~1.2 nm above the fluid phase and that correspond to the larger dye-excluded regions in the fluorescence image (several of these areas are marked with arrows in Fig. 5A–C). Interestingly these new raised regions are more clearly visible in the lateral deflection AFM images (Fig. 5C and F); the lateral deflection of the cantilever is a measure of the friction between the tip and sample and can thus show differences in the physical properties of the sample that reflect variations in chemical composition. The domains, including the raised edges, are also detectable in the lateral deflection images, but show much lower contrast than the raised regions that exclude dye. In some areas, the new dye-excluded regions show occasional raised areas with size and shape consistent with the original domains (data not shown). The correlated images in Fig. 5 suggest that the large, dark, dye-excluded regions correspond to areas where original domains have been replaced with membrane regions that are higher than the original DOPC fluid phase; although their thickness is similar to that of the original domains, they clearly have a different composition based on the strong contrast in lateral deflection. Note that for Fig. 5, the AFM parameters have been optimized for lateral deflection imaging which requires higher force and may lead to compression of the fluid membrane regions. This may explain the height variability observed in the AFM images and the large height of the ceramide-rich regions in the domains. In previous work we have argued that flip-flop of ceramide to the lower leaflet may also partly contribute to the height observed for ceramide-rich regions (Ira and Johnston, 2008).

Fig. 5. Correlated AFM and fluorescence imaging of a DOPC/ESM/cholesterol (2:2:1) bilayer with 0.5% TR-DHPE after incubation with 0.3 U/ml SMase for 37 min, followed by washing to remove the enzyme. (A) shows a fluorescence image, with the area in the box corresponding to the region shown in the AFM images (B, topography; C, lateral deflection). Circles identify the same area in the three images. (D, E, F) Smaller scale topographic (D) and lateral deflection (F) images for a region in the centre of the larger AFM images, and a cross section for the line in image D, showing heights of the domains and the new raised regions. Arrows in the various images identify three regions that exclude dye, are approximately 1.2 nm thicker than the surrounding fluid phase and show increased friction in lateral deflection AFM scans.

To further probe the relationship between the original SM-cholesterol-rich domains and the new regions that exclude dye and have strong contrast in lateral deflection, we performed a correlated imaging experiment in which the same bilayer area was imaged before and after enzyme addition. The results are illustrated in Fig. 6; the mica substrate had several defects (see lines in the upper left corner of images A and B), thus facilitating the correlation of the AFM and optical images. Comparison of AFM images before (A) and ~55 min after (D) enzyme addition indicates that new regions of intermediate height (marked with arrows) have grown in around existing domains. These areas correspond to new dye-excluded regions in the fluorescence image recorded in the presence of enzyme (Fig. 6E). The effects of enzyme treatment on the domains are more clearly illustrated in AFM scans of smaller areas before and after SMase treatment (Fig. 6C and F). Examination of the region marked with the blue box demonstrates that the new irregularly-shaped raised areas are adjacent to the initial domains. Some of the initial domains are similar in size and shape after enzyme treatment whereas others have partly or completely disappeared. Inspection of the entire image indicates that it is frequently the smaller domains that show large changes in size or disappear, whereas the larger domains are similar in size but significantly changed in height after SMase incubation.

The same area of the bilayer shown in Fig. 6 was imaged after washing (Fig. 7). Note that the domain marked with an arrow in Fig. 7 is visible in the centre of the images in Fig. 6, confirming that the same area is imaged in both cases. The dye-excluded areas in the fluorescence image (Fig. 7A) have become considerably larger and no longer overlap with the new phase detected in the AFM topographic image (Fig. 7B), but do correspond well to the large regions of high adhesion in the lateral deflection image (Fig. 7C). Note that the larger patches observed after washing are similar to those observed at long times in the TIRF images shown in Figs. 1 and 2. The time required to wash the bilayer and find the same area for correlated fluorescence and AFM imaging makes it difficult to conclude how much of the change between Fig. 6D–F and Fig. 7

is due to washing vs continued evolution of the bilayer. However, this experiment demonstrates that the dye-excluded patches observed at various time points are bilayer regions that have a different composition, as indicated by the high lateral contrast, and that retain many of the initial domains.

An additional AFM experiment confirms that there are areas of lower phase that cannot be distinguished from the original fluid phase in topographic images of enzyme-treated bilayers, but which do show modified properties. Areas of bilayers encompassing both domain and fluid phase were scanned at high force before and after enzyme treatment, in attempts to create bilayer defects (Fig. S4). It was not possible to create a defect in the original bilayer because areas where parts of an ordered domain were removed rapidly re-filled with the lower fluid phase. By contrast, defects could be maintained in enzyme-treated bilayers, indicating that the area adjacent to the domains had a significantly decreased fluidity, although there was no detectable change in height from the original fluid phase.

4. Discussion

4.1. Membrane restructuring

A combination of TIRF and AFM has been used to investigate the bilayer restructuring induced by enzymatic generation of ceramide in bilayers with co-existing fluid and liquid-ordered domains. The two techniques are complementary with TIRF allowing detailed time course studies and examination of multiple large regions of the bilayer, whereas AFM provides more detailed information on the nanoscale organization. TIRF experiments using Texas Red-DHPE to label the fluid phase show the formation of dye-excluded regions around or adjacent to the initial ordered domains. Our initial hypothesis was that these dye-excluded regions corresponded to the raised regions that we assigned to a new bilayer phase in an earlier AFM study (Ira and Johnston, 2006). Nevertheless, the complexity of the membrane restructuring makes it problematic to ex-

Fig. 6. Correlated AFM and fluorescence imaging of a DOPC/ESM/cholesterol (2:2:1) bilayer with 0.5% TR-DHPE before (A–C) and after (D–F) treatment with 0.3 U/ml SMase. The AFM and fluorescence images shown after enzyme incubation were recorded at ~55 (D), ~65 (F) and ~70 (E) minutes after addition of enzyme. The regions circled in green indicate the same area in the individual images. (C, F) show AFM images of a small region near the centre of the larger images, with a boxed region to draw attention to one area where new raised regions have grown in and some of the original domains have become smaller or disappeared. Blue arrows show areas of fluid phase before enzyme treatment (empty arrows) where a new raised region that excludes dye appears after enzyme treatment (solid arrows).

Fig. 7. Fluorescence (A) and AFM (B, topography; C, lateral deflection) images of the bilayer shown in Fig. 6, after washing to remove the enzyme. The bilayer was washed immediately after recording the image in Fig. 6E and the images shown here were recorded between 10 and 60 min later. Although the bilayer morphology in the dye-excluded regions may change slightly during this time period, the original domains retain their original shape and location. Some regions that show up as higher areas in the topographic images and have enhanced contrast in the lateral deflection images are outlined in green in B and C; a common feature in AFM and fluorescence images is outlined in blue. An arrow marks a large domain with a distinctive shape that is also visible in the centre of the images in Fig. 6.

clude other possibilities, such as membrane defects, based solely on comparison of TIRF and AFM imaging of different samples. However, correlated AFM and fluorescence images provide unequivocal evidence that the new dye-excluded patches formed by enzyme activity correspond to bilayer areas of increased height (1.2 nm above the surrounding fluid phase) that are larger and have more irregular boundaries than the original domains and exhibit increased adhesion in lateral deflection scans. These regions either form around existing domains which largely retain their original shape and size, but are heterogeneous in height, or appear in areas from which the original domains have completely or partly disappeared. Interestingly, the dye-excluded areas cannot always be detected in topographic images although they are clearly visible in lateral deflection scans.

Two-color TIRF experiments in which the liquid-ordered domains were labeled with GM1–Ctx-B indicated that the domains remained intact during the growth of the dye-excluded regions. However, this experiment is complicated by effects of protein, based on the qualitative observation of reduced membrane reorganization in AFM experiments for bilayers in which domains were labeled with GM1–Ctx-B and the disappearance of some domains in the correlated imaging experiments. Control experiments for enzyme-treated bilayers with and without GM1 indicated that the changes are due to the presence of Ctx-B, not GM1. The reduced bilayer reorganization in the presence of Ctx-B may reflect restricted access of the enzyme to its SM substrate, resulting in lower ceramide production and a concomitant decrease in membrane restructuring. The mobility of individual lipids may also be lower in the presence of Ctx-B, which will reduce the ability of the membrane to rearrange in response to ceramide generation. In this regard, a recent study has shown that Ctx-B partitions strongly into ceramide-rich regions of liquid-ordered domains when ceramide is incorporated in a ternary lipid mixture prior to vesicle and bilayer formation (Chiantia et al., 2008).

In our earlier study (Ira and Johnston, 2006), we suggested that the new raised areas that formed around existing domains resulted from expulsion of cholesterol from the domains into the DOPC-rich fluid phase in response to ceramide generation. These conclusions are consistent with reports that ceramide incorporation leads to displacement of cholesterol from ordered domains in both model membranes and cells (Ali et al., 2006; Chiantia et al., 2006, 2007; Megha et al., 2006; Megha and London, 2004; Yu et al., 2005b) and with the reported ability of ceramide to substitute for cholesterol in liquid-ordered phases (Carrer et al., 2006; Silva et al., 2007). However, the correlated imaging results suggest that the membrane restructuring initiated by SMase may be more complex than simple loss of cholesterol from a ceramide-rich domain. In

some areas, the new raised regions form a “platform” around pre-existing domains and it is possible that this is largely due to displacement of cholesterol from the domains as ceramide-rich islands form. This is consistent with the expected increased thickness and decreased fluidity of the DOPC-rich fluid phase upon cholesterol addition. In other areas some of the original domains disappear completely, leading to new raised regions that have higher adhesion and also exclude dye. We hypothesize that the cholesterol and ceramide/SM from the original domains must be incorporated in the original DOPC phase, since we see no evidence for significant enlargement of adjacent domains or of defects caused by loss of material from the bilayer surface. One would not expect strong interaction between cholesterol and ceramide; although one could speculate that the two are segregated in different areas of the new dye-excluded regions, we have no direct evidence for this. Exclusion of Texas Red from ceramide-enriched regions is expected based on observations that dye-labeled lipids have very low affinity for ceramide-enriched bilayer domains (Chiantia et al., 2008; Sot et al., 2008).

Although the present results do not lead to definitive conclusions on the composition of the new dye-excluded regions, it is likely that these regions have a range of lipid compositions and are not at equilibrium. This is based on the continued slow evolution of the bilayer morphology after enzyme removal and the observation that some areas of dye-excluded phase do not show a clear height difference from the original DOPC phase, but do exhibit differences in adhesion and fluidity. Since both ceramide and cholesterol are expected to lead to an increased order/thickness of the DOPC-rich phase, it is likely that the regions with higher adhesion but no detectable height change have a lower overall concentration of additional material in the DOPC phase than do areas that are both thicker and have higher adhesion. We believe that the different lipid compositions for the dye-excluded regions are caused by varying levels of enzyme activity in specific areas of the bilayer, as discussed in more detail below. This spatial variation in the enzyme concentration leads to significant differences in the amount of SM hydrolyzed and to varying levels of ceramide and cholesterol incorporation in the surrounding DOPC-rich phase. The initial domain size may also modulate the extent of membrane restructuring since lipids in smaller domains may more rapidly migrate into the surrounding fluid phase after ceramide generation.

There are two additional factors that could contribute to some of the heterogeneity in the enzyme-restructured bilayers. The first is ceramide chain heterogeneity (ESM is 84% C₁₆ and 16% longer saturated chains); the ability of ceramides to stabilize liquid-ordered phases and to form gel phase domains has been shown to depend on chain length (Chiantia et al., 2007; Megha et al., 2007;

Pinto et al., 2008). The second is the asymmetric distribution of ceramide between bilayer leaflets; ceramides have a strong propensity to undergo inter-leaflet flip-flop, and we have previously suggested that negative membrane curvature induced by flipping of ceramide to the lower leaflet may contribute to the apparent height of the ceramide-rich domains (Ira and Johnston, 2008). The complex membrane morphology illustrates the necessity of employing multiple imaging modes for understanding enzyme-mediated reorganization, even in a relatively simple bilayer with only three initial lipid components. We have recently shown that chemically-selective imaging using time-of-flight mass spectrometry can be used to identify ceramide-rich domains in supported monolayers (Popov et al., 2008) and a similar approach could be used to probe the lipid composition of enzyme-restructured bilayers. A detailed study of the incorporation of ceramide in DOPC/SM/cholesterol bilayers using a combination of lateral and topographic imaging and force spectroscopy will also aid in understanding the effects of ceramide incorporation.

The correlated imaging results allow us to rationalize some puzzling observations from our earlier study of the effects of SMase on bilayers prepared from DOPC/ESM mixtures with 9% cholesterol (Ira and Johnston, 2008). We had observed apparently homogeneous regions of the bilayer (i.e., no domains) that were similar in height to the original DOPC fluid phase, but exhibited a decreased fluidity based on their ability to maintain bilayer defects. At the time the lack of a detectable height change for an apparently new, less fluid region of the membrane was puzzling. However, the additional information available from correlated fluorescence and AFM, including lateral deflection imaging, now makes it easier to rationalize these results.

4.2. Kinetics of membrane restructuring

Studies of the mechanism and kinetics for SMase hydrolysis of SM have concluded that the enzyme binds with high affinity to SM vesicles and remains tightly bound to the interface during catalytic turnover in the so-called scooting mode (Yu et al., 2005a, 2002). As a consequence, equilibration of enzyme between vesicles is slow compared to establishment of steady state kinetics (Yu et al., 2005a, 2002). Similarly, studies of SMase activity for monolayers at the air–water interface have concluded that all interfacial-activated enzyme is associated irreversibly with the interface (Fanani et al., 2002; Hartel et al., 2005).

The kinetics for the growth of the dye-excluded areas observed by TIRF were analyzed by plotting the normalized area increase as a function of time. The area increase is normalized to the initial domain area (before enzyme addition), which provides an estimate for the initial amount of SM-containing phase for each region analyzed. An analogous approach was employed recently to examine the activity of several lipases by using AFM to follow the formation

of bilayer defects created by enzymatic lipid hydrolysis (Balashev et al., 2007, 2003; Grandbois et al., 1998). The plots in Fig. 8A show an initial fast increase in dye-excluded area followed by a plateau region where the area increases at a much slower rate. These are assigned to a rapid and constant rate of enzymatic activity at early time, followed by a sharp decrease in activity that is due to product buildup and/or decreased substrate accessibility. Both the initial slopes and the plateau values are different for each region; for example, the slopes for the plots in Fig. 8A vary by a factor of approximately 50. This indicates a variable number of active enzymes per unit area of SM-rich ordered phase for various regions of the bilayer. Similar stochastic behavior has been observed in a previous AFM kinetic study of PLA₂ (Balashev et al., 2007). The experiments with 0.1 U/ml SMase also show different initial slopes and plateau values for various bilayer regions (Fig. 8B), although the difference in slopes is much smaller, a factor of approximately four, and there is a longer lag time before the initial rapid expansion of dye-excluded area. Several interfacial enzymes exhibit comparable behavior, with the lag time attributed to factors such as the promotion of lateral phase separation and the requirement for an interfacial activation step.

Both the number of active enzymes/area analyzed and the number of molecules of SM hydrolyzed are required to calculate a hydrolysis rate from the observed area increase. As noted above, we attribute the increase in area to a sequence of events in which the initial production of ceramide by SM hydrolysis results in movement of cholesterol and/or ceramide into the DOPC-rich fluid phase with a concomitant loss of Texas Red-labeled lipid. Thus, the increase in dye-excluded area cannot be directly related to the extent of SM hydrolysis. The situation is different for SM monolayers where changes in surface area due to enzymatic production of the smaller ceramide molecule are proportional to the extent of SM hydrolysis (Fanani et al., 2002; Hartel et al., 2005). Previous studies of PLA₂ have employed conditions where initial bilayer changes were attributable to single enzymes (Balashev et al., 2007). We see no evidence for this at low enzyme concentrations, where we do not observe significant membrane restructuring by TIRF. This suggests that the changes that we detect by TIRF, and to a lesser extent by AFM, require a threshold level of ceramide.

The initial rapid expansion of the dye-excluded patches levels off within 2–3 min and is followed by a subsequent slower change in area. There are two possible explanations for the second slow increase in area. The first is that there is slow exchange of enzyme between various bilayer regions after SM depletion and/or product accumulation has caused the initial rapid rate of enzyme activity to drop significantly. The second possibility is that the bilayer continues to re-organize after enzyme activity has stopped. Consistent with this, we observed changes in bilayer morphology, even after samples have been extensively washed or treated with EDTA to remove or deactivate the enzyme.

Fig. 8. Increase in area of dye-excluded regions in Figs. 1 and 2 (A and B, respectively) after normalization to initial domain area. The numbers on the plots correspond to the regions shown in Figs. 1 and 2. The different slopes and plateau values for each region indicate activity of variable numbers of enzymes.

4.3. Summary

In conclusion, the combination of AFM and fluorescence imaging provides a detailed understanding of the structural reorganization initiated by *in situ* enzymatic generation of ceramide in supported lipid bilayers with initial co-existing fluid and liquid-ordered phases. The membrane restructuring has two main features. The first is the formation of heterogeneous domains with many small ceramide-enriched gel domains that are readily visualized by AFM. The second is a larger scale reorganization of the membrane that results in formation of new regions that exclude dye. In some cases these new areas form around the modified domains, whereas in other cases the domains have completely disappeared. We hypothesize that the new dye-excluded regions vary in composition for different membrane areas and are formed by incorporation of cholesterol and/or ceramide into the original DOPC-rich fluid phase. Detailed TIRF studies indicate that variable numbers of enzyme molecules are active in specific regions of the bilayer and also indicate that there is continued lipid reorganization after enzyme activity is stopped. Both of these observations are consistent with the variable properties of the new dye-excluded regions. The results presented here and in our earlier work clearly indicate that membrane restructuring is very sensitive to the bilayer composition and domain size. This is relevant to enzymatic generation of ceramide in cell membranes where subtle changes in membrane composition may lead to markedly different responses to ceramide production. Finally, the results highlight the utility and complementarity of correlated AFM and fluorescence imaging for *in situ* imaging of enzyme-induced changes in supported membranes.

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Appendix A. Supplementary data

Four figures showing additional TIRF, AFM and correlated imaging experiments are provided as supporting information. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2009.03.014.

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