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Organization and synergistic binding of copine I and annexin A1 on supported lipid bilayers observed by atomic force microscopy

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ABSTRACT

The transduction of signals across the plasma membrane of cells after receptor activation frequently involves the assembly of interacting protein molecules on the cytoplasmic face of the membrane. However, the structural organization and dynamics of the formation of such complexes has not been well defined. In this study atomic force microscopy was used to monitor the assemblies formed in vitro by two classes of calcium-dependent, membrane-binding proteins that participate in the formation of signaling complexes on membranes – the annexins and the copines. When applied to supported lipid bilayers composed of 25% brain phosphatidylserine and 75% dioleyl phosphatidylcholine in the presence of 1 mM Ca^{2+} both human annexin A1 and human copine I bound only to specialized domains that appeared to be 0.5 to 1.0 nm lower than the rest of the bilayer. These domains may be enriched in phosphatidylserine and have a more disordered structure allowing probe penetration. Confinement of the binding of the proteins to these domains may be important in the process of concentrating other signaling proteins bound to the copine or annexin. The binding of the annexin promoted the growth of the domains and created additional binding space for the copine. This may reflect a general ability of annexins to alter membrane structure in such a way that C2 domain-containing proteins like copine can bind. Copine I formed a reticular lattice composed of linear elements approximately 45 nm long on the specialized domains. This lattice might provide a scaffold for the assembly and interaction of copine target proteins in signaling complexes.

1. Introduction

Hormones and neurotransmitters that act on cell surface receptors typically initiate a cascade of events that result in the transfer of information into the interior of the target cell. In many cases this "signal transduction" process involves sequential protein–protein interactions of high specificity. Receptor activation may also result in an increase in the concentration of calcium in the cytoplasm of the cell. The calcium ion activates calcium-binding proteins that may participate directly in the signaling cascade, or modulate the individual steps. A number of cytosolic proteins are recruited to the cytoplasmic face of the plasma membrane to participate in these interactions. These proteins might form reversible multi-protein complexes that are ordered in such a way as to maximize the transfer of information in the signal transduction process. These complexes might exist only briefly in time, or they might remain organized for some period of time after the initial signaling event and thereby alter the rates of response to subsequent signaling events. However, such signaling complexes have not been visualized at a molecular level of resolution. In this study atomic force microscopy (AFM) has been used to visualize the structure and dynamics of complexes formed in vitro by representatives of two important and ubiquitous classes of calcium-dependent, membrane-binding proteins that are involved in signal transduction at the cell membrane: the copines and the annexins [1–6]. Both of these classes of proteins have recently been demonstrated to recruit other signaling or membrane trafficking proteins to membrane surfaces [5,7].

Copine was originally identified in extracts of the important model secretory cell, *Paramecium tetraurelia* [4,6]. In contrast to either mammals or green plants, in which the majority of calcium-dependent, membrane-binding proteins are annexins, paramecium extracts contain only a single major membrane-binding protein, now called copine. Sequencing of this protein revealed that it binds to membranes through a pair of C2 domains, homologous to the domain of protein kinase C that binds calcium and phospholipids. EST and genomic sequencing databases revealed the presence of families of homologous proteins in plants, animals, and slime molds. The sequence of the C terminal half of the copine molecule shows a

Abbreviations: AFM, atomic force microscopy; DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine; HBS, HEPES-buffered saline [100 mM NaCl and 20 mM HEPES (pH 7.5)]; PS, l-α-phosphatidylserine

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distant relationship to the "A domain" (or von Willebrand domain) in the extracellular portion of integrins [8]. In the integrin protein family this domain is responsible for the binding of extracellular matrix proteins. A hypothesis suggested by this sequence similarity is that the copine A domain may be a site for protein–protein interactions. This was tested by screening for proteins that interact with the copine A domain in yeast two hybrid screens. A number of interacting proteins were identified and the ability of these proteins to interact directly with copine was tested in in vitro assays using recombinant proteins in which the interactions were found to be of high affinity [5]. Furthermore, the copines were found to be able to recruit the interacting proteins to phospholipid surfaces in vitro [5].

A notable characteristic of the binding partners identified for copines is that the majority are proteins that are involved in intracellular signal transduction pathways. These proteins could be grouped into several categories [5], including regulators of protein phosphorylation, transcription, ubiquitination/NEDDylation, cytoskeletal organization, and calcium-binding proteins. This extensive array of potential binding proteins suggests the copines may be involved generally in providing calcium regulation of intracellular signaling pathways. In addition, by virtue of binding to membranes as well as the target proteins, the copines may be able to specifically localize signaling pathway components to certain membranes in the cell. A biological role of copines in signaling is supported by the demonstration that expression of a dominant-negative copine construct that blocks the ability of copine to recruit target proteins to membranes was found to completely abrogate the ability of calcium to enhance signaling from the tumor necrosis factor alpha receptor in HEK293 cells [9]. Since there are multiple copines expressed in a given cell, and since the copines have both overlapping and unique specificities for given target proteins [5], it is intriguing to consider that a group of different copines might work together to assemble several components of a signaling pathway at a single membrane site. In the present study we address the ability of human copine I to form supramolecular structures on membrane surfaces that could potentially provide such a scaffold for signaling pathway components.

Similar to the copines, the annexins represent a family of calcium-dependent, membrane-binding proteins present in most eukaryotes [3]. Their interaction with membranes, however, is mediated by the unique annexin (or endonexin [10]) fold. Their N-terminal domains are sites for possible interaction with other proteins and in this way they could perform a function similar to that of the copines in recruiting soluble proteins to membrane surfaces in a calcium-dependent fashion. For example, recently the annexins have been shown to recruit the μ subunits of the clathrin assembly proteins to membranes in vitro [7]. Such an interaction in vivo might be important in conferring calcium sensitivity to clathrin coat assembly.

It has also been suggested that annexins or other proteins that bind acidic phospholipids might recruit proteins to membranes by altering membrane lipid organization [11–13]. Specifically, because the annexins promote the formation of membrane domains enriched in acidic phospholipids, the annexins could promote the binding of other proteins, such as C2 domain-containing proteins, that also have specific affinity for acidic phospholipids. However, such a mechanism is conceptually problematic since the annexins would be expected to compete with the other proteins for the same phospholipids. In the present study we demonstrate that domains created by human annexin A1 in supported bilayers extend beyond the "footprint" of the annexin and provide binding sites for the C2 domains of copine I. This synergistic action of the annexins may have broad importance in the assembly of membrane signaling or structural complexes involving calcium-regulated membrane-binding proteins such as copine, protein kinase C, or synaptotagmin.

2. Materials and methods

2.1. Proteins

Recombinant human copine I and human annexin A1 were expressed in yeast and isolated by calcium-dependent binding to lipid vesicles as previously described [5,14].

2.2. Supported lipid bilayers

Supported bilayers were prepared by the vesicle fusion technique [15–17]. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and brain l-α-phosphatidylserine (PS), obtained from Avanti Polar Lipids (Birmingham, AL) as chloroform stocks, were mixed as appropriate. The chloroform was evaporated under a stream of nitrogen gas, and the lipids were rehydrated overnight in water (from a Millipore water purification system) to give a total lipid concentration of 2 mg/ml. The lipid mixture was vortexed to produce large multilamellar vesicles, from which small unilamellar vesicles were prepared by sonication with a probe sonicater for 1 min. For the imaging of supported lipid bilayers, 50 μl of the vesicle suspension was added to 50 μl of HEPES-buffered saline (HBS) [100 mM NaCl and 20 mM HEPES (pH 7.5)], containing 1 mM $CaCl_2$. The resulting suspension was deposited onto freshly cleaved mica (12.7 mm diameter disks; Agar Scientific) fixed by epoxy (Aron Alpha type 102, Agar Scientific) to 15-mm steel specimen discs (Agar Scientific). After a 30-min adsorption on mica,

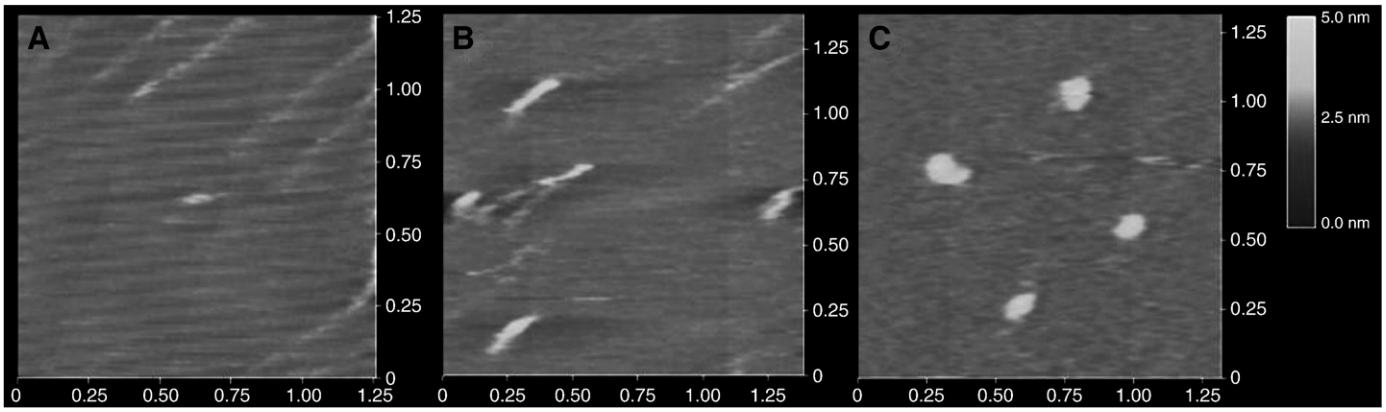

Fig. 1. Imaging copine multimers in a calcium-containing buffer. The copine (1 μg, final concentration approximately 10 μg/ml) was injected above a 25% brain PS/75% DOPC bilayer. In Panel A it is seen that the copine is not bound to the bilayer and is pushed ahead of the scanning probe. Increasing the scan rate allows the probe to over run the copine. Panel A, scan rate 2 Hz; Panel B, 4 Hz; Panel C, 8 Hz. Image size, 1.25 × 1.25 μm (zoomed with software from original 5 × 5 μm scan). Height scale, 0 to 5 nm.

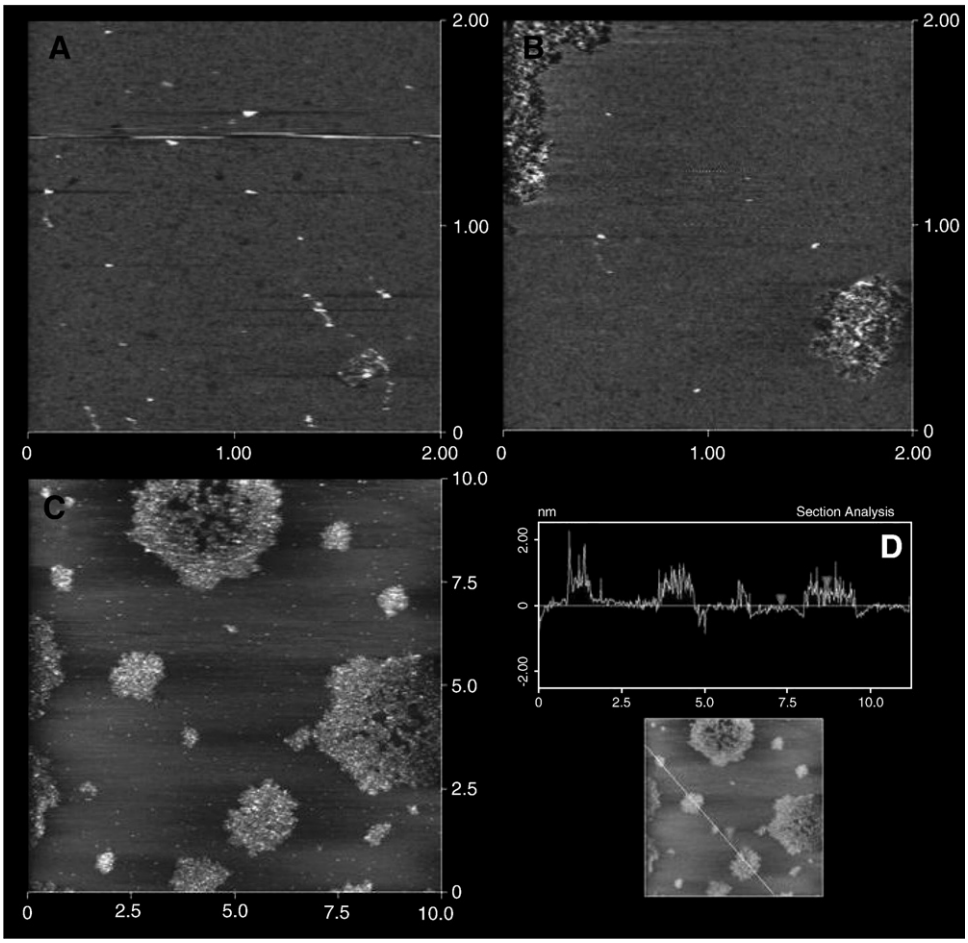

Fig. 2. Formation of copine assemblies on a 25% brain PS, 75% DOPC bilayer. Panel A: Only after 30 min of incubation were small patches of copine initially seen (lower right part of panel). Panel B: After another 20 min, the patch has expanded and a larger patch appears on the left part of the panel. Panel C: At lower magnification it is seen that at this time (50 min) a large portion of the bilayer is coated with copine patches. Panels A and B are the same area as seen in the bottom left portion of Panel C. Panel D: Section analysis of the image in Panel C reveals that the patches have a height of 0.5to 1.0 nm. The height scale for these images is the same as in Fig. 1 (0 to 5 nm). Panels A and B, scan size 2 × 2 μm; panel C, scan size 10 × 10 μm.

the sample was rinsed with HBS to remove unadsorbed liposomes and then transferred to the atomic force microscope. Proteins were imaged in HBS containing 1 mM $CaCl_2$ after injection above the bilayer in the fluid cell used for imaging. A 1 μl aliquot of protein at a concentration of 100 or 1000 μg/ml was injected into the approximately 100 μl volume of the cell, followed by 5 μl of buffer to push the bolus of

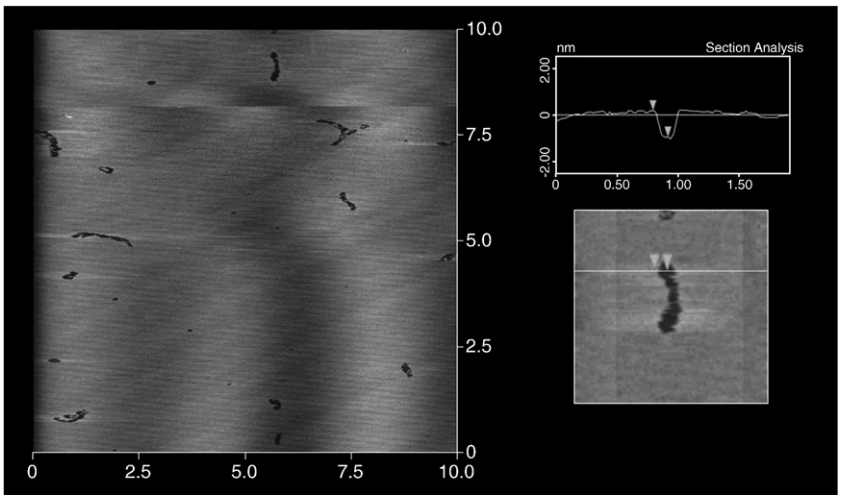

Fig. 3. Domains of apparent depth 1 nm present in a 25% brain PS, 75% DOPC bilayer. Section analysis of the domain at the top of the panel on the left is seen in the right panel, indicating a height difference of 1 nm. Scan size 10 × 10 μm, height scale 0 to 5 nm, as shown in Fig. 1.

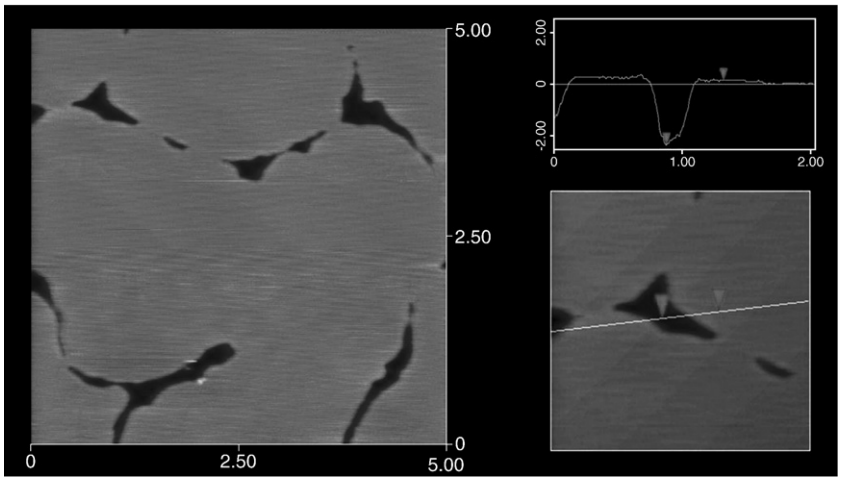

Fig. 4. Cracks in an incompletely formed bilayer of 25% brain PS, 75% DOPC. Lipids were washed off of the mica substrate after only 5 min of incubation, before a complete bilayer could form. These defects can be distinguished from the domains seen in Fig. 3 because of their greater depth, 2.5 nm as shown in the section analysis (on the right part of the figure) of the defect on the upper left of the left panel. This depth corresponds to the full bilayer thickness as measured with the probe used in this experiment (see Materials and methods). Scan size on the left, 5×5 μm; height scale 0 to 5 nm, as in Fig. 1.

protein through the dead volume of the injection port. The resulting final protein concentrations over the bilayer were approximately 1 or 10 μg/ml.

2.3. Atomic force microscopy

AFM imaging was carried out at room temperature (20 °C) using a Digital Instruments Multimode atomic force microscope equipped with an E-scanner and a Nanoscope IIIa controller with an in-line electronics extender module (Veeco/Digital Instruments, Santa Barbara, CA). Images of bilayers were collected using tapping mode in fluid with etched silicon probes (Mikromasch NSC18 with Cr–Au backing) with a typical resonant frequency in fluid of 30 to 35 kHz and a spring constant of 3.5 N/m. Images were captured continuously at a scan rate of 2 Hz (unless otherwise noted), with 512 scan lines per area. Typical scanning areas ranged from 2 by 2 μm to 10 by 10 μm. Some of the images in the figures in the Results section were magnified by software to give a higher magnification view, as indicated in the figure legends. Data analysis was performed using commercially or publicly available software (NanoScope III software, Digital Instruments; and Image J (NIH: http://rsbweb.nih.gov/ij/ for planimetry).

2.4. Probe characteristics

After completion of the experiments described in this report a direct comparison was made of the apparent bilayer thickness in a single sample of an incompletely formed bilayer when visualized using two different probes: a Veeco DNP-S silicon nitride probe and a Mikromasch NSC18 etched silicon probe. A bilayer thickness of 4 nm was seen with the Veeco probe, as previously reported [17], while the Mikromasch probe, used for the experiments described in this report, gave an apparent thickness of 2.5 nm. This may be an indication of partial penetration of the bilayer by the Micromasch probe which has a stiffer spring constant, 3.5 N/m, than the Veeco probe, 0.58 N/m. Such penetration may have enhanced the visualization of the PS-containing domains (see Discussion).

3. Results

3.1. Imaging copine in aqueous buffers on supported lipid bilayers

In order to visualize copine in association with a lipid bilayer, supported bilayers were obtained by the standard technique of vesicle

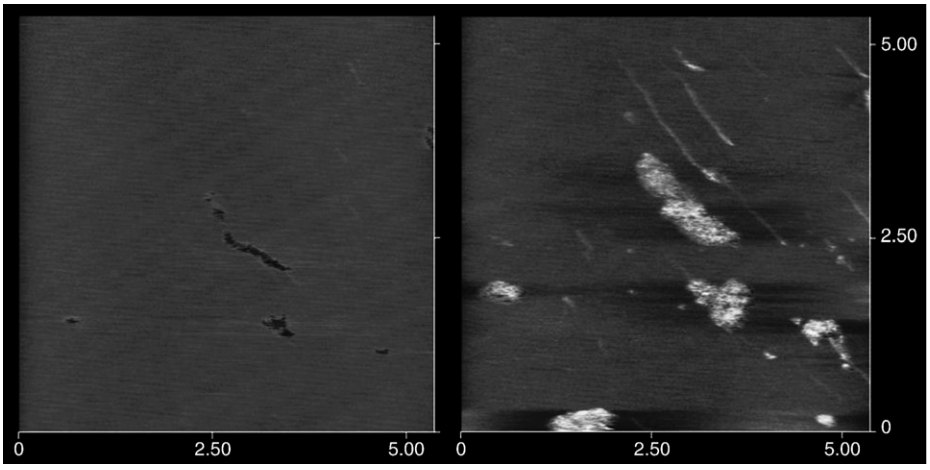

Fig. 5. Copine rapidly binds to domains of lower height in the supported bilayer. Left panel, small domains in a 25% brain PS/75% DOPC bilayer imaged immediately before addition of copine. Right panel, copine (1 μg, final concentration approximately 10 μg/ml) was added and a scan of the same area initiated from the top. The scan from top to bottom was completed in 4 min and 16 s. Some copine aggregates are pushed ahead by the probe, creating streaks. However, copine is also seen in patches coinciding with the positions and geometry of the domains seen on the left. The copine patches are larger than the domains, possibly because of growth of the domains during the binding of copine. The height scale for these images is the same as shown in Fig. 1 (0 to 5 nm). Scan size 5.3×5.3 μm.

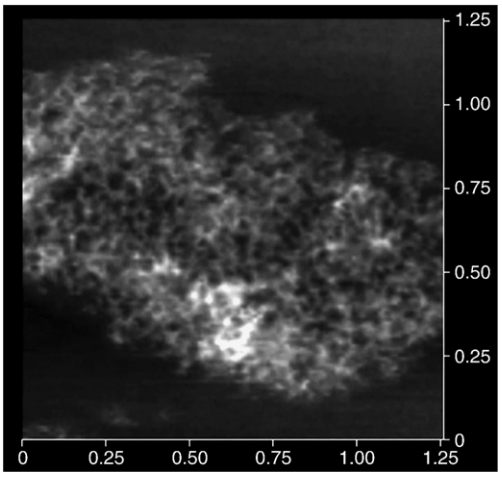

Fig. 6. Higher magnification image of a copine patch that has formed on top of one of the domains in a 25% brain PS/75% DOPC bilayer. The pattern is made up of linear elements that are about 45 nm long. The height scale for this image is the same as shown in Fig. 1 (0 to 5 nm). Image size 1.25×1.25 μm (zoomed with software from original 5×5 μm scan).

fusion [15–17]: suspensions of small unilamellar lipid vesicles composed of 25% brain PS and 75% dioleyl-PC (DOPC) were applied to a freshly cleaved mica surface in the presence of a buffer containing 1 mM Ca^{2+}. After 30 min at room temperature and washing away excess lipid, continuous bilayers were obtained that were generally featureless except for the presence of small areas of depression (described further below) which may represent compositional or organizational domains.

In the absence of membranes, calcium is known to promote the self association of isolated copine into larger, fairly monodisperse structures composed of approximately 10 monomers as detected by dynamic light scattering [18]. In regions where the bilayer was featureless, copine did not readily bind to the bilayer, even in the presence of 1 mM Ca^{2+}, but instead appeared to form the larger aggregates seen by dynamic light scattering from copine solutions. Streaks were observed as in Fig. 1A resulting from the scanning probe pushing copine particles ahead of it during the imaging process. However, in these cases, if the scanning rate were increased, then the particles could be imaged, as seen in Fig. 1C. When the scan rate is increased the frequency of vertical oscillation of the probe remains the same. Therefore, when the probe passes over a copine aggregate it strikes it a smaller number of times at the higher scan rate than at the lower scan rate. Therefore less total force is imparted to the particle and it is not swept in front of the probe. In this case the copine multimers appeared to be 2.16+/−.07 nm high, and 124+/−15 nm in diameter. These observations indicate that the copine did not bind to the membrane as rapidly as it associated with itself to form a larger particle, and that the larger particle did not have a high affinity for the bilayer. The observed height of the particles is likely to be artificially low because of deformation of the particle by pressure from the probe, as well as possible attractive interactions between the probe tip and the protein. The observed horizontal diameter of the particles greatly exceeds the hydrodynamic radius measured by

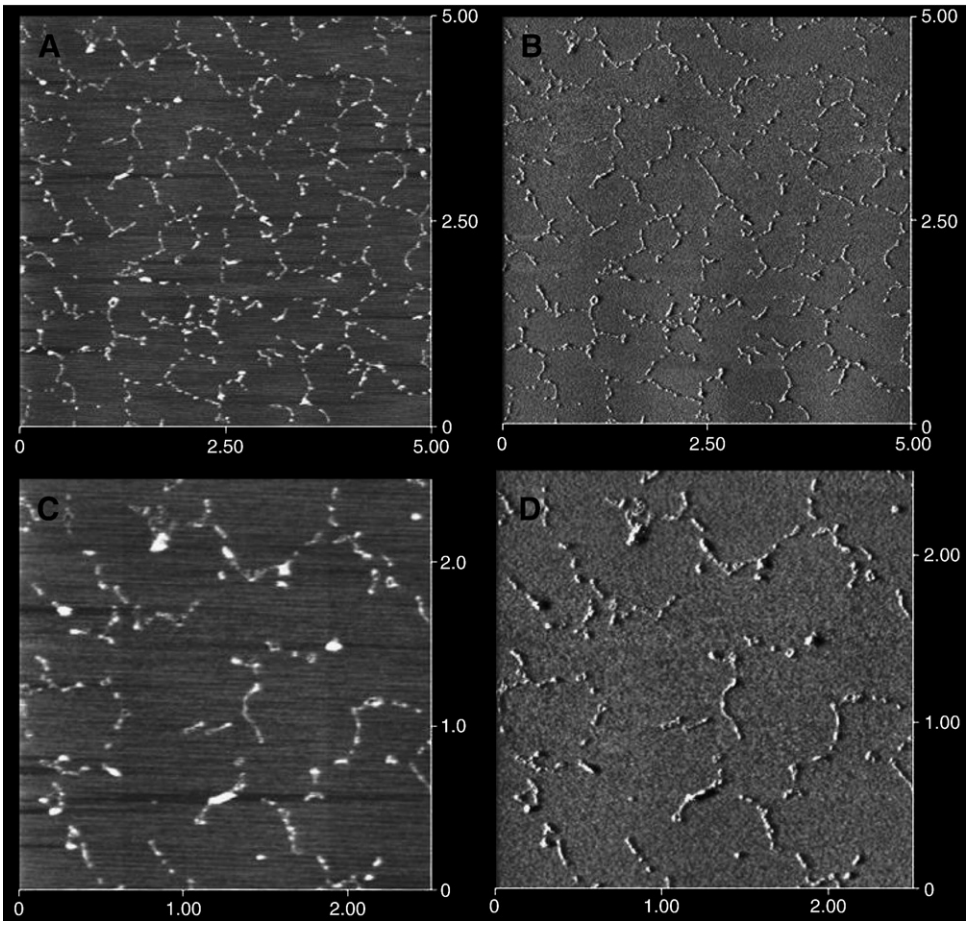

Fig. 7. Pattern of copine molecules that forms when the copine is preincubated with 25% brain PS/75% DOPC small unilamellar vesicles prior to vesicle fusion to form the supported bilayer. Panels A and B, lower magnification (scan size 5×5 μm), Panels C and D, higher magnification (2.5×2.5 μm, zoomed with software from original 5×5 μm scan). Panels A and C, height image, Panels B and D, phase image.

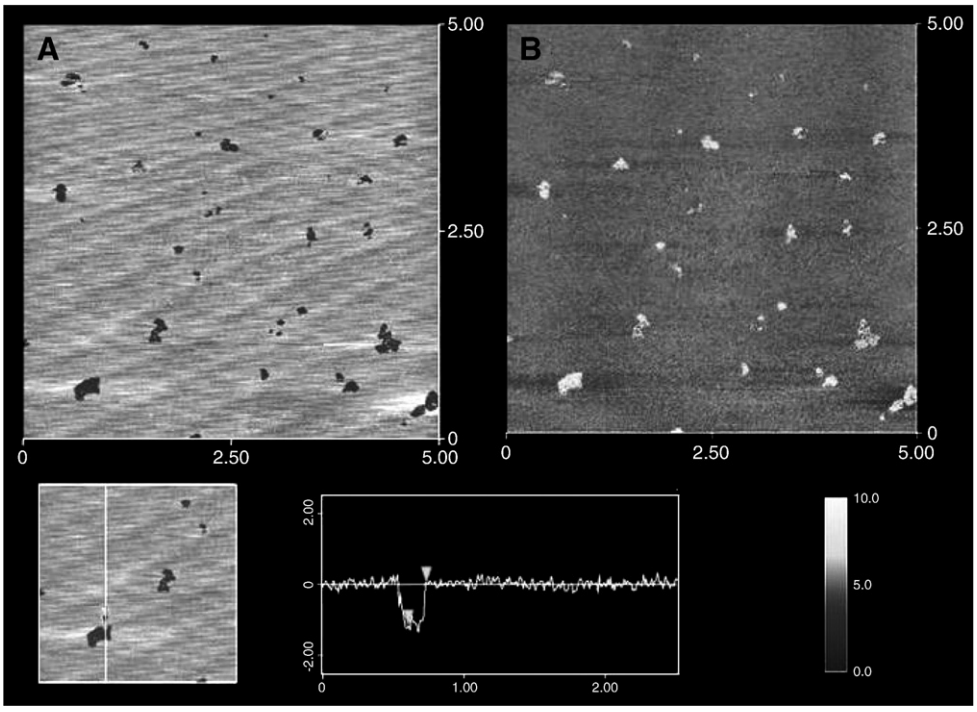

Fig. 8. Height and phase images of annexin-binding domains in a 25% brain PS, 75% DOPC bilayer. Panel A, height image, height scale as shown in Fig. 1, 0 to 5 nm. Panel B, phase image, phase scale on bottom right, 0 to10°. A cross sectional analysis of the height image of the domain at the bottom left of panel A is shown at the bottom of the figure. Domain depth, 0.8 nm. Scan size, 5×5 μm.

dynamic light scattering of 11.4 nm [18,19]. This is likely due to the finite radius of the probe tip (nominally 10 nm) as well as possible mobility of the particles during imaging. In the absence of calcium, copine could not be visualized, consistent with its failure to interact with membranes under these conditions [18].

Although the copine did not rapidly associate with the bilayer, after variable periods of time – 30 min in the experiment illustrated in Fig. 2 – some copine was typically seen to form patches on the bilayer that slowly expanded. The cross sectional analysis (Fig. 2D) suggests that the large multimeric structures formed by copine in solution did not bind the membrane, but instead reassembled so that the copine bound in patches with a height of 0.5 to 1.0 nm. The protein layer could actually be thicker if there is any insertion of the protein into the bilayer which does appear to occur with the C2 domains of synaptotagmin [20]. Single copine molecules were not seen bound to the membrane, suggesting the binding is a cooperative event that requires interactions between copine molecules on the bilayer to form a stable structure that can be imaged. In control experiments without lipids copine did not bind to the mica substrate in the presence or absence of calcium.

In the course of these studies it was found that the copine would bind immediately to the bilayer if there were specific domains in the bilayer that were manifest as regions that were 0.5 to 1.0 nm lower than the overall surface. Domains of this nature are seen in Fig. 3. Their

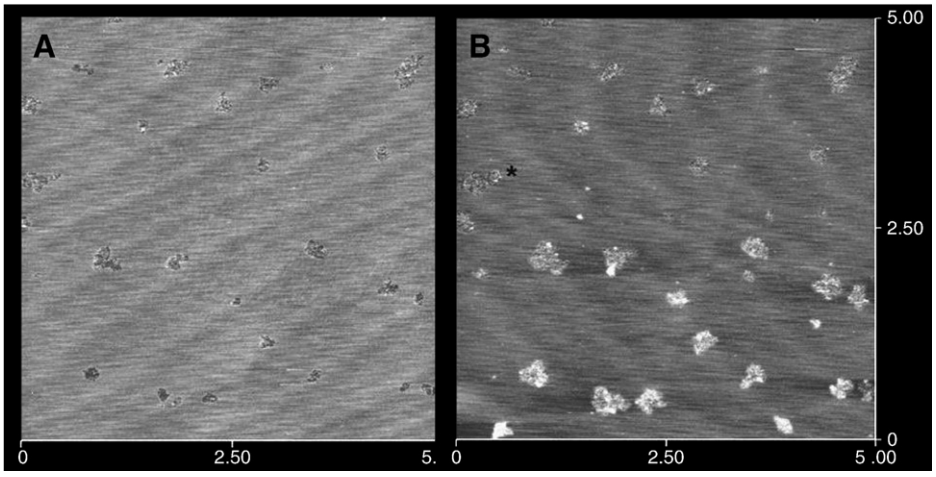

Fig. 9. Annexin A1 binds to domains of lower height in a 25% brain PS/75% DOPC bilayer. Panels A and B represent consecutive images 4 min and 16 s apart of an adjacent area of the same sample as shown in Fig. 8. Scan in Panel A begins at the bottom, immediately after addition of 1 μg (final concentration approximately 10 μg/ml) of annexin A1. As the scan progresses upwards over 4 min and 16 s the annexin is seen to occupy the domains. Panel B, continuing the scan of the same area as in Panel A, starting from the top, annexin A1 continues to accumulate in the same domains which have now become expanded in size. The domain marked with an asterisk (*) is shown in higher magnification in Fig. 10. Scan size 5×5 μm, height scale as shown in Fig. 1, 0 to 5 nm.

presence or absence was variable under the conditions used, and they were present in only about half of the samples when initially examined. Imaging of the depressions sometimes led to their reduction or elimination indicating that they are not highly stable. It is likely that these depressions represent domains enriched in PS which are known to form in the presence of calcium in mixed PS/PC bilayers [21,22]. They could be distinguished from defects or holes in the bilayer because they were several fold shallower than holes penetrating the bilayer. Richter and Brisson [23] have shown that if the process of the formation of the supported bilayer is interrupted by washing away the lipid vesicles before the bilayer is complete, defects remain between patches of membrane. In order to compare the putative PS enriched domains with such defects, a bilayer was prepared with defects according to the method of Richter and Brisson [23] using 25% brain PS and 75% DOPC. As seen in Fig. 4 these defects have a depth of 2.5 nm, which is the measured thickness of incomplete bilayers using the Mikromasch probes used in this study (see Materials and methods).

When domains similar to those in Fig. 3 were present at the time copine was added to the sample, the copine immediately bound to the domains and assembled into structures similar to the patches in Fig. 2 (see Figs. 5 and 6). It is possible that the binding of copine seen in Fig. 2, following a significant delay, was dependent on the transient formation of similar domains that were not visualized during the AFM experiment. At high magnification the copine patches were found to have an interesting reticular structure composed of elements that are of variable length, averaging 44.9 +/− 13.9 nm ($n = 20$ measurements) (Fig. 6).

3.2. Preincubation of copine with vesicles reveals more detailed fine structure of copine aggregates

It has been demonstrated that copine binds to acidic lipid vesicles in a calcium-dependent manner in co-sedimentation assays [4,18]. Therefore, in some experiments copine was incubated with vesicles first, and then the vesicles were applied to the mica substrate and allowed to fuse. After formation of the bilayer in this way copine was seen to be bound to the membrane in a pattern possibly reflecting the borders between the membrane patches that had fused during the formation of the continuous bilayer (Fig. 7). It is known that when the copine is incubated with vesicles, it aggregates the vesicles in a calcium-dependent manner, similar to the characteristic ability of annexins to aggregate membranes [4]. It may be that the membrane contacts formed by the copine are regions where the copine has stabilized a lipid domain structure similar to the structures appearing as depressions in the planar bilayer. After fusion, the copine may remain bound to these domains as seen. It is interesting that the copine in these images also appears to consist of linear elements with globular varicosities spaced at an average of 42.4 +/− 8.3 nm apart ($n = 20$ measurements). This is similar to the lattice spacing seen in the reticular patch of Fig. 6, although there is considerable variability in shape of the elements. In between the varicosities the copine fibrils

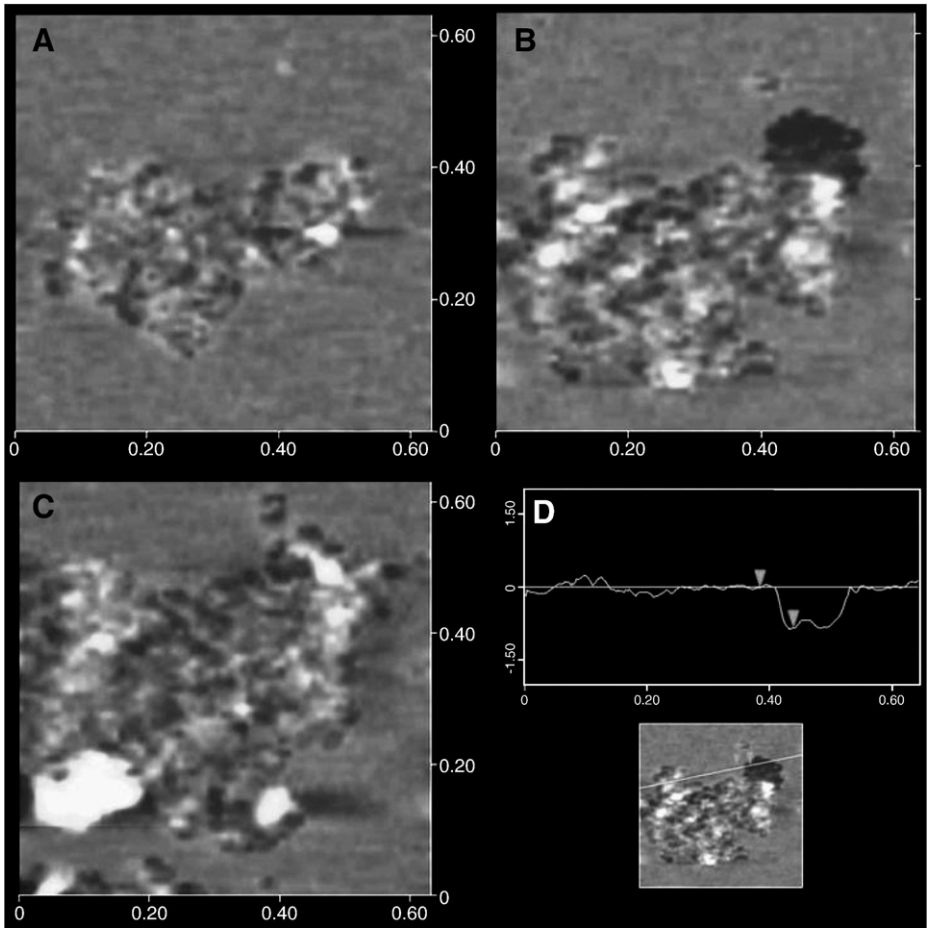

Fig. 10. High magnification images of the expansion of an annexin-binding domain. The domain imaged here is the one labeled with an asterisk on the left side of Panel B in Fig. 9. Images A, B, and C were taken 4 min and 16 s apart, starting with Panel A which is from the same scan as Fig. 9, Panel B. Notice the overall growth of the domain through this sequence of images. In particular, notice the dark area protruding from the upper right of the domain in panel B. This represents expansion of the modified lipid domain evidently due to the action of the annexin on the adjacent membrane area. In the next image, C, this new part of the domain is now seen to have been occupied by the annexin. A section analysis of the new domain area in Panel B is presented in panel D, the depth of this region is 0.8 nm. Image size, 0.62 × 0.62 μm (zoomed with software from original 5 × 5 μm scan).

had an apparent minimum thickness of 30 nm. Both the height image and the corresponding phase images are shown in Fig. 7. The phase image results from a phase delay in the oscillating response of the probe tip relative to the driving frequency and is sensitive to compositional and visco-elastic differences and can therefore potentially provide better visualization of borders between (or within) protein molecules.

3.3. Binding of annexin A1 to supported bilayers

The behavior of annexin A1 was similar to that of copine in that it did not bind to the mica substrate in the absence of lipids but would readily bind 25% PS bilayers if domains of lower height were present, as seen in Figs. 8 and 9. Phase imaging of these domains (shown in Fig. 8B) indicated that they cause a positive phase shift in the probe response, indicating a differential interaction of the probe tip with the lipids in the domain versus the rest of the bilayer. Fig. 9 represents an adjacent location of the same sample as in Fig. 8, immediately after injection of the annexin, and shows the rapid association of the protein with the domains of lower height.

During the monitoring of the association of annexin A1 with the bilayer it was observed that the annexin appeared to be responsible for expanding the size of the domains, and, as they expanded, more annexin was recruited into the newer parts of the domains. This is particularly clear in the images in Fig. 10, representing the expansion of a single domain from Fig. 9 over time.

Interestingly, and in contrast to the behavior of copine, if domains were not initially visible it appeared that the annexin rapidly promoted the formation of domains. It may be that small domains that were not possible to visualize were stabilized and then expanded by the binding of the annexin. Subsequently the domains filled with bound annexin. This process is seen in Fig. 11. It is intriguing that apparently only a few molecules of annexin were necessary to form the domains initially since the annexin molecules appear quite sparse. That is, the annexin did not initially form the sort of contiguous protein structures seen with copine that covered the membrane domain.

3.4. Annexin A1 creates membrane domains that promote the binding of copine I

The ability of the annexin to form domains that extend beyond the protein itself suggested that the annexin might be able to promote the binding of copine if the domains formed by the annexin are similar in nature to the domains that bind copine. In order to test this hypothesis a smaller amount of annexin A1 was added (0.1 μg versus 1 μg used before, resulting in a final concentration of approximately 1 μg/ml) to a featureless bilayer. A small number of domains were immediately created which were then stable (Fig. 12). As in the case of the spontaneous domains, these domains had a positive phase difference from the rest of the bilayer (Fig. 12A). After monitoring the stable domains for 30 min, copine was added (1 μg, final concentration approximately 10 μg/ml), and it was found to immediately occupy the domains created by the annexin (Fig. 12C and D).

4. Discussion

4.1. Copine and annexin associate with specialized domains in 25%PS/75%PC bilayers

The association of either copine I or annexin A1 with the supported bilayers was found to occur only on specialized domains of the bilayers. These domains were visualized by AFM as regions of 0.5 to 1 nm lower height in the bilayer, and they imparted a characteristic phase change to the response of the probe. The presence or absence of these domains was variable under the conditions that were used.

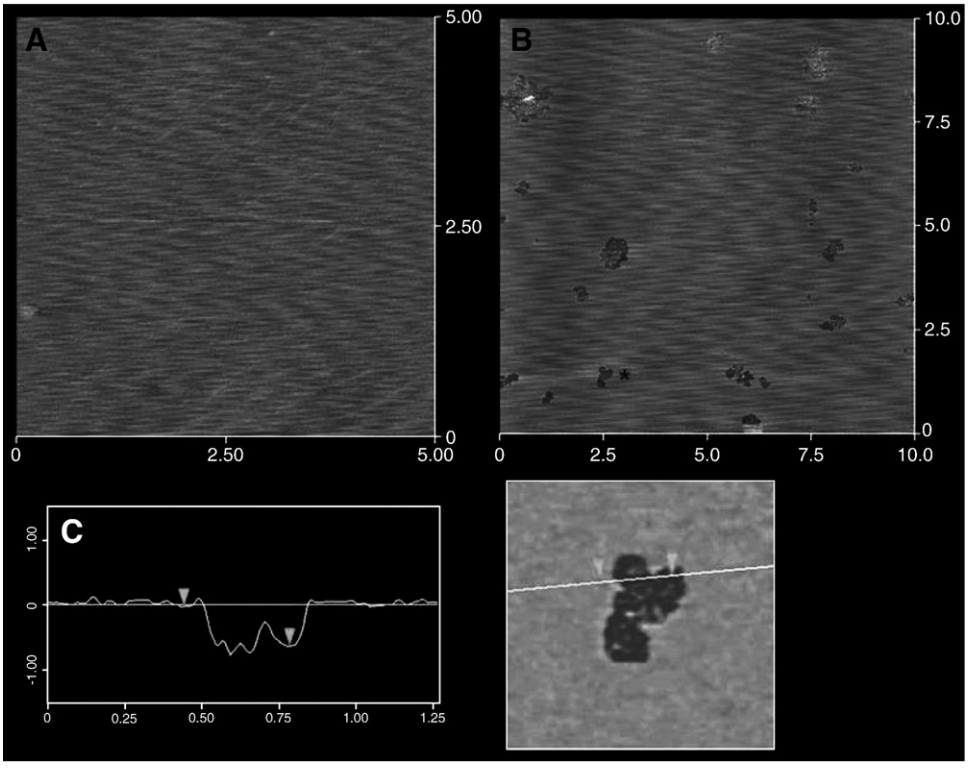

Fig. 11. Annexin A1 promotes the formation of domains that are then occupied by the protein. Panel A, image of 25% brain PS bilayer prior to addition of annexin A1 (scan size 5×5 μm). Panel B, same bilayer immediately after addition of 1 μg (final concentration approximately 10 μg/ml) annexin A1 (scan size 10×10 μm). Scan starts from the bottom and traverses the field to the top in 4 min and 16 s. Notice the appearance of small domains at the beginning of the scan, which grow as the scan continues, then become occupied with annexin at the top. Panel C, section analysis of the early domain marked with an asterisk in Panel B; depth 0.5 nm.

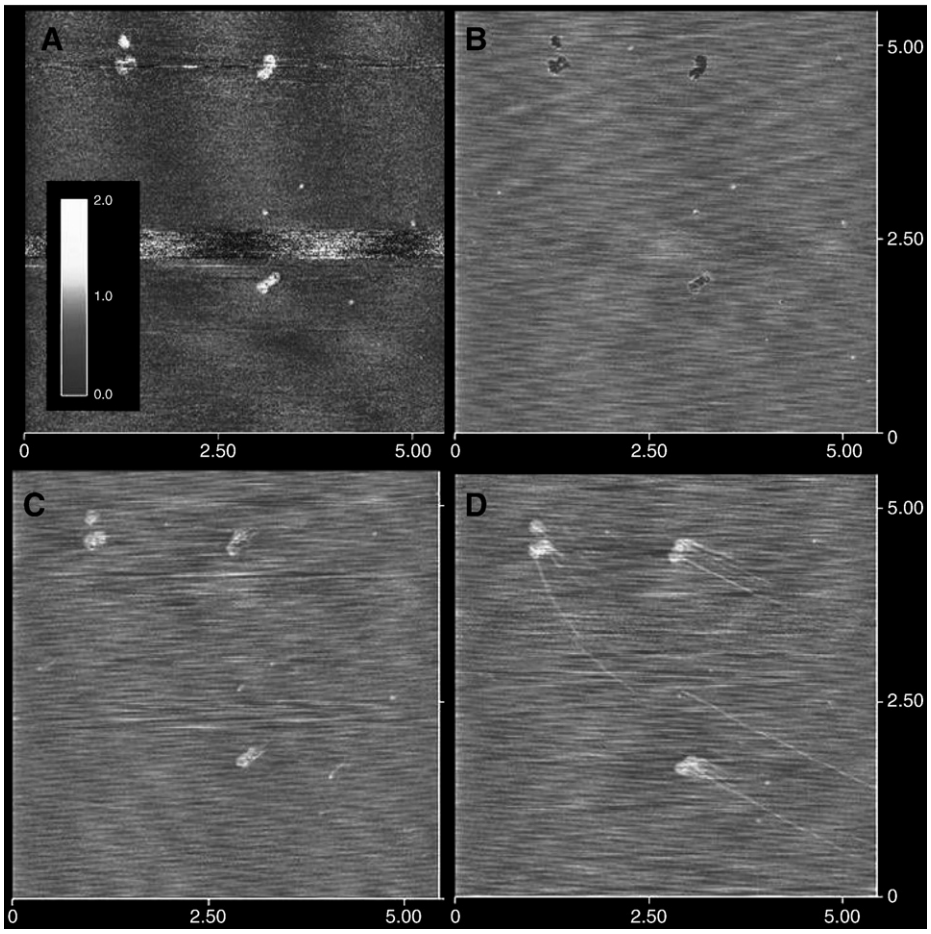

Fig. 12. Domains formed in a 25% brain PS bilayer by annexin A1 provide binding sites for copine I. Panels A and B: 0.1 μg annexin A1 was injected (final concentration approximately 1 μg/ml) and small domains were formed. A, phase image, scale, 0 to 2°. B, height image, scale as shown in Fig. 1, 0 to 5 nm. The domains seen in this image were formed immediately after the injection of the annexin and have, at this point in time, remained stable in size for 30 min. Panel C, height image, immediately after injection of 1 μg copine (final concentration approximately 10 μg/ml), the domains formed by the annexin now become occupied by the copine. Four minutes and 16 s later, panel D, as the copine continues to accumulate in the domains, some copine is displaced by the scanning probe creating the visible streaks. Scan size 5.4 × 5.4 μm.

Although the samples were nominally prepared the same way for each experiment, the domains were present in only about half of the bilayers when initially visualized. When present, their abundance and size was variable, and imaging of the depressions sometimes lead to their reduction or elimination indicating that they were not highly stable. These domains were not holes through the bilayer, which would have a depth on the order of 2.5 to 4 nm, depending on the probe type (see "Probe characteristics" in Materials and methods). Furthermore, in experiments in buffer but without lipids the copine or annexin did not adhere to the substrate suggesting they do not have affinity for the bare mica. Similar height differences (about 1 nm) have been seen by AFM in the case of either compositional domains in supported bilayers, or domains reflecting the state of order of the lipids [24–29]. In the present case it is unclear if the domains reflect compositional or order differences or both. Indeed, it is unclear if the height difference actually exists; it may be that the properties of these domains are such that the AFM probe penetrates into the bilayer to a greater depth resulting in the appearance of an area of lower height. Such an increased penetration might also contribute to the phase difference seen in the probe response. Subtle differences in the viscoelastic responses of surfaces have frequently been found to lead to phase changes in tapping mode AFM [30].

Since it was found that the copine and annexin bound to these domains without delay (Figs. 5, 9, and 12), it is likely the domains may contain increased concentrations of PS because of the well known affinity of these proteins for acidic lipids [3,4]. It has been reported that in bilayers composed of the defined lipids POPC and POPS, in the presence of calcium the PS forms raised domains — rather than the lower domains observed here [22]. The depressions seen in the present experiments may reflect a more disordered nature of the brain PS used in these experiments which has a mixed composition of saturated and unsaturated fatty acids. Visualization of the domains may have been enhanced by the use of a fairly stiff probe that tends to partially enter the membrane. In addition to showing specificity for acidic lipids, annexins have been reported to interact preferentially with disordered versus ordered lipid phases. In particular, Patel et al. have shown that a hydra annexin homolog will bind to vesicles composed of phospholipids (PC and PS) if the lipids are in the liquid crystalline phase at higher temperatures, but will not bind below the transition temperature at which the lipids enter the gel state [31]. In a related study, Fischer et al. demonstrated that the hydra annexin will bind to vesicles of high curvature even in the absence of calcium, but will not bind larger vesicles unless calcium is present [32]. Both of these results suggest that lipid organization also plays an important role in promoting annexin binding.

However, the exact composition and structural characteristics of the domains seen in these experiments are unknown. Recently techniques have been developed for combining AFM and measurements of the order parameter of a fluorescent probe in supported bilayers to correlate topography with physical properties of lipid phases [33,34], and combining time-of-flight secondary-ion mass spectrometry (TOF-SIMS) imaging with AFM to correlate topography with composition [35]. Application of such techniques to the system studied here might prove useful. In addition, it will be important to

extend these studies to more biologically relevant lipid mixtures and calcium concentrations. DOPC and brain PS were used here primarily because of the extensive use of simple mixtures of PC and PS in prior studies of calcium-dependent, membrane-binding proteins in the annexin and C2 domain classes. Cellular membranes contain other lipid components, some of which have been shown to specifically modulate the binding of annexins to membranes including phosphatidylethanolamine [36], phosphatidylinositol 4,5-bisphosphate [37], ceramide [38], and cholesterol [39–41]. Techniques have recently been developed for forming supported bilayers where the lipid compositions of the two leaflets of the bilayer reflect the asymmetry seen in biological membranes [42]. Furthermore, the coupling of domain formation between the leaflets has been observed in such systems [43,44]. Clearly it would be of interest to use these more sophisticated model systems to examine the behavior of annexins and copines and their effects on membrane organization, and to conduct the studies in the lower concentrations of calcium more likely to occur in vivo.

4.2. Structure of copine aggregates bound to membranes

Previous studies using dynamic light scattering have demonstrated that copine I in solution in the absence of membranes forms a larger particle in the presence of calcium [18]. The particle has a hydrodynamic radius consistent with the assembly of 10 molecules of copine if the copine monomer and the copine complex are both essentially globular. However, the actual morphology of the oligomer is not known. Furthermore, it was not known if this oligomer will form in the presence of membranes or whether the copine molecules might bind to the membrane as individual species. The present results using AFM confirm that copine forms a large calcium-dependent complex when not attached to a membrane, but forms a reticular, largely flat array on membranes through interactions of copine with the membrane and evidently between copine molecules. The confinement of the copine arrays to the specialized membrane domains may be an important driving element in the formation of the inter-copine contacts.

The reticular copine array on membranes approaches a roughly square lattice of approximately 45 nm spacing, although considerable variation from an ideal lattice is apparent (Fig. 6). When the bilayer was formed from vesicles that had been preincubated with small amounts of copine, linear elements were formed, but these were not assembled into a lattice (Fig. 7). The linear elements tended to form longer, flexible strings interrupted by globular varicosities. The distance between these varicosities was similar to the lattice dimension of 45 nm. Therefore, the varicosities may represent material present in the nodes of the lattice. Possibly these are points of intermolecular contact in the copine assemblies. However, the length of these structural elements at 45 nm would appear to be too large to be accounted for by single copine molecules which are 55 kDa and consist of domains of well known structure — two C2 domains and one integrin A domain. Even if these three globular domains were aligned in a linear fashion the copine molecule would not likely be longer than 12 nm. Therefore the linear elements visualized here by AFM likely represent small assemblies of copine molecules.

In interpreting the AFM images of the copine fibrils and the copine lattice it is important to consider the contribution of the finite geometry of the AFM probe tip to the image. The image is a convolution of the shape of the tip and the shape of the object being observed. The nominal radius of the probe tip is 10 nm, as indicated by the manufacturer, so simple geometric considerations, illustrated in Fig. 13A, indicate that the apparent width of the copine

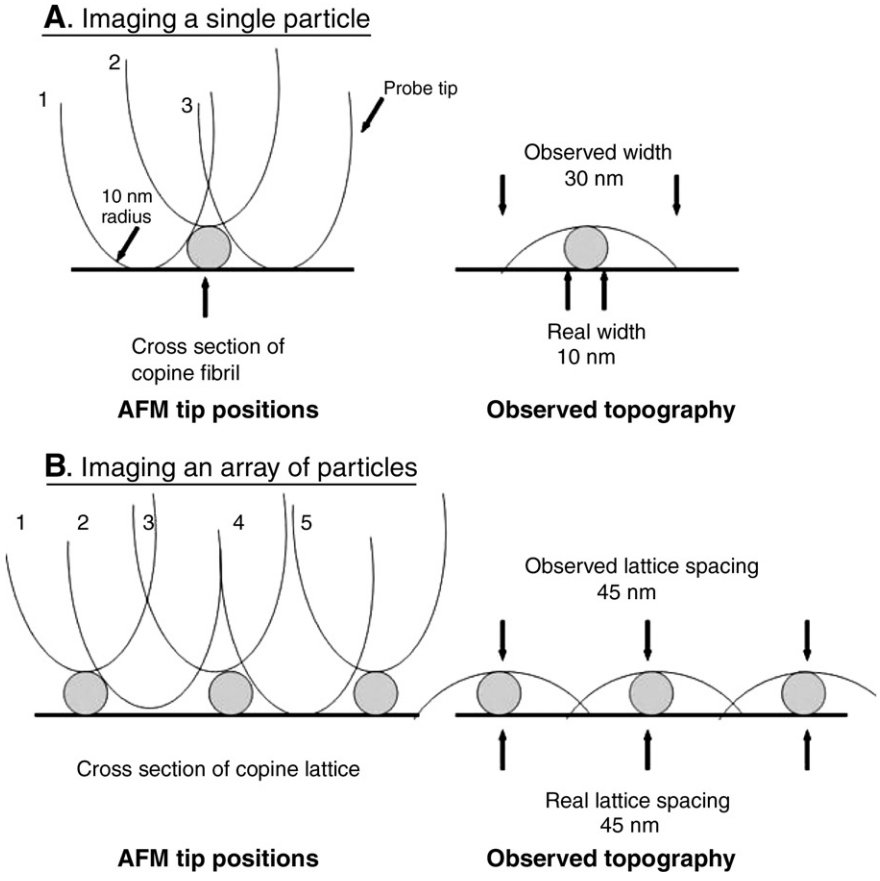

Fig. 13. Schematic illustration of the effects of AFM tip convolution on the images of copine fibrils and the copine lattice. The numbers on the left side of the figure indicate different positions of the probe tip as it scans from left to right. As seen in part A, the finite radius of the probe tip (10 nm) may add as much as 20 nm to the observed width of the copine fibrils seen in Fig. 7. However, as seen in part B, the basic spacing of the copine fibrils in the lattice seen in Fig. 6 is accurately observed.

fibrils may be overestimated by as much as 20 nm. In addition, significant additional broadening of the image can occur due to deformation and spreading of the protein due to the force of the probe. Therefore, although the minimal width of the fibrils appeared to be on the order of 30 nm, the true width of the fibrils is likely to be less than 10 nm. On the other hand, as illustrated in Fig. 13B, the basic lattice spacing as observed (45 nm) is likely to be correct because it is not modified by tip convolution.

The reticular copine arrays might provide ideal scaffolds for the assembly of the signaling proteins bound by copine. However, it is not known if the copine when bound to a "target" protein component of a signaling pathway would assemble into patches similar to the ones seen with copine alone. It is possible that the formation of the reticular patches may depend upon the binding of the A domain of one copine molecule to a section of another copine molecule. In that case, the presence of the target protein bound to the A domain might make it impossible for copine to self associate. In order to test the hypothesis that copines can promote interactions between signaling molecules it will be important to determine the structural characteristics of the complexes formed by copine and associated target proteins when bound together to membranes.

4.3. Annexin A1 promotes the formation of domains that bind copine I

In cases where domains were not initially evident in the supported bilayer the introduction of annexin A1 appeared to actively promote the formation of domains that had similar properties to the spontaneous domains that bound either copine or annexin. Both types of domains had a similar apparent depth and caused a positive change in the phase of the probe response suggesting they have similar compositional and structural features. The annexin itself did not appear to form well ordered structures within these domains. This is similar to the observations of Steinem's group using AFM of annexin A1 bound to POPC/POPS supported bilayers [45], and contrasts significantly from behavior of annexin A5 which forms well ordered two dimensional crystals on supported bilayers after prolonged incubation [46].

The variable quantity and size of the domains under the conditions of the experiments described here, as well as the sensitivity of the domains to imaging, suggest the bilayers are poised at a point where the domain formation can readily occur with minimal changes in environment. The binding of the annexin appears to strongly tip the balance in favor of the demixing or changes in structure represented by the domains. The copine presumably has a similar effect on the membrane since the patches of copine that were observed also expanded over time. However, it is not clear why the annexin promoted the formation of domains that extended well beyond the apparent footprint of the annexin molecules themselves, while this did not happen with copine. It may be that the annexin has a higher affinity for the PS, or imposes a change in membrane structure that favors further demixing of the lipid components. This ability to catalyze domain formation and create binding sites for other proteins may be a fundamental biological function of annexins in general because of the similar structures and lipid specificities of the multiple members of this protein family. Fig. 14 provides a schematic overview of the hypothetical role the annexin plays in promoting domain formation and recruiting copine to the newly formed domains. Although the action of the annexin in recruiting copine to the membrane may depend only on the changes induced in lipid organization by the annexin, the experiments described here do not rule out a possible direct binding interaction between the annexin and copine during the nucleation of the copine lattice.

From planimetry of the images of the domains observed in Fig. 9 over a 16 min period it was possible to calculate the rate at which the annexin promotes the formation of these domains. The domains were monitored continuously in a series of scans in alternating directions (up, followed by down, etc.). The time each domain was imaged was determined from the scan number, the direction of the scan, and the position of the domain in the image. The perimeters and areas of the domains were measured in each image and a rate of growth calculated for each time interval as the increase in area $(A2-A1)$ divided by the average perimeter during the time interval $((P1+P2)/2)$, normalized to the time interval $(T2-T1)$:

$$\text{Growth rate parameter} = 2 \times (A2 - A1) / ((P1 + P2)(T2 - T1))$$

This parameter can be visualized as representing the "flux" of area across the boundary as the domain grows (units nm^2/nm-s), or as the rate of linear growth of the domain perpendicular to its edge (same units reduced to nm/s). This analysis revealed that the area of each domain expanded at a rate of $0.15+/-0.05$ nm/s on average (15 domains measured at 4 time points each). This value was roughly independent of the area of the domain as it was similar for both large and small domains and for individual domains at different times during their growth for the first 16 min after adding the annexin. Eventually the growth parameter declines due to depletion of the annexin and/or a requisite lipid component, likely the PS. Observation by AFM of the growth of domains during lipid phase separation after thermal cooling of mixed two-component supported lipid bilayers reveals a similar pattern of growth [27].

In order for the annexin to create space in a domain for a C2 domain protein like copine with an approximate diameter of 6 nm to bind would require 40 s based on an expansion of the border of the domain at an average rate of 0.15 nm/s. However, growth at specific points on the borders of the irregular domains was not isotropic and

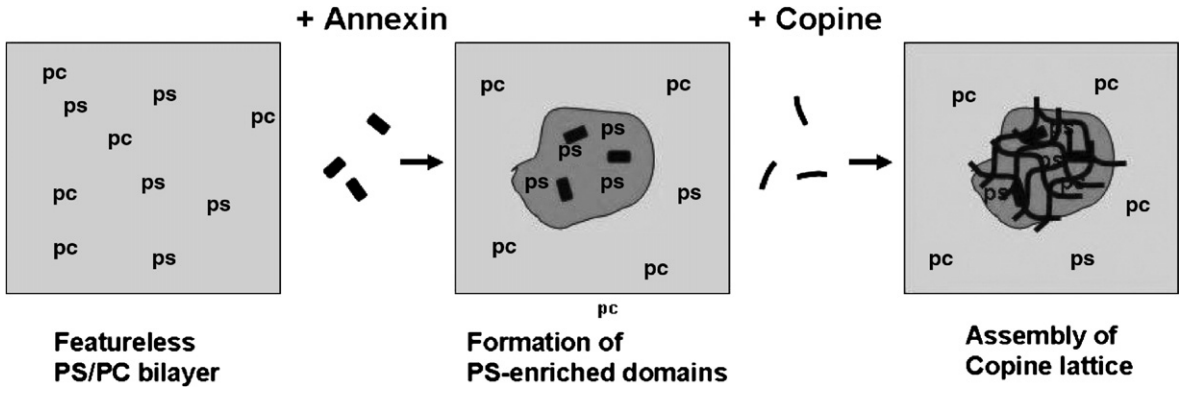

Fig. 14. Schematic illustration of the synergism of the binding of annexin and copine to a bilayer. In the presence of calcium the annexin creates or stabilizes specialized domains enriched in PS. These domains extend beyond the "footprint" of the annexin and create space for copine to bind and form a structured lattice. The copine lattice may subsequently provide a scaffold promoting the interaction of signaling proteins bound to copine.

frequently highly divergent from the average value. For example, the bulge in the domain in Fig. 10B was formed in 4 min indicating an expansion rate of at least 0.45 nm/s at this point. At this rate a binding site for a copine molecule would be created in 13 s or less. This value would be compatible with the time course of many cellular signaling processes, but is too long, for example, to be associated with fast exocytosis in neurotransmitter release if that were dependent on the formation of a domain to accommodate the binding of synaptotagmin. However, assessment of the true physiological significance of these domain growth characteristics must await further experiments with membranes of more natural composition.

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