Characterization of lipid domains in erythrocyte membranes

(fluorescence microscopy/digital image analysis)

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ABSTRACT Fluorescence digital imaging microscopy was used to study the lateral distribution of the lipid components in erythrocyte membranes. Intact erythrocytes labeled with phospholipids containing a fluorophore attached to one fatty acid chain showed an uneven distribution of the phospholipids in the membrane thereby demonstrating the presence of membrane domains. The enrichment of the lipotropic compound chlorpromazine in domains in intact erythrocytes also suggested that the domains are lipid-enriched regions. Similar membrane domains were present in erythrocyte ghosts. The phospholipid enrichment was increased in the domains by inducing membrane protein aggregation. Double-labeling experiments were done to determine the relative distributions of different phospholipids in the membrane. Vesicles made from extracted lipids did not show the presence of domains consistent with the conclusion that membrane proteins were responsible for creating the domains. Overall, it was found that large domains exist in the red blood cell membrane with unequal enrichment of the different phospholipid species.

Since it was proposed almost 20 years ago, the Fluid Mosaic Model has served as a foundation for understanding membrane structure and function (1). The model describes biological membranes as a mixture of lipid and protein components in a phospholipid bilayer where all the components can undergo free lateral motion. This results in a random distribution of the membrane components with no long-range order. One refinement to this model is the role of the cytoskeleton in anchoring transmembrane proteins (2). Another change is that biological membranes may be more heterogeneous than depicted by the Fluid Mosaic Model with the existence of relatively large domains with compositions different from the bulk membrane.

The occurrence of lipid domains in phospholipid bilayers has been extensively characterized *in vitro*. A classical phase separation occurs when a fluid phospholipid bilayer is cooled to give rise to the formation of gel-phase lipids (3). In special cases *in vivo*, such as for *Acholeplasma laidlawii* or *Escherichia coli* supplemented with saturated or trans-unsaturated fatty acids, substantial gel-phase lipids can exist at the growth temperature (4–6).

Another type of phase separation that has been well characterized *in vitro* occurs when exogenous compounds or ions, such as Ca^{2+} , are added to a phospholipid bilayer containing acidic phospholipids (7–14). A similar phenomenon occurs when cytochrome *c*, or other basic proteins, bind to membranes containing acidic phospholipids (15–22).

Evidence for the existence of intrinsic membrane domains in biological membranes has been increasing in recent years (8, 23–43). In some cases, the domains are clearly present since they are morphologically distinct, such as the apical and basolateral domains of polarized epithelial cells, with each region having a different lipid and protein composition (23).

Tight junctions give rise to the different regions and appear to be responsible for the compositional differences (43). This is different from the type of domain referred to here.

Within a single type of membrane, which is the topic of this paper, there is also evidence that domains can be fairly large. For example studies with Rous sarcoma virus and vesicular stomatitis virus were consistent with the existence of membrane domains at least the size of the virus, $\approx 0.1 \mu\text{m}$ (26). Fluorescence photobleaching recovery experiments of labeled human fibroblast cells produced evidence of micrometer-size lipid domains (41). Using similar techniques, domains were also measured in soybean protoplasts but not in 3T3 fibroblasts (8). Studies using differential polarization microscopy have shown large protein domains to be present in thylakoid membranes (27). Many of the studies on domains in biological membranes are interpreted in terms of the existence of gel-phase domains. However, the lipid compositions of most biological membranes indicate that gel-phase lipids are largely absent from membranes at the growth temperature (44, 45). Lipids in domains of nonbilayer phases also have been postulated to occur in biological membranes (46), and additional evidence is needed to determine how extensive these phases are under physiological conditions.

Direct visualization of membrane domains using fluorescence digital imaging microscopy offers the best means for demonstrating their existence (7, 15, 50). This paper reports studies using this method to collect and analyze images of fluorescently labeled erythrocyte membranes. Erythrocytes from most species have only one type of membrane, the plasma membrane, which is a significant advantage for these types of experiments in that internalization or metabolism of the label is not a problem. By using improved methods of image enhancement, large lipid domains have been visualized, and their degree of lipid enrichment has been characterized. Preliminary reports of this work have been presented (48, 49).

MATERIALS AND METHODS

Sample Preparation. Rabbit erythrocytes were isolated, and the ghosts were prepared as described (7). Samples for labeling and microscopy were suspended in 137 mM NaCl/3 mM KCl/6 mM Na_2HPO_4 /2 mM KH_2PO_4 , pH 7.4 (PBS) or, for double-labeling experiments, 10 mM Tris/150 mM NaCl, pH 7.4. All samples were used immediately after labeling. The cells and ghosts were labeled for microscopy by monomer transfer from ethanol-injected donor vesicles as described (7). All lipids used for donor vesicles were purchased

Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; NBD-phosphatidylcholine, -serine, and -ethanolamine, 1-acyl-2-[6-(N-NBD)amino]hexanoyl phosphatidylcholine, -serine, and -ethanolamine; dansyl-phosphatidylcholine and -serine, 1-acyl-2-[4-(N-dansyl)amino]butanoyl phosphatidylcholine and -serine.

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or synthesized, purified, and stored as described (7, 50). The donor vesicles were composed of cholesterol and phospholipid at a molar ratio of 0.9. The fluorescent phospholipid composed 20–35% of the total lipid in the donor vesicles, with the remaining phospholipid fraction consisting of dioleoyl phosphatidylcholine. For fluorescent phospholipids, phosphatidylcholine, -serine, and -ethanolamine were labeled with NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) or dansyl (5-dimethylaminonaphthalene-1-sulfonyl) on the fatty acid residue in position 2—e.g., NBD-phosphatidylcholine is 1-acyl-2-[6-(N-NBD)amino]hexanoyl phosphatidylcholine and dansyl-phosphatidylcholine is 1-acyl-2-[4-(N-dansyl)amino]butanoyl phosphatidylcholine. After labeling, donor vesicles were removed by washing. For chlorpromazine imaging, a 1.0 mM stock solution of chlorpromazine in PBS was added to a 5% hematocrit of intact cells to a final chlorpromazine concentration of 100 μ M. To induce aggregation of intramembrane particles, ghosts were first pretreated as described by Elgsaeter and Branton (51) and suspended in 10 mM Mes/150 mM NaCl, pH 5.5.

Vesicle Preparation. The erythrocyte lipids used for forming large liposomes for microscopy were extracted by the method of Rose and Oklander (52). The phospholipid concentration was determined by colorimetric assay after acid digestion (53). The large liposomes were formed as described (7) and fluorescently labeled by incubation with both NBD-phosphatidylserine and dansyl-phosphatidylcholine donor vesicles.

Instrumentation. All fluorescence microscopy was done with the instrument described previously (7) except that the computer was further modified to allow the image-processing operations presented here (IBM 9000 modified by Quantex, Sunnyvale, CA). For NBD imaging, excitation filters between 420 and 460 nm were used, and for dansyl and chlorpromazine imaging, excitation was between 340 and 380 nm. Emission wavelengths > 430 nm (dansyl and chlorpromazine) and 515 nm (NBD) were used for image collection. The instrument response was determined to be linear over a broad range of concentrations using fluorescent microbead standards (Coulter Immuno-Brite Microspheres). Phospholipid separations were done by using either HPLC or TLC (54, 55). Fluorescence measurements were made with a SLM 8000 fluorometer. The wavelength for excitation was 350 nm for dansyl and 470 nm for NBD.

Image Processing. All fluorescence images shown were processed as follows. In the first step, the background field of illumination was subtracted from each image collected. During the subtraction operation, the gray scale of 0–255 was redefined so that a difference of 0 equaled a gray value of 128, and all positive differences had gray values from 129 to 255.

After the background subtraction, the resulting images were normalized by enhancing the images so that their final mean gray level values were 100 ± 4 . Enhancement of the images included setting 128 equal to 0, followed by multiplying the gray value of each pixel in the image by a common integer so that the entire image had the desired average gray value. The area used for normalization was a box enclosing the edges of the membrane.

RESULTS

Erythrocyte Membrane Domains. Lipid domains in erythrocyte membranes can be visualized by using fluorescence digital imaging microscopy as illustrated in Figs. 1 and 2. In the fluorescence image of an NBD-phosphatidylcholine-labeled erythrocyte (Fig. 1A), there are patches of greater intensity, or lipid enrichment, illustrating membrane domains. The bright-field image of the cell (Fig. 1A Right) shows that the areas of enrichment do not correspond to any distinctive morphological feature of the cell and therefore are not an artifact created by the geometry of the erythrocyte. Each cell examined had a distinctive pattern of domains with different sizes and degrees of enrichment. The morphology as shown in Fig. 1A was the same for each cell. Similar types of domains were visualized in whole cells labeled with NBD-phosphatidylserine.

The fluorescent compound chlorpromazine was also examined to see if it were uniformly distributed in erythrocyte membranes or localized in domains. The chlorpromazine fluorescence as shown in Fig. 1B appears enhanced in domains analogous to what is seen with the NBD-phosphatidylcholine labeling. The hydrophobic nature of chlorpromazine causes it to partition into lipid-rich regions (56). Erythrocytes labeled with the concentration of chlorpromazine used for fluorescence microscopy (100 μ M) acquired a spherical shape as revealed by bright-field illumination (57, 58). Ghosts labeled with the same concentration of chlorpromazine also showed domains in their membrane.

FIG. 1. Visualization of membrane lipid domains. (A Left) Fluorescence image of an NBD-phosphatidylcholine-labeled erythrocyte. (A Right) Image of the same erythrocyte collected by bright-field illumination at the same plane of focus with the same objective. (B) A chlorpromazine image of an intact erythrocyte. (C) A NBD-phosphatidylcholine image of an erythrocyte ghost. (D) A NBD-phosphatidylcholine image of an erythrocyte ghost after treatment to induce the aggregation of intramembrane particles. (E) The pseudo-color-scheme applied to the images (Left) is shown with its equivalent gray level values (Right). The gray level values range from 0 (blue) to 255 (red). The two grey bands in the pseudo-color-scale result from the color mixing by the television monitor on which the images are displayed. The images were normalized by enhancing them to a mean gray level value of 100 ± 4 . The white bar equals 2.5 μ m.

FIG. 2. Double-labeled erythrocyte ghosts. Shown here are four separate ghosts double-labeled with a NBD-phospholipid (Left) and dansyl-phosphatidylcholine (Right). The NBD-phospholipids used were NBD-phosphatidylcholine (A), NBD-phosphatidylethanolamine (B), and NBD-phosphatidylserine (C and D). CaCl_2 (10 mM) was added to the ghost in D. (E) Vesicle made from extracted erythrocyte lipids and double-labeled with NBD-phosphatidylserine and dansyl-phosphatidylcholine. The white bar equals 2.5 μm . The images were normalized by enhancing them to a mean gray level value of 100 ± 4 .

Membrane domains that are intrinsic to erythrocytes were also present in ghosts as illustrated in Fig. 1 C and D. When aggregation of intramembrane particles was induced there was an increase in the lipid-enriched domains (Fig. 1D). In the experiments with ghosts, the membranes appeared featureless when viewed with phase-contrast microscopy. Consequently, to further characterize the membrane domains, ghosts rather than whole cells were used, since the geometry of the sample would not compromise the results other than an edge effect arising from the curvature of the membrane.

Membrane Domain Composition. Erythrocyte ghosts were double-labeled with dansyl-phosphatidylcholine and either NBD-phosphatidylcholine, NBD-phosphatidylethanolamine, or NBD-phosphatidylserine. The double-labeling experiments were done to ascertain the amount of enrichment in the domains for the different phospholipid species. Dansyl-phosphatidylcholine could be used to label the membrane since the dansyl fluorescence was no longer quenched by hemoglobin. Companion images of each ghost were generated by selective excitation of the dansyl-phosphatidylcholine and the NBD-phospholipid (Fig. 2). To compare the companion

images of each double-labeled ghost, identical areas were used during normalization so that any differences that occurred between the final normalized NBD and dansyl images corresponded to differences in the relative distribution of the phospholipids in the membrane (59).

Fig. 2 shows the NBD and dansyl images of four double-labeled ghosts showing variable amounts of relative phospholipid enrichment in the membrane domains. For example, in the ghost labeled with NBD-phosphatidylcholine and dansyl-phosphatidylcholine (Fig. 2A), a similar distribution of the two probes occurred in all areas of the ghost. A similar distribution of the NBD-phosphatidylcholine and the dansyl-phosphatidylcholine would be expected because both fluorophores are phosphatidylcholine analogs and therefore serve as a valuable control for the double-labeling experiments. The domains were stable during the time necessary to collect the two images (30–60 sec). After 5–10 min, some changes in the enrichment and size of the domains could be observed, but the majority of the domains were stable. Further studies are necessary to characterize the changes in more detail.

In the ghost double-labeled with NBD-phosphatidylserine and dansyl-phosphatidylcholine to which Ca^{2+} was added (Fig. 2D), there was one domain more enriched in phosphatidylserine (domain on the right in Fig. 2D Left) and one domain more enriched in phosphatidylcholine (domain on the left). Though this ghost was incubated with 10.0 mM Ca^{2+} , enrichment of NBD-phosphatidylserine also was seen in experiments with 5.0 mM Ca^{2+} . This concentration of Ca^{2+} is close to the concentration of Ca^{2+} in whole blood (60) and is below the concentration sequestered in mitochondria (61), for example. Finally, the ghosts that were double-labeled with dansyl-phosphatidylcholine and either NBD-phosphatidylethanolamine or NBD-phosphatidylserine (Fig. 2 B and C) had domains with intermediate enrichment of the phospholipids compared with the domains observed in the ghosts labeled with dansyl-phosphatidylcholine and either NBD-phosphatidylcholine or NBD-phosphatidylserine with Ca^{2+} . Importantly, the different amounts of dansyl-phosphatidylcholine and NBD-phosphatidylethanolamine or NBD-phosphatidylserine enrichment in the domains show that the visualized domains are not an artifact created by morphology or instrumentation since such an artifact would result in apparent equal enrichment of the phospholipids in the domains.

Fig. 2E shows a vesicle made from extracted erythrocyte lipids double-labeled with NBD-phosphatidylserine and dansyl-phosphatidylcholine. The images show the vesicle to be evenly labeled with no significant lipid domains and indicate the level of background noise. This also provides a good control for the other images that show domains. The absence of membrane domains in the liposome strongly implicates membrane-associated proteins in having a vital role in formation of the domains seen in the erythrocytes and ghosts.

The amount of fluorescent phospholipids transferred to the membranes was measured and found to be no more than 0.5% of the total membrane lipid. To ascertain whether metabolism of the fluorophore had occurred, labeled ghosts were incubated for 6–8 hr at 4°C , which was the maximum time samples were maintained. The lipids were extracted from the ghosts (62) and were separated by HPLC and TLC. With the NBD-phosphatidylcholine, NBD-phosphatidylethanolamine, and dansyl-phosphatidylcholine samples, no metabolism of the fluorescent phospholipid was found, while slight metabolism (5–7%) of the NBD-phosphatidylserine was evident by the appearance of free 6-(N-NBD)amino hexanoic acid. All samples showed no additional metabolism when Ca^{2+} was added (10.0 mM final concentration).

To determine if the domains were created by labeling the membranes, a series of double-labeling experiments were conducted with dansyl-phosphatidylcholine and NBD-

phosphatidylcholine. The dansyl-phosphatidylcholine concentration was held constant ($\approx 0.5\%$), and the relative concentration of NBD-phosphatidylcholine was increased from 0 to 7 mol % of the total membrane phospholipid. The dansyl-phosphatidylcholine was selectively excited, and no differences were observed in the extent of domain formation between the samples. Thus, the domains were independent of the label concentration and appeared to be an intrinsic property of the membrane itself.

Although the lipid donor vesicles used for labeling the membranes are too small to be visualized in the microscope, the possibility of contribution to the fluorescence image by membrane-bound liposomes remaining after washing was addressed with experiments similar to those described by Pagano and Sleight (63). Erythrocytes and ghosts were incubated with 1% head group-labeled dipalmitoyl *N*-dansyl-phosphatidylethanolamine vesicles, which does not undergo monomer transfer (64). The remaining lipids consisted of dioleoyl phosphatidylcholine and cholesterol in the proportions used for the donor vesicles. In one set of experiments, 5% dioleoyl phosphatidylserine was substituted for 5% dioleoyl phosphatidylcholine to mimic the NBD-phosphatidylserine donor vesicles. Any image obtained in these experiments would result from fluorescence generated by vesicles bound to the cell or ghost that was not removed by washing. After incubating the membranes with the same vesicle concentrations used for the other labeling experiments, none of the cells or ghosts incubated with the head group-labeled dipalmitoyl *N*-dansyl-phosphatidylethanolamine vesicles were visible with digital imaging microscopy.

Larger populations of double-labeled erythrocyte ghosts were examined to determine the significance of the differences that are illustrated in Fig. 2. First, a difference image of each double-labeled ghost was generated by subtracting the normalized dansyl-phosphatidylcholine image from the normalized NBD-phospholipid image. That is, each pixel in the dansyl-phosphatidylcholine image was subtracted from the corresponding pixel in the NBD-phospholipid image. Then a histogram was made of the numbers of pixels in the difference images versus the intensity, and a standard deviation (SD) was calculated. The SD value of the difference images' histogram was used to quantitate the relative distribution of the two fluorescent phospholipids in the ghost. Increasing dissimilarity between the companion images results in greater detail in the difference image and therefore a broader histogram. For example, the difference images for the companion images in Fig. 2 *A* and *D* have histogram SD values of 7.2 and 11.4, respectively.

For the double-labeling experiments shown in Fig. 2, the number of ghosts with a given SD in the difference image is shown in Fig. 3. The distribution of SD values shifted to progressively greater values in the order of ghosts labeled with dansyl-phosphatidylcholine and NBD-phosphatidylcholine < NBD-phosphatidylethanolamine < NBD-phosphatidylserine < NBD-phosphatidylserine plus Ca^{2+} . These increases in SD values for the four double-labeling experiments were found to be reproducible in another set of experiments. Thus, these values show that the phospholipids are distributed differently in the membrane depending on their polar head group. The trimodal character of the distribution for the ghosts labeled with dansyl-phosphatidylcholine and NBD-phosphatidylserine to which Ca^{2+} was added arises from three separate populations: ghosts with little additional Ca^{2+} -induced patching, and two subpopulations of ghosts with different degrees of Ca^{2+} -induced patching. These three populations could arise from different accessibilities of phosphatidylserine for patching (47). Extracted lipid vesicles double-labeled with NBD-phosphatidylserine and dansyl-phosphatidylcholine (Fig. 2*E*) had a distribution similar to ghosts double-labeled with NBD-phosphatidylcholine and

FIG. 3. The number of ghosts with a given SD for the difference image in a double-labeling experiment. The sample sizes were 30, 38, 37, and 36 for the ghosts labeled with dansyl-phosphatidylcholine (dansyl-PC) and either NBD-phosphatidylcholine (NBD-PC) (○), NBD-phosphatidylethanolamine (NBD-PE) (△), NBD-phosphatidylserine (NBD-PS) (■), or NBD-phosphatidylserine and Ca^{2+} (◆), respectively. All images used for processing were normalized to a mean gray level value of 100 ± 4 . The distributions were normalized to a sample size of 38.

dansyl-phosphatidylcholine with a maximum SD between 6 and 7. The maximum SD value of 7 (rather than 0) for these vesicles or ghosts can be attributed largely to the noise produced during collection and enhancement of the separate images. Control experiments using dansyl solutions gave SD values of ≈ 7 for the difference images.

The images in Fig. 2 represent images with the most common values in the distributions in Fig. 3. The extent of variation in the size and enrichment of the domains can be seen by comparing the dansyl-phosphatidylcholine images in Fig. 2 *A*, *B*, and *C* and reflects the variability in the samples in general. This variability helps to illustrate that the domains are areas of lipid heterogeneity rather than sharply defined regions with a fixed concentration of components.

The amount of lipid enrichment in the domains was determined by measuring the gray value of the domains relative to the entire ghost. In the absence of Ca^{2+} , the extent of enrichment for a particular phospholipid in a domain was 60–80% for the population of ghosts. The enrichment of NBD-phosphatidylserine in the domains of membranes to which Ca^{2+} had been added was 240%.

The relative enrichment of different phospholipids in the domains of double-labeled ghosts also was determined. An equal enrichment of NBD-phosphatidylcholine and dansyl-phosphatidylcholine occurred in the domains so that the average ratio of their concentrations was the expected value of 1.0. In ghosts double-labeled with NBD-phosphatidylserine and dansyl-phosphatidylcholine to which Ca^{2+} was added, the NBD-phosphatidylserine was enriched an average of 70% more than dansyl-phosphatidylcholine in the domains more enriched in phosphatidylserine. For ghosts without Ca^{2+} and double-labeled with dansyl-phosphatidylcholine and either NBD-phosphatidylethanolamine or NBD-phosphatidylserine, the NBD-phospholipids had intermediate values of relative enrichment in the domains, ≈ 20 –30%. There were no significant differences in the distributions of the sizes and enrichment of the dansyl-phosphatidylcholine domains for the three different double-labeling experiments without Ca^{2+} , showing that the presence of a second labeled phospholipid did not alter the domains. However, in the experiment using ghosts double-labeled with dansyl-phosphatidylcholine and NBD-phosphatidylserine with Ca^{2+} , the average size of the dansyl-phosphatidylcholine domains increased by $\approx 50\%$.

To ascertain if the phosphatidylserine enrichment values were too low because of NBD self-quenching in the domains, ghosts were double-labeled with NBD-phosphatidylcholine and dansyl-phosphatidylserine. The measured enrichment values of the dansyl-phosphatidylserine, which does not self quench like the NBD fluorophore, agreed well with the NBD-phosphatidylserine data.

DISCUSSION

The use of fluorescence microscopy coupled to digital image processing has allowed direct visualization of lipid domains in erythrocyte membranes (Fig. 1). These domains represent an intrinsic structural motif in the membrane and are not "gel-phase domains." Chlorpromazine imaging of erythrocytes also demonstrated the presence of large lipid-enriched regions.

Since domains were not visible in images of vesicles made from lipids extracted from ghosts, it appears that the membrane proteins are responsible for the creation of the lipid domains. The increased size and enrichment of the lipid domains in membranes after protein aggregation is consistent with the role of the proteins in forming the domains. It has been shown that some proteins can cause nonrandom distributions of lipids in a bilayer (15, 55). It is possible that certain proteins in the erythrocyte membrane are not uniformly distributed, perhaps through interactions with the cytoskeleton, and that the proteins induce the formation of the lipid domains.

Normalizing the companion images of double-labeled membranes to a given average radiance value followed by subtraction of the normalized companion images provides an unambiguous quantitative assignment of differences in phospholipid distribution. The changing distributions toward greater SD values for the ghosts double-labeled with dansyl-phosphatidylcholine and either NBD-phosphatidylethanolamine or NBD-phosphatidylserine show that the relative distributions of the phospholipids varied in a headgroup-dependent fashion. The distribution of phospholipid species is quite heterogeneous, with each cell or ghost having a distinct pattern.

It seems reasonable to assume in these studies that the labeled phospholipids mix freely with the endogenous phospholipids and that domains of endogenous lipids are reflected by measurements on the labeled lipids. Labeling of ghosts, however, most likely resulted in labeling both leaflets of the bilayer, while the endogenous lipids are asymmetrically distributed (65). Further studies are needed to sort out the contributions of the two leaflets to the domains. This does not compromise the major conclusions, however, that large domains are present in the erythrocyte membrane and that the domains are not enriched equally in the different phospholipid species. Thus, domains represent an important structural element of biological membranes.

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