

# Regionalization and Lateral Diffusion of Membrane Proteins in Unfertilized and Fertilized Mouse Eggs

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**ABSTRACT** The unfertilized mouse egg has a round and highly villated main body and a “nipple” that is unvillated and buds off on fertilization to form the second polar body. Fluorescent markers stain the body more intensely than the nipple, which has been assumed to result from surface amplification due to microvilli. Using fluorescence recovery after photobleaching and microfluorescence photometry, we have measured the membrane protein diffusion and concentration on the main body and nipple region of unfertilized and on fertilized CD-1 mouse eggs. Two general membrane protein labels were used: rhodamine-labeled succinylated concanavalin A and trinitrobenzene sulfonate visualized with a rhodamine Fab fragment of a sheep anti-trinitrophenyl. We found that while the diffusion coefficient was the same on the nipple and main body, considerably higher recovery was observed on the nipple for both probes. The ratio of intensity of fluorescence on the nipple to main body was significantly lower for the concanavalin A stain than for the trinitrophenyl stain, indicating that true concentration gradients exist beyond those that result from surface amplification. The effect of fertilization was not general. No effect was observed for the concanavalin A stain for either diffusion coefficient or percent recovery. For the trinitrophenyl stain, percent recovery decreased approximately twofold while diffusion coefficient increased approximately threefold.

The ability of membrane lipids and proteins to move freely in the plane of the membrane is fundamental to membrane function in many cases (20). Considerable experimental effort has been directed at the issue of how the motion of membrane components is altered by membrane transformations such as fertilization and differentiation (6, 9, 14, 21, 22, 26, 35, 38). The development of direct biophysical measures of lateral diffusion of membrane components, such as fluorescence recovery after photobleaching (FPR),<sup>1</sup> has contributed considerably to our understanding of the motion of membrane molecules. Recent experiments have suggested that the lipids in membranes are not mixed homogeneously but rather sequester into an ensemble of microenvironments or domains (16, 17, 29, 35, 38) and, therefore, that the membrane does not have a single bulk viscosity. It is also apparent that plasma membrane protein diffusion is in general too slow to be controlled by lipid

“viscosity.” Rather, interactions with other cellular components, such as the cytoskeleton, appear to control membrane protein diffusion (32, 42). The nature both of the lipid domains in membranes and the various interactions that control membrane protein diffusion are just beginning to be understood.

An excellent system for the study of the effect of cellular transformation on the organization and motion of membrane components is fertilization of the mammalian egg. As seen in Fig. 1, the unfertilized mouse egg has two regions: a round and highly villated main body, and a protruding unvillated nipple (8). When the egg is fluorescently tagged for a surface component such as concanavalin A (Con A) receptors, the stain is “polarized” with the main body staining more intensely than the nipple (13). Sperm bind and fuse to the main body of the egg (13), initiating the cortical reaction (10), and rendering the egg refractory to further sperm egg fusion (10, 40, 41). The nipple region subsequently constricts and buds off to form the second polar body (see Fig. 1).

Data of Wolf et al. (35, 38) suggest that fertilization in both echinoderms and mice is accompanied not by a change in bulk membrane viscosity but rather by an alteration in the ensemble of lipid domains. In this paper we consider the effect of fertilization on the diffusion of membrane proteins in the mouse egg. Specifically, we will address three questions: (a) Is

<sup>1</sup> Abbreviations used in this paper: C<sub>16</sub> diI,3,3'-dihexadecylindocarbocyanine iodide; Con A, concanavalin A; D, diffusion coefficient; DTAF, 4,6-dichloro-s-triazin-2-yl amino fluorescein; FI, fluorescence intensity; FPR, fluorescence recovery after photobleaching; hCG, human chorionic gonadotrophin; %R, percent recovery; S-Con A, rhodamine-conjugated succinylated Con A; TNBS, trinitrobenzene sulfonate; TNP, trinitrophenyl; TRITC-sFab  $\alpha$ TNP, rhodamine-labeled Fab fragment of sheep IgG against TNP.

there a general effect of fertilization on the diffusion of membrane proteins? (b) In the unfertilized mouse egg is there a difference in the diffusibility of membrane proteins on the main body vs. the nipple? (c) Do differences in fluorescence staining between the nipple and main body merely reflect surface amplification due to microvilli or are there true differences in the concentration of membrane proteins in these two regions of a continuous plasma membrane?

## MATERIALS AND METHODS

**Embryos:** Female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) 3–6 wk of age, were induced to ovulate synchronously by an injection of 10 IU i.p. of pregnant mare's serum (Intervet, Cambridge, United Kingdom) followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Intervet). For experiments where fertilized eggs were required, the hormonally primed females were mated with CD-1 males. The presence of a vaginal plug was taken as an indication of successful mating.

At 15–20 h post hCG, unfertilized and fertilized egg masses were popped from the ampullae of excised oviducts into Hanks' balanced salt solution containing 4 mg/ml bovine serum albumin (HBSS plus BSA). The cumulus cells were removed by a 5–10-min exposure to 0.1% wt/vol hyaluronidase (Sigma Chemical Co., St. Louis, MO). Eggs were then washed through three changes of HBSS plus BSA. Zonae pellucidae were removed by a brief exposure (10–30 s) to prewarmed acid Tyrode's solution (23). The completeness of this method of zona removal has been demonstrated by Bleil and Wassarman (5). Most unfertilized eggs had one polar body and an observable nipple. Eggs were judged to be fertilized if they contained two pronuclei and/or two or three polar bodies.

**Fluorescent Labeling of Embryos:** Amino groups on the surface of zonafree unfertilized and fertilized eggs were labeled covalently with 1 mM trinitrobenzene sulfonate (TNBS) in HBSS plus BSA for 20 min at 37°C. Eggs were washed through three changes of HBSS plus BSA and incubated for 10 min at room temperature in a rhodamine-conjugated Fab fragment of sheep immunoglobulin G (IgG) directed against trinitrophenyl (TNP) (TRITC-sFab  $\alpha$ TNP). To ensure that the rabbit antibody was free of Fc fragments, eggs were labeled first with TNBS, then with the TRITC-sFab  $\alpha$ TNP, a whole sheep IgG  $\alpha$ TNP or no antibody, and lastly with a 4,6-dichloro-5-triazin-2-yl amino fluorescein (DTAF)-labeled protein A (which binds to the Fc portions of antibody molecules). No DTAF-protein A fluorescence was seen on embryos when the TRITC-sFab  $\alpha$ TNP fragment or no antibody was used, but fluorescence was seen when the intact sheep IgG  $\alpha$ TNP was used.

Zonafree unfertilized and fertilized eggs were incubated for 10 min at room temperature in rhodamine-conjugated succinylated concanavalin A (S-Con A; 100  $\mu$ g/ml in HBSS plus BSA; Vector Laboratories, Inc., Burlingame, CA) and washed through three changes in HBSS plus BSA. As described above, both S-Con A and TRITC-sFab  $\alpha$ TNP are general membrane protein labels. Mouse embryos do not exhibit exocellular matrices at this stage (12).

Fluorescently-labeled embryos were taken up into 100- $\mu$ m pathlength microslides (Vitro Dynamics, Inc., Rockaway, NJ) for examination in the fluorescence microscope and for FPR measurements.

**Fluorescence Recovery after Photobleaching:** The technique for FPR has been described in detail elsewhere (2). FPR provides us with two measures of diffusion: first, the fraction of the component that is free to diffuse (percent recovery, %R) and second, the diffusion coefficient ( $D$ ) of that fraction. Our instrument is similar to that of published designs. It consists of a Lexel 95-2 Argon Laser (Lexel Corp., Palo Alto, CA), a beam splitter attenuator similar to that described by Koppel (18), a Leitz Dialux fluorescence microscope with I2, D2, and N2.1 epillumination filter systems, and Leitz MPV photometry system (Kramer Scientific Corp., Yonkers, NY), modified to accept an EM1 9568 photomultiplier tube in a Products for Research, Inc. (Danvers, MA) dry-ice-cooled housing with amplifier discriminator and electronic shutter from EMI. The image plane diaphragm of the Leitz MPV was always set to insure that light was collected only from a single plasma membrane. This procedure is discussed in detail by Wolf and Edidin (34). Photons are counted on a custom built scaler, which also interfaces the instrument to a Technico SS16 computer (Columbia, MD), which stores and analyzes the data on dual 8" floppy disks. Data are fitted by nonlinear least squares programs after Bevington (4) according to algorithms described by Barisas and Leuther (3) and Wolf and Edidin (34). Measurements were made using a Leitz 63  $\times$  1.4 numerical aperture phase plano achromat. The beam exp(-2) radius was determined (28) to be  $(0.63 \pm 0.10)$   $\mu$ m. Bleaching times were ~5 ms at ~10 mW at 514.5 nm. Monitoring intensities were ~1  $\mu$ W. Typically, we used a counting interval of 500 ms. In all cases it was determined that no major faster components ( $\geq 10^{-7}$  cm<sup>2</sup>/s) of diffusion were present.

**Photomicrography:** Photomicrographs were made using either phase

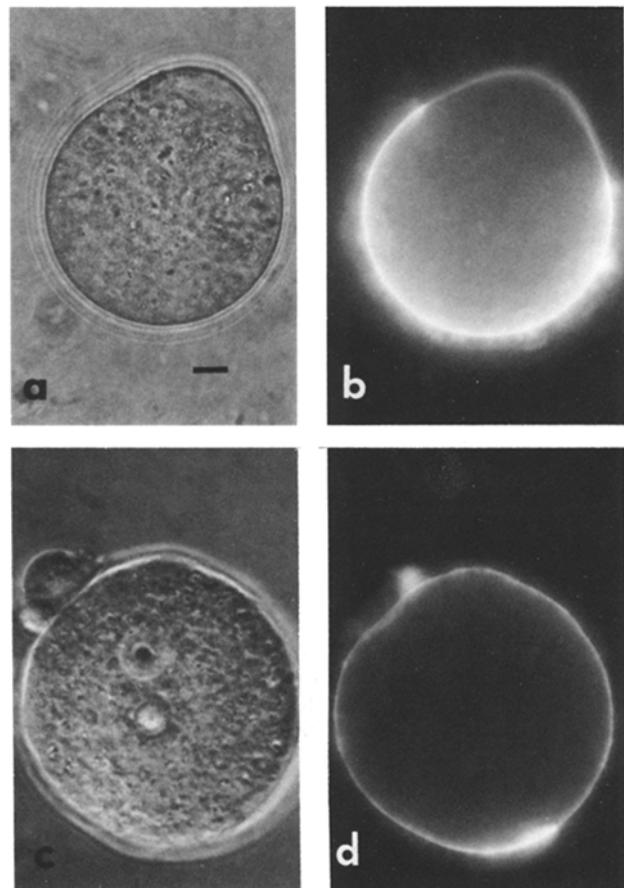


FIGURE 1 (a) Phase-contrast micrograph of unfertilized mouse egg showing round main body and protruding nipple (upper right). (b) Same egg as in a showing fluorescence staining of TNBS-labeled sites. Considerable less fluorescence is observed on the nipple as compared with the main body of the egg. Egg was labeled for 20 min in 1 mM TNBS in HBSS plus BSA at 37°C; washed three times in HBSS plus BSA; and incubated for 10 min in TRITC-sFab  $\alpha$ TNP at room temperature and washed three times in HBSS plus BSA. (c) Phase-contrast micrograph of a fertilized mouse egg, showing two pronuclei (center) and second polar body (upper left). (d) Same egg as in c showing fluorescence staining with S-Con A. While the ring staining of the body is intense, staining of the second polar body, which buds from the nipple, is faint. The bright area diametrically opposed to the second polar body is the site of sperm entry. Egg was incubated with S-Con A at 100  $\mu$ g/ml in HBSS plus BSA for 10 min at room temperature, then washed three times with HBSS plus BSA. Bar, 10  $\mu$ m.  $\times$  450.

or standard epillumination of the Dialux system. We used Ilford XP1-400 film "pushed" to an effective ASA of 800.

## RESULTS

### Membrane Protein Diffusion on Unfertilized Mouse Eggs

We have measured the diffusion of two general membrane protein labels S-Con A and TNBS on the main body and nipple of unfertilized mouse eggs. The results are summarized below in Tables I and II. For comparison, we have included in these tables data for the lipid probe 3,3'-dihexadecylindocarbocyanine iodide (C<sub>16</sub>dI), which has been published elsewhere (36). Diffusion measurements on the nipple were made only on clear and well-developed nipples. We found for the TNBS label that:  $D$  (body) =  $(7.3 \pm 1.0) \times 10^{-11}$  cm<sup>2</sup>/s and %R (body)

=  $39 \pm 3$ , while  $D$  (nipple) =  $(7.2 \pm 0.7) \times 10^{-11}$  cm<sup>2</sup>/s and %R (nipple) =  $71 \pm 4$ . FPR recovery curves showing TNBS recovery on the body and nipple of a typical egg are shown in Fig. 2. For the S-Con A label we found that  $D$  (body) =  $(1.7 \pm 0.4) \times 10^{-10}$  cm<sup>2</sup>/s and %R (body) =  $22 \pm 3$ , while  $D$  (nipple) =  $(1.3 \pm 0.8) \times 10^{-10}$  cm<sup>2</sup>/s and %R (nipple) =  $57 \pm 22$ . Thus, while each label exhibits the same diffusion coefficients on the body vs. nipple, they exhibit significantly greater recoveries on the nipple as compared with the main body ( $P < 0.01$  using either an unpaired or paired Student's  $t$  test). Similar results were obtained by Wolf et al. (36) for the lipid probe C<sub>16</sub>diI.

### Comparison of Fluorescence Intensity (FI) Ratios (Body/Nipple) for Different Labels on Unfertilized Mouse Eggs

Our FPR instrument also enables us to measure FI on the body and nipple of the unfertilized mouse egg. For TNBS we found FI (body)/FI (nipple) =  $2.0 \pm 0.3$  ( $n = 21$ ), while for S-Con A FI (body)/FI (nipple) =  $4.1 \pm 1.1$  ( $n = 8$ ). The ratio obtained for S-Con A is significantly greater than that for TNBS ( $P < 0.01$  using either an unpaired or paired Student's  $t$  test). Furthermore, while the value for TNBS is accurate, that for S-Con A is only a lower limit, since in most cases the sensitivity of our instrument precluded making measurements on the nipple (despite the fact that body FI levels were the same as those observed for TNBS).

### Membrane Protein Diffusion on Fertilized Mouse Eggs

On fertilized mouse eggs we found for TNBS,  $D = (2.2 \pm 0.5) \times 10^{-10}$  cm<sup>2</sup>/s with %R =  $21 \pm 2$ ; and for S-Con A,  $D = (2.1 \pm 0.5) \times 10^{-10}$  cm<sup>2</sup>/s with %R =  $21 \pm 5$ . We may compare these to values for the main body of unfertilized eggs. While

fertilization has no significant effect on either  $D$  or %R for S-Con A, we observed an increase in  $D$  and a decrease in %R for TNBS ( $P < 0.01$  using the unpaired Student's  $t$  test; see Table II).

## DISCUSSION

Using FPR, we have measured the diffusion of two general protein labels TNBS and S-Con A on the villated main body and unvillated nipple region of unfertilized eggs and on fertilized mouse eggs.

Our results on unfertilized eggs show that while there is no difference in the diffusion coefficients between the nipple and main body, there is a significant ~30% absolute increase in the diffusing fraction. One might expect these differences to result from artifacts introduced by topological differences due to microvilli. However, a theoretical treatment of this problem by Aizenbud and Gershon (1) shows that for the size and density of microvilli in the two regions and for the size of the laser beam used, microvilli will not significantly perturb FPR measurements. This is further substantiated by measurements of the microvilli effect by Dragsten et al. (7) on lymphocytes, and by Wolf et al. (36) using the lipid probe C<sub>16</sub>diI on the same system that we studied here. Measurements with C<sub>16</sub>diI on these eggs indicate that microvilli should not affect  $D$  and maximally will decrease %R by ~10%. Thus, the differences that we have observed here appear to reflect a true difference in membrane protein diffusibility in the two regions. A complete understanding of the factors that govern the diffusion of membrane proteins has yet to evolve. Clearly, factors other than the viscosity of the lipid matrix must be invoked, since membrane protein diffusion rates are typically two orders of magnitude or more slower (25) than is predicted by fluid dynamics (27). Recently, Tank et al. (32) have shown on membrane blebs and Wu et al. on bulbous lymphocytes (42) that when the membrane is detached from major elements of the cytoskeleton, membrane protein diffusion approaches the fluid dynamic limit, both in that 100% recovery is observed and in that  $D$  becomes  $\sim 10^{-8}$  cm<sup>2</sup>/s. The nature of the cytoplasmic factor and possibly other factors involved remain to be elucidated. Our results are interesting in this regard: first, because they represent a naturally rather than artificially induced relaxation of restrictions to protein diffusion, and, second, unlike the observations on blebs and bulbous lymphocytes, only the diffusing fraction is affected; diffusion coefficients remain unchanged. It may be that separate factors govern diffusion rate and diffusing fractions. Differences have been reported in the distribution of cortical components in the nipple and main body of mammalian eggs (24, 31). A comparison of the distribution of cytoskeletal elements in these two regions

TABLE I  
Diffusion on Unfertilized CD-1 Mouse Eggs

Probe	$D \times 10^{10}$ cm <sup>2</sup> /s		%R	
	Body	Nipple	Body	Nipple
TNBS	$0.73 \pm 0.10$ (33)	$0.72 \pm 0.07$ (21)	$39 \pm 3$ (33)	$71 \pm 4$ (21)
S-Con A	$1.7 \pm 0.4$ (29)	$1.3 \pm 0.8$ (8)	$22 \pm 3$ (39)	$57 \pm 22$ (8)
C <sub>16</sub> diI*	$64 \pm 6$ (22)	$71 \pm 8$ (22)	$77 \pm 2$ (22)	$85 \pm 1$ (22)

\* Data for the lipid probe C<sub>16</sub>diI, given here for comparison, are from Wolf et al. (36). All data are given as mean  $\pm$  1 SEM. Values given below each datum in parentheses are the number of measurements made.

TABLE II  
Effect of Fertilization on Membrane Protein Diffusion in the CD-1 Mouse Egg

Probe	$D \times 10^{10}$ cm <sup>2</sup> /s		Effect	%R		Effect
	Unfertilized	Fertilized		Unfertilized	Fertilized	
TNBS	$0.73 \pm 0.10$ (33)	$2.2 \pm 0.5$ (16)	Increase	$39 \pm 3$ (33)	$21 \pm 2$ (16)	Decrease
S-Con A	$1.7 \pm 0.4$ (29)	$2.1 \pm 0.5$ (15)	NE	$22 \pm 3$ (39)	$21 \pm 5$ (24)	NE

All data are given  $\pm$  1 SEM values. Values given below each datum in parentheses are the number of measurements made. Effects shown are to a significance of  $P < 0.01$  using the Student's  $t$  test. NE, no effect.

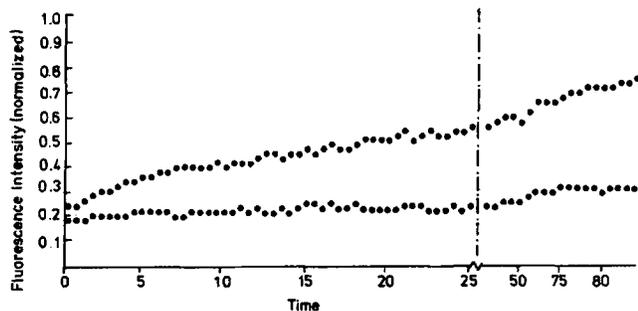


FIGURE 2 FPR recovery curves for membrane proteins labeled with TNBS and then R-sFab  $\alpha$ TNP on the nipple (upper) and main body (lower) of an unfertilized mouse egg. Both curves have been normalized to a prebleach value of 1.0. While the halftime (in seconds) for recovery and therefore the diffusion coefficient is the same on both regions, there is a higher diffusing fraction (reflected in a greater fractional recovery) in the nipple.

would contribute to our understanding of the factors governing diffusion.

Further evidence for greater restriction of diffusion comes from our observation that the ratio of FI (body)/FI(nipple) is  $(2.0 \pm 0.3)$  for TNBS and is  $\geq(4.1 \pm 1.1)$  for S-Con A. If all proteins were homogeneously distributed everywhere, FI (body)/FI (nipple) would always be equal to the ratio of surface area (body)/surface area (nipple) within the beam. Thus, while we can not say what the value of the surface area ratio is, we can conclude that the polarity of unfertilized eggs for some proteins represents a true concentration difference between the two regions in addition to surface amplification due to microvilli. The ability of the cell to localize membrane components to specific regions appears to be essential to membrane function in a number of systems (15, 19, 30). In the case of unfertilized mouse eggs it is of significance to note that sperm binding always occurs on the main body (38). Thus, restrictions to free diffusion of membrane proteins may play a major role in proper egg function.

As the result of new membrane added by the cortical reaction or of other membrane changes during fertilization (10, 11, 14, 40), one might expect changes in the diffusion of egg membrane components. Our results on fertilized eggs show that there is no general effect of fertilization on the diffusion of membrane proteins in the mouse. TNBS shows an increase in  $D$  and a decrease in  $\%R$ , while S-Con A shows no significant change in either parameter. This result is not surprising since one would expect that a process such as fertilization would involve specific proteins not all membrane proteins. It is useful to point out that despite the reported increase in the ability of Con A receptors to be patched by ligand in fertilized eggs (11), there is no change in the diffusibility of Con A receptors with fertilization. The failure of ease of patching to correlate with diffusibility has been observed in a number of systems (7, 9, 33, 37, 39). The ability to patch does not a priori reflect the ability to diffuse. Other mechanisms not requiring diffusion may be a factor (39).

In summary, three conclusions may be drawn from this work: (a) There is no generalizable effect of fertilization on the diffusion of membrane proteins in the CD-1 mouse egg. (b) The diffusion of general membrane proteins is freer on the nipple as compared with the main body of unfertilized CD-1 mouse eggs. (c) Differences in FI for general membrane proteins on the body as compared with the nipple of unfertilized CD-1 mouse eggs in some cases reflect true concentration

differences in addition to surface amplification due to microvilli.

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