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Diacylglycerol Induces Fusion of Nuclear Envelope Membrane Precursor Vesicles*

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Purified membrane vesicles isolated from sea urchin eggs form nuclear envelopes around sperm nuclei following GTP hydrolysis in the presence of cytosol. A low density subfraction of these vesicles (MV1), highly enriched in phosphatidylinositol (PtdIns), is required for nuclear envelope formation. Membrane fusion of MV1 with a second fraction that contributes most of the nuclear envelope can be initiated without GTP by an exogenous bacterial PtdIns-specific phospholipase C (PI-PLC) which hydrolyzes PtdIns to form diacylglycerides and inositol 1-phosphate. This PI-PLC hydrolyzes a subset of sea urchin membrane vesicle PtdIns into diglycerides enriched in long chain, polyunsaturated species as revealed by a novel liquid chromatography-mass spectrometry analysis. Large unilammelar vesicles (LUVs) enriched in PtdIns can substitute for MV1 in PI-PLC induced nuclear envelope formation. Moreover, MV1 prehydrolyzed with PI-PLC and washed to remove inositols leads to spontaneous nuclear envelope formation with MV2 without further PI-PLC treatment. LUVs enriched in diacylglycerol mimic prehydrolyzed MV1. These results indicate that production of membrane-destabilizing diglycerides in membranes enriched in PtdIns may facilitate membrane fusion in a natural membrane system and suggest that MV1, which binds only to two places on the sperm nucleus, may initiate fusion locally.

At the end of each mitosis in eukaryotes, the nuclear envelope is typically reconstituted by membrane fusion, forming the nuclear compartment and segregating the chromosomes from the cytoplasm. A similar process encloses sperm chromatin in egg cytoplasm following fertilization. A number of studies emphasizing the role of proteins have addressed the mechanism of nuclear envelope assembly, many utilizing cell-free systems derived from eggs or somatic cells (1–4). However, relatively little attention has been paid to the essential role(s) played by membrane lipids in this process.

Male pronuclear or somatic nuclear envelope formation involves binding of nuclear membrane precursors to the chromatin surface followed by fusion to create a double membrane enclosing the chromatin (1, 5–7). We have previously reported that envelope formation in a cell-free system derived from sea urchin eggs requires the fusion of three egg membrane vesicle populations and remnants of the sperm nuclear envelope at the tip and base of the conical nucleus (8–10).

One of the egg vesicle populations (MV1)[3] is particularly unusual. It is a low density fraction highly enriched in the membrane lipid phosphatidylinositol (PtdIns) (9, 11). MV1 binds at the tip and base of the sperm nucleus and is required for nuclear envelope formation, which can be induced by addition of GTP or a bacterial PtdIns-specific phospholipase C (PI-PLC) (9, 12). The endogenous sea urchin PI-PLC activity probably resembles a typical eukaryotic enzyme whose substrate is PtdIns(4,5)P_2. GTP-initiated envelope formation is inhibited by GTPγS and by the PI 3-kinase inhibitors, wortmannin and LY294002 (12, 13). Initiation of the fusion process by exogenous bacterial PI-PLC or human recombinant PI-PLCγ can be inhibited by the PI-PLC inhibitors ET-18-OCH_3 or U73122 (12, 14).

PtdIns hydrolysis is best known as an intermediate step in G-protein signaling pathways in which PtdIns(4,5)P_2 is hydrolyzed by PI-PLC to form diacylglycerol (DAG) and inositol-1,4,5 triphosphate ($InsP_3$). Typically, such signaling occurs in membranes containing 3–10% PtdIns with much lower amounts of PtdIns(4,5)P_2 (15). However, the large amount of PtdIns present in MV1 (up to 80% of the phospholipid) suggested to us that the PtdIns hydrolysis may be important in altering membrane structure rather than in initiating a signaling pathway (12). Since MV1 binds to the tip and base of the nucleus, hydrolysis at these points might lead to fusion initiation through localized formation of DAG. The hydrolysis products of PtdIns catalyzed by the bacterial PI-PLC are diglycerides (normally DAG) and inositol 1-phosphate (from the intermediate D-myo-inositol-1,2-cyclic phosphate) (16). DAG produced enzymatically by phospholipases acting on synthetic membranes has been shown to be membrane destabilizing and induce membrane fusion (17–20).

We show here that under fusion-stimulating conditions, bacterial PI-PLC treatment of sea urchin egg membranes results in large increases of a small subset of diradylglyceride (diacylglycerol, alkylacylglycerol, and alkenylacylglycerol) molecular species, in particular, DAG 18:0/20:4. To test whether fusion of natural membranes induced by PI-PLC in our cell-free system might result from localized production of DAG, we took two complementary approaches. First, MV1 was hydrolyzed with PI-PLC and washed to remove the water-soluble inositol

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[3] The abbreviations used are: MV, membrane vesicle population; DAG, diacylglycerol; DRG, diradylglyceride; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; $InsP_3$, inositol 1,4,5-triphosphate; PI-PLC, PtdIns-specific phospholipase C; LC-MS, liquid chromatography-mass spectrometry; LUV, large unilammelar vesicle; GTPγS, 5-guanosine-5′-(γ-thio)triphosphate; ET-18-OCH_3 or ET, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine; LB, egg lysis buffer; S150, cytosolic egg extract 150,000 × *g* supernatant; S10, cytoplasmic egg extract 10,000 × *g* supernatant; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; MWB, membrane wash buffer; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; NE, nuclear envelope.

products. The resulting prehydrolyzed MV1 when added to a cell-free system containing other nuclear envelope precursor MVs and cytosol led to nuclear envelope formation with no added inducer. Second, we used synthetic large unilamellar membrane vesicles (LUVs) to substitute for MV1 and varied the phospholipid composition of these membranes. LUVs containing 75% PtdIns mimicked MV1. In the presence of cytosol these LUVs bound to the tips of nuclei and initiated fusion when exogenous PI-PLC was added. Furthermore, LUVs containing 75% DAG led to fusion without exogenously added PI-PLC, mimicking prehydrolyzed MV1.

These results indicate that production of substantial amounts of diglycerides from the PtdIns of MV1 can lead to nuclear envelope formation and offer a possible role for PtdIns-rich membranes in local initiation of nuclear envelope formation.

MATERIALS AND METHODS

Buffers and Reagents—Egg lysis buffer (LB), nuclear preparation buffer, membrane wash buffer (MWB), and Tris/HCl, pH 7.4, buffer (TN) were prepared as previously described (21). *Lytechinus pictus* and *Paracentrotus lividus* (sea urchins) were obtained from Marinus (Long Beach, CA) and Universidade Lusófona, Portugal, respectively (*L. pictus* was used for all experiments unless otherwise stated). The ATP generation system was 1 mM ATP, 20 mM creatine phosphate, and 1 mg/ml creatine kinase (Type I), all from Sigma in LB. GTP (Sigma, Type II) stock was 1 mM in LB buffer. The stock of lipophilic membrane probe 3,3′-dihexylocarbocyanine iodide ($DiOC_6$) from Molecular Probes was 0.1 mg/ml in methanol. Synaptojanin1 (Syn1-5ptase) phosphatase construct (S470-R962; lacking the Sac phosphatase and proline-rich domains) from R. Woscholski (Imperial College London). PI-PLC from *Bacillus cereus* was from Sigma. PI-PLC stocks (3.14 units/ml) were made by dissolving 0.8 mg in 1 ml of 144 mM NaCl, 10 mM Tris/HCl, pH 7.4, 0.02% (w/v) bovine serum albumin, 600 μl of glycerol were added, and aliquots were stored up to 1 month at 4 °C. 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH_3) stock solution was 9.6 mM dissolved in Me_2SO, aliquoted, and stored at −20 °C up to 3 months. Lipids for LUVs were D-myo-phosphatidylinositol (Di-C_{16}) from Echelon, egg yolk dioleoylglycerol (Grade 1) from Lipid Products, and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylcholine from Avanti Polar Lipids.

Lipid Extraction of Cytoplasmic Membrane Vesicles Hydrolyzed with PI-PLC—MV0 from *P. lividus* was isolated from 1 ml of S10 and resuspended in 400 μl of LB. The suspension was divided into two equal parts and either left untreated or treated with 0.16 unit/ml bacterial PI-PLC for 2 h at room temperature. Total lipids were extracted from each using a modified Folch procedure (11). An internal standard mix containing 12:0/12:0 species of DAG, phosphatidic acid, phosphatidylcholine (PtdCho), phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine (500 ng each) was added to each sample followed by 1.5 ml of methanol. Chloroform (3 ml) was then added to each, and samples were mixed and left for 10 min. To split the phases, 1.5 ml of 0.88% KCl was added. The upper aqueous phase was removed and the lower organic phase containing total lipids was dried under a stream of nitrogen, dissolved in 150 μl chloroform/methanol (2:1 v/v), transferred into a silanized autosampler vial insert, dried again on a rotary vacuum evaporator, and dissolved in 15 μl of chloroform.

LC-MS Separation of Diradylglycerols (DRGs)—Isolation of diradylglycerols (diacylglycerol, alkylacylglycerol, and alkenylacylglycerol) was by gradient separation on a Luna silica column (3 μm, 1.0 × 150 mm; Phenomenex) using 100% chloroform/hexane/propan-2-ol/water (30:70:1.5:0.025), held for 3 min, then changed to 100% acetonitrile/chloroform/methanol/water (30:30:35:5) containing 5 mM ethylamine over the next 37 min at 100 μl/min. Post-column addition of 10 mM ammonium formate in methanol at 50 μl/min was required for diradylglycerol ionization. The 1,2-diradylglycerols eluted at 2–2.5 min were detected as sodiated adducts ($[M + Na]^+$) by positive electrospray ionization on a Shimadzu QP8000α single quadrapole MS (probe voltage, 1 kV; nebulizer gas, 4 liters/min N_2; curved desolvation line temperature, 275 °C).

Sperm Nuclei Permeabilization, Fertilized Egg Extracts, and MV Preparation—Sperm nuclei of *L. pictus* were permeabilized with 0.1% Triton X-100 as described previously (21). Demembranated nuclei were washed and resuspended at 10^8 nuclei/ml. Nuclei were diluted 1:25 and added to egg extracts to a final ratio of approximately one sperm nucleus per egg equivalent. Eggs and sperm were collected and eggs fertilized as described (21). Fertilized eggs were washed twice in Millipore HAWP-filtered sea water at 100 × g for 1 min in a 5403 Eppendorf swinging bucket microcentrifuge at 15 °C. At 13 min post-fertilization, 2.5 ml of packed eggs were washed twice with an equal volume of cold LB buffer and homogenized by passing twice vigorously through a 22-gauge needle. The lysate was cleared at 10,000 × *g* for 10 min in a 5417R Eppendorf microcentrifuge at 4 °C. The recovered supernatant, referred to as cytoplasmic extract or S10, includes cytosol and cytoplasmic membrane vesicles. S10 was used directly or frozen and stored in small aliquots at −80 °C.

Cytosol (S150) was prepared by fractionating the S10 at 150,000 × *g* for 3 h in a Beckman Ti50 rotor at 4 °C. S150 supernatant was used immediately or frozen in aliquots at −80 °C. The pellet of membrane vesicles (MV0) was washed twice in MWB with phenylmethylsulfonyl fluoride added freshly to a final concentration of 1 mM for 10 min at 45,000 × *g* in a Ti50 rotor. MV0 was resuspended in 0.10 of the volume of the original S10 and used immediately or quick frozen in aliquots at −80 °C.

To prepare MV1 and MV2, MV0 from 2.5 ml of packed eggs was carefully resuspended in 100 μl of TN, and then 900 μl of MWB buffer was added. Complete suspension was achieved by passing through a series of increasingly smaller plastic micropipette tips (1 ml to 20 μl). MVs were stained with $DiOC_6$ at a final concentration of 10 μg/ml and observed in a fluorescence microscope with a fluorescein filter set to confirm the absence of MV aggregates. A linear sucrose gradient of 0.1–2.0 M sucrose (15 ml) in TN buffer was made in a 16-ml Ultra-Clear Beckman centrifuge tube. The MV0 suspension was carefully applied to the top of the gradient and overlaid with mineral oil. MVs were subfractionated by sedimentation to density equilibrium at 150,000 × *g* for 20 h at 4 °C. Each band was recovered by side puncture with a 22-gauge needle on a 5-ml syringe. Median densities of MV1 and MV2 were 1.02 and 1.04 g/ml, respectively. Each band was diluted with 4 volumes of ice-cold MWB and concentrated at 150,000 × *g* for 30 min in an SW28 rotor. Each pellet was suspended in 250 μl of MWB, and the samples were aliquoted and frozen at −80 °C.

Binding, Fusion, and Inhibition Assays with MVs—To 20 μl of S10 and 1.2 μl of ATP-generating system, demembranated nuclei at a final concentration of 8×10^5 were added as described (12). After 1 h at room temperature, a 0.10 volume of $DiOC_6$ stock was added and samples observed using a Zeiss Neofluar 100× oil-immersion objective and a fluorescein filter set (λ_{ex} 460 ± 20 nm; λ_{em} > 500 nm). Images were captured in gray scale with a Hamamatsu Photonics C2400 SIT video camera using a frame image averaging and background subtraction with a Hamamatsu Argus-10 image processor.

Decondensed nuclei with bound MVs were underlaid with 0.5 M sucrose in nuclear preparation buffer and centrifuged for 20 min at 500 × g at 4 °C. Pellets were resuspended in 20 μl of S100 or S150, and

the appropriate inducer was added. Samples were incubated for 2 h. Nuclear envelope formation was scored as a continuous fluorescent rim, in contrast to the patchy appearance of bound MVs.

GTP inducer was added from the stock solution to a final concentration of 0.25 mM. PI-PLC was added to a final concentration of 0.07 unit/ml. Inhibition reactions were performed by addition of inhibitors to S150. The final concentration of ET-18-OCH_3 (Sigma) was 19.6 μM and of wortmannin (Sigma) was 25 nM. Each experiment was repeated at least three times, 100 nuclei were counted in each sample and standard deviations calculated.

For the synaptojanin1 experiments, MVs from S10 were assembled around chromatin in the presence of ATP for an hour. Fusion was induced with 1 mM GTP for 2 h. Alternatively, nuclei were treated with 1 μg/ml of the Syn1-5ptase protein for 15 min prior to the addition of GTP, and a further 1 μg/ml Syn1-5ptase was added simultaneously with GTP. An average of 24 nuclei were scored on three independent occasions and the mean and S.E. of these results calculated.

Pretreatment of MV1—MV1 was diluted in MWB, and either the inducer alone or the inducer and inhibitor was added to the reaction mix as above. Reactions were incubated for 1 h at room temperature. Vesicles were stained with $DiOC_6$ as described above and were pelleted by centrifugation at 45,000 × *g* for 15 min. The supernatant was removed, and the pellet was washed in MWB and resuspended. The pretreated MV1 was added to MV2 and S150 in presence of the ATP-generation system and decondensed nuclei. The reaction was incubated for 2 h and observed as described.

LUV Preparation—LUVs were made by extrusion with a mini-extruder from Avanti Polar Lipids, Inc. Lipids were dissolved in chloroform and mixed in the desired proportions by weight. The organic solvent was evaporated with an argon stream in a fume hood to yield a lipid film, which was hydrated in LB buffer by agitation at 4 °C. The lipid suspension was successively extruded with an Avanti Mini-Extruder (Avanti Polar Lipds) through polycarbonate filters of pore sizes 1.0 and 0.4 μm, the latter approximately the size of MVs in S10.

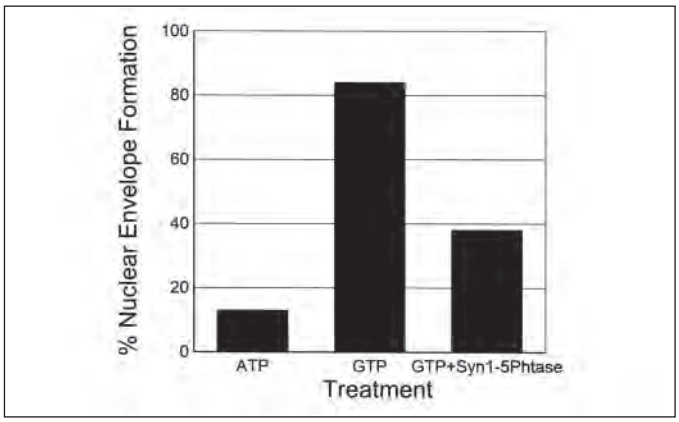

FIGURE 1. **Nuclear envelope formation is inhibited by Syn1 5-phosphatase.** After binding of membrane vesicles to sperm nuclei in cytoplasmic extracts (S10) in the presence of an ATP-generating system, aliquots were untreated or treated with a construct of the 5-phosphatase domain of synaptojanin1. GTP was added to one treated and one untreated aliquot and nuclear envelope formation scored. The phosphatase, specific for the D5-phosphate of the inositol ring, severely blocked nuclear envelope formation induced by GTP.

RESULTS

GTP-induced Nuclear Envelope Formation Is Blocked by Depletion of the Putative Substrate of the Endogenous PI-PLC—Nuclear envelope formation in a cell-free system can be induced by adding GTP or PI-PLC (12). GTP induction of nuclear envelope formation is inhibited by the PI-PLC inhibitor U73122 (14). To further demonstrate that GTP induction of nuclear envelope formation in the cell-free system requires endogenous PI-PLC activity, GTP was added to cytoplasmic extracts depleted of the PI-PLC substrate phosphatidylinositol bisphosphate.

We used a construct of synaptojanin1 (Syn1-5ptase) phosphatase (S470-R962; lacking the Sac phosphatase and proline-rich domains), which has a strong specificity for the D5-phosphate of the inositol ring. Enzyme kinetics have shown that this construct has the greatest preference for PtdIns(4,5)P218:0/20:4 over other phosphoinositide substrates (22). Nuclear envelope formation by GTP was severely inhibited by treatment with this phosphatase indicating that an endogenous PI-PLC is required for GTP induction (Fig. 1.).

Bacterial PI-PLC Treatment of Sea Urchin Membrane Vesicles Produces a Subset of Diacylglycerol Species—Bacterial PI-PLC, which hydrolyzes unphosphorylated PtdIns and has been reported to have no activity toward PtdIns(4,5)P_2 (16), was chosen for our experiments to minimize complications of rates of PtdIns phosphorylation upon the kinetics of DAG production from PtdIns(4,5)P_2. In addition this enzyme does not produce inositol 1,4,5-trisphosphate and therefore a resulting increase in free Ca^{2+} associated with this effector. Hydrolysis of sea

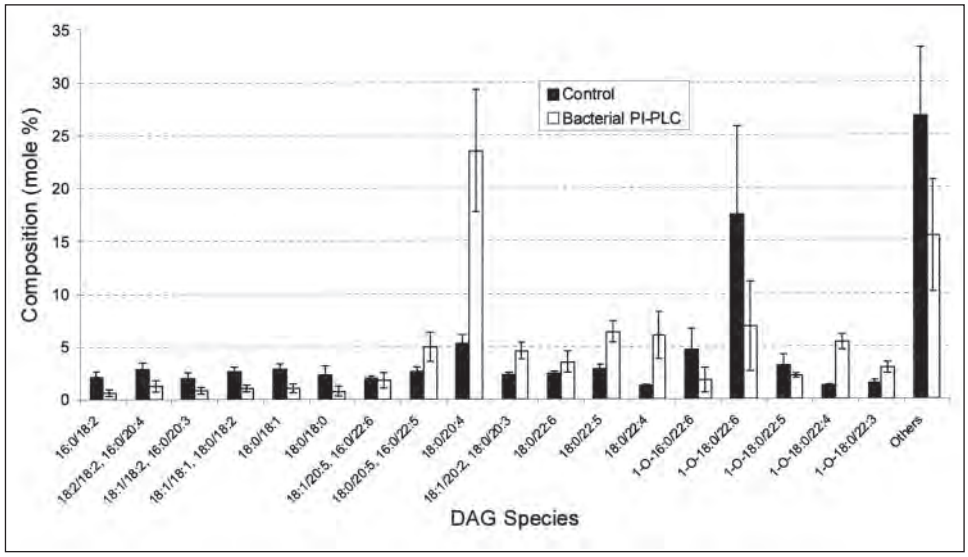

FIGURE 2. **Mass spectrometry analysis of diacylglycerol species present in isolated sea urchin cytoplasmic membrane vesicles before and after bacterial PI-PLC treatment.** *P. lividus* MV0 was isolated from 1.0 ml of S10 and resuspended in 400 μl of LB. Half was untreated, and half was treated with 0.16 unit/ml bacterial PI-PLC for 2 h at room temperature. Total lipid extracts were made and 64 diglyceride species analyzed as described under "Materials and Methods." Species that contributed less than 2% of total diglycerides in control and treated samples have been grouped as "others." Data are presented as mean mole % of the total DAG pool ± S.E. ($n = 4$).

TABLE ONE

Nuclear envelope formation *in vitro* requires MV1

Nuclear envelopes (NEs) were formed around membrane-stripped sperm nuclei in a cell-free system. MV0 contained total cytoplasmic membrane vesicles from a 10,000g supernatant of fertilized egg extract. MV1 and MV2 were separated by buoyant density and contain all of the MV0 membrane precursors necessary to form the NE (9). Formation is dependent on MV1, stimulated by GTP hydrolysis or PI-PLC activity, and inhibited, respectively, by wortmannin and ET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system. Some data points are keyed to Fig. 3.

Membranes present	Inducers	Envelope formation	Inhibitors		Envelope formation
			ET-18-OCH_3	Wortmannin	
MV0	GTP	95 ± 3%	−	+	<1%
	PI-PLC	98 ± 2%	+	−	<1%
MV1 + MV2	GTP	95 ± 6% (Fig. 3*A*)	−	+	<1% (Fig. 3*B*)
	PI-PLC	93 ± 6% (Fig. 3*C*)	+	−	<1% (Fig. 3*D*)
MV2	GTP	9% ± 7	−	−	
	PI-PLC	15% ± 7	−	−	

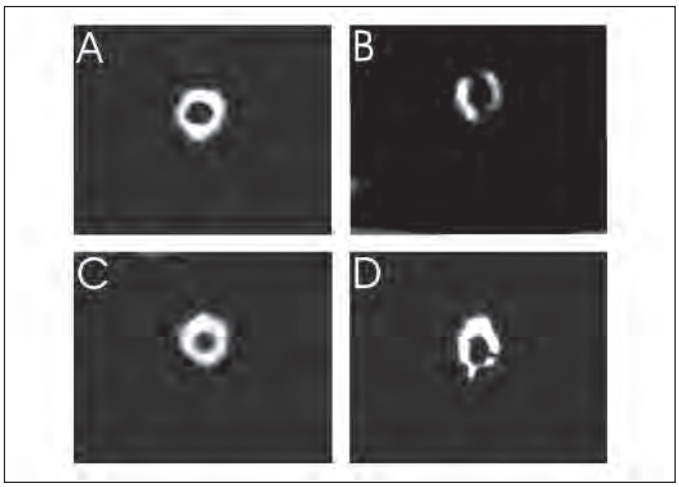

FIGURE 3. **Representative examples of nuclear envelope formation in a cell-free system.** *A*, GTP-induced nuclear envelope formation with purified MV1 and MV2. *B*, inhibition by wortmannin. *C*, bacterial PI-PLC-induced nuclear envelope formation with purified MV1 and MV2. *D*, inhibition by ET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system (see TABLE ONE).

urchin cytoplasmic membrane vesicles by bacterial PLC resulted in ~75% increase in total DAG content of the vesicles (41 ± 14 to 68 ± 18 nmol/200 μl MV0; ±S.E., $n = 4$).

The DRG species were resolved using a novel LC-MS procedure. Direct detection of underivatized DRG in very low amounts by mass spectrometry has previously been difficult to achieve, primarily because of extremely poor ionization of the lipid. To address this we developed a novel HPLC separation of the diradylglycerols with post-column addition of ammonium formate, which permitted the formation of positively charged adducts that could then be detected by ESI-MS.

In untreated membrane vesicles, the major DRG species was the alkylacyl structure 1-*O*-18:0/22:6 representing ~18% of total species (Fig. 2). Following PI-PLC hydrolysis, the species profile changed, with almost a 5-fold increase in the proportion of DAG 18:0/20:4, which became the predominant species representing ~24% of the total.

Several other species initially present in lower proportions also increased, but only two of these to comparable degrees (18:0/22:4 and 1-*O*-18:0/22:4). A few others showed increases of ~2-fold (18:0/20:5, 16:0/22:5; 18:1/20:2, 18:0/20:3; 18:0/22:5, and 1-*O*-18:0/22:4). Most species, including the predominant 1-*O*-18:0/22:6 present in untreated vesicles, decreased in relative amount ~2-fold, which would be expected if their masses had not changed, while total DAG mass almost doubled. Most of the species whose relative proportions increased after PI-PLC treatment contained relatively long chain, polyunsaturated fatty acids.

These result show that under the conditions used here, bacterial PI-PLC produces a large increase in a minor subset of diglycerides present in sea urchin membrane vesicles, in particular DAG 18:0/20:4.

Pretreatment of MV1 with PI-PLC Renders It Fusogenic—We have previously hypothesized that, upon hydrolysis, high levels of PtdIns in MV1 provide sufficient DAGs to facilitate fusion of nuclear envelope precursors (12). We tested this idea by pretreating MV1 with PI-PLC to produce diglycerides, then washing the membranes to remove soluble inositol phosphates and incubating pretreated MV1 in extracts containing the remaining egg MV nuclear envelope precursors (collectively termed MV2).

As shown in TABLE ONE, nuclear envelope formation can be induced by either GTP or PI-PLC in a complete system containing decondensed sperm nuclei, cytosol (S150), an ATP generating system and MV0 (cytoplasmic MVs, which include MV1, MV2, and other unnecessary MV fractions (9)). Similar levels of NE formation were seen in a more defined system when purified MV1 and MV2 were substituted for MV0. If MV1 was omitted, basal levels of envelope formation were detected, indicating that MV1, although it contributes a minor fraction of total nuclear envelope, is required for envelope formation. GTP-induced fusion was blocked by wortmannin (a PI 3-kinase inhibitor), and PI-PLC induced fusion by ET-18-OCH_3 (a PI-PLC inhibitor). Representative nuclei corresponding to some experiments in TABLE ONE (and keyed there) are shown in Fig. 3.

If the relevant activity of exogenous PI-PLC is the production of diglycerides in MV1, pretreatment of MV1 should make subsequent PI-PLC treatment unnecessary. As shown in TABLE TWO, MV1 prehydrolyzed with PI-PLC led to nuclear envelope formation even in the absence of the PI-PLC inducer. Envelope assembly using pretreated MV1 was no longer sensitive to ET-18-OCH_3 inhibition during incubation in extract, although ET-18-OCH_3 blocked the effect of PI-PLC pretreatment. MV2 was required, since it provides most of the nuclear envelope (9). Representative nuclei corresponding to some experiments of TABLE TWO (and keyed there) are shown in Fig. 4.

These data indicate that PI-PLC pretreatment renders MV1 fusigenic and are consistent with the notion that exogenous PI-PLC acts to induce fusion in the cell-free system by production of diglycerides in the PtdIns-rich MV1 membrane fraction at sufficient levels to induce fusion.

PtdIns-rich LUVs Can Substitute for MV1—To better define the role of MV1 in the formation of nuclear envelopes, we prepared protein-free model membranes with a phospholipid composition mimicking MV1 (75% PtdIns/25% PtdCho (w/w)). These vesicles, when added to a system containing cytosol, an ATP-generating system and sperm nuclei but no MV2, bound to the nuclei at two positions corresponding to the sites of the sperm nuclear envelope remnants, thus mimicking MV1

TABLE TWO

Pretreatment of MV1 with PI-PLC renders it fusogenic

NEs were formed around membrane-stripped sperm nuclei in a cell-free system. MV1 was pretreated with bacterial PI-PLC before addition. Formation no longer required inducer and was not inhibited by post-treatment withET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system. Some data points are keyed to Fig. 4.

MV1 pretreatment	MV2	Inducers	Envelope formation	Inhibitors ET-18-OCH_3	Envelope formation
PI-PLC	+	PI-PLC	97 ± 3%	−	
	+	−	93 ± 7% (Fig. 4*A*)	+	90 ± 8% (Fig. 4*C*)
	−	−	<1%	−	
PI-PLC + ET-18-OCH_3	+	−	<1% (Fig. 4*B*)	+	<1%

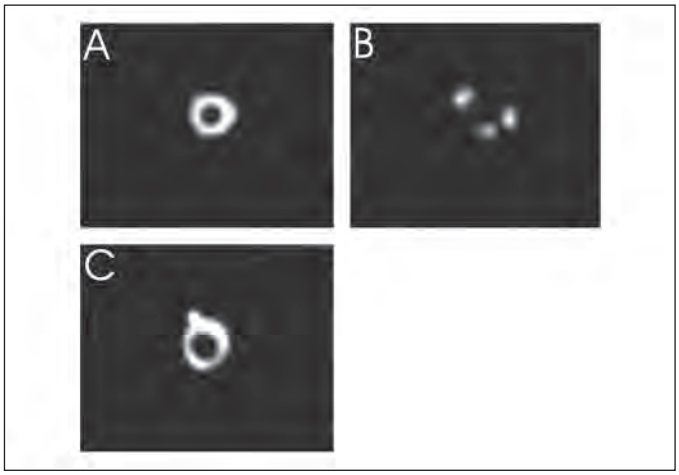

FIGURE 4. **Representative examples of nuclear envelope formation with MV1 pretreatment.** *A*, MV1 pretreated with PI-PLC, washed and added to MV2 without PI-PLC. *B*, MV1 pretreated with PI-PLC and ET-18-OCH_3 and added to MV2 without PI-PLC. *C*, MV1 pretreated with PI-PLC, washed, and added to MV2 and ET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system (see TABLE TWO).

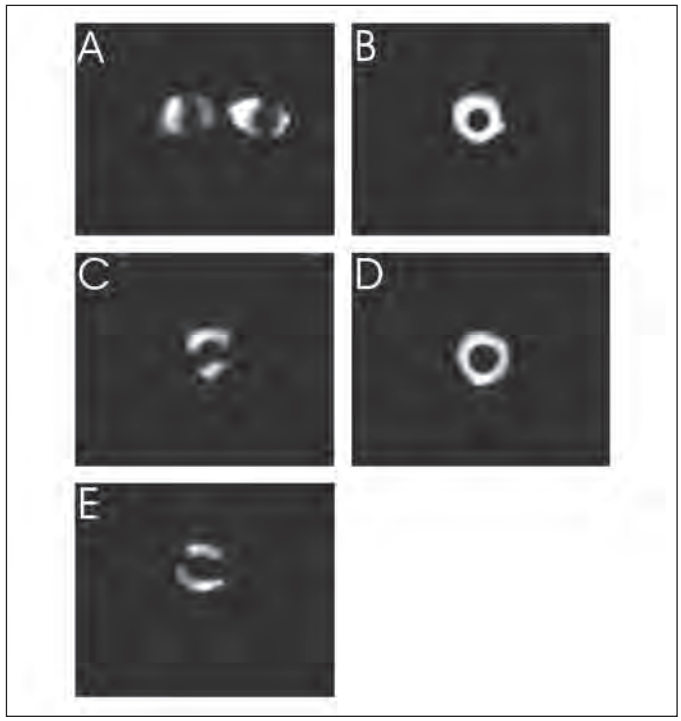

FIGURE 5. **Representative examples of nuclear envelope formation with 75% PtdIns/25% PtdCho LUVs substituted for MV1.** *A*, LUVs (75% PtdIns/25% PtdCho (w/w)) added to sperm nuclei with no MV2 or inducer added (two nuclei shown). *B*, LUVs (75% PtdIns/25% PtdCho) added to MV2 and GTP. *C*, LUVs (75% PtdIns/25% PtdCho) added to MV2, GTP, and wortmannin. *D*, LUVs (75% PtdIns/25% PtdCho) added to MV2 and PI-PLC. *E*, LUVs (75% PtdIns/25% PhCho) added to MV2, PI-PLC, and ET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system (see TABLE THREE).

(Fig. 5*A*). Binding did not occur in LB buffer (data not shown), presumably because proteins in the S150 (cytosol) are necessary to mediate specific binding.

That these LUVs are capable of assuming the role of MV1 in membrane fusion was tested as shown in TABLE THREE. TABLE ONE shows that induction by PI-PLC requires the MV1 fraction. TABLE THREE shows that when MV1 is substituted by PtdIns-rich LUVs, either GTP or PI-PLC will initiate fusion, each subject to the appropriate inhibition. No envelope formation was seen when the LUV, MV2, or inducer were omitted. Some representative examples of induction of nuclear envelope formation by GTP and PI-PLC of PtdIns-rich LUVs in the presence of MV2 are shown in Fig. 5, *B–E* (keyed in TABLE THREE).

These data indicate that synthetic membranes of 75% PtdIns/25% PtdCho can mimic many properties of MV1. These vesicles bound to the same regions as MV1 and conferred GTP or PI-PLC regulation of nuclear envelope formation.

DAG-rich LUVs Are Fusigenic—The experiments in TABLE TWO suggest that diglycerides in the hydrolyzed MV1 fraction are responsible for fusion. To directly test this idea, we made LUVs in which DAG was quantitatively substituted for PtdIns. When 75% DAG/25% PtdCho (w/w) LUVs were added to a system containing MV2, cytosol, and an ATP-generating system, envelope formation occurred with or without the inducers GTP or PI-PLC (TABLE FOUR). The percent of nuclei showing fusion was in all cases well above the background levels in the absence of LUVs or MV1 (TABLE ONE). No envelopes were formed without MV2 so that the LUVs by themselves did not form an envelope. Fusion was not altered by the inclusion of inhibitors of the normal inducers. Fig. 6 shows some representative examples (keyed in TABLE FOUR). These data indicate that DAG vesicles are fusigenic in a natural membrane system and suggest a mechanism for the fusion of PtdIns-rich vesicles upon hydrolysis by PI-PLC.

DISCUSSION

A cell-free system derived from fertilized sea urchin eggs supports nuclear envelope assembly on added membrane-stripped sperm nuclei induced by GTP hydrolysis (4, 10). Exogenously added bacterial PI-PLC can also induce nuclear membrane formation (12, Fig. 7). Each is dependent on a minor membrane fraction MV1, highly enriched in PtdIns (12). MV1 contributes only 10% of the nuclear membrane vesicle precursor population and binds exclusively to the regions of the sperm nucleus containing remnants of the sperm nuclear envelope (9). Most of the nuclear membrane is contributed by the major fraction MV2 enriched in a marker enzyme of the endoplasmic reticulum (9).

The biological function of MV1 is not known. A natural membrane fraction so rich in PtdIns had never been reported previously (11). MV1

TABLE THREE

PtdIns-rich LUVs can substitute for MV1 in nuclear envelope assembly

NEs were formed around membrane-stripped sperm nuclei in a cell-free system. MV1 was replaced by LUVs of 75% PtdIns/25% PtdCho. These LUVs mimicked MV1. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system. Some data points are keyed to Fig. 5.

LUVs	MV2	Inducers	Envelope formation	Inhibitors		Envelope formation
				Et-18-OCH_3	Wortmannin	
75% PtdIns/25% PtdCho	−	−	<1% (Fig. 5*A*)	−	−	
	+	−	<1%	−	−	
	+	GTP	90 ± 9% (Fig. 5*B*)	−	+	<1% (Fig. 5*C*)
	−	GTP	<1%	−	−	
	+	PI-PLC	93 ± 5% (Fig. 5*D*)	+	−	<1% (Fig. 5*E*)
	−	PI-PLC	<1%	−	−	

TABLE FOUR

DAG-containing LUVs are fusigenic

NEs were formed around membrane-stripped sperm nuclei in a cell-free system. MV1 was replaced by LUVs of 75% DAG/25% PtdCho. Formation no longer required inducer and was not inhibited by wortmannin or ET-18-OCH_3. These LUVs mimicked MV1 pretreated with PI-PLC. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system. Some data points are keyed to Fig. 6.

LUVs	MV2	Inducers	Envelope formation	Inhibitors		Envelope formation
				Et-18-OCH_3	Wortmannin	
75% DAG/25% PtdCho	+	−	93 ± 6% (Fig. 6*A*)	−	−	
	+	GTP	95 ± 5%	−	+	93 ± 5% (Fig. 6*B*)
	−	GTP	<1% (Fig. 6*C*)	−	−	
	+	PI-PLC	97 ± 4%	+	−	90 ± 6% (Fig. 6*D*)
	−	PI-PLC	<1%	−	−	

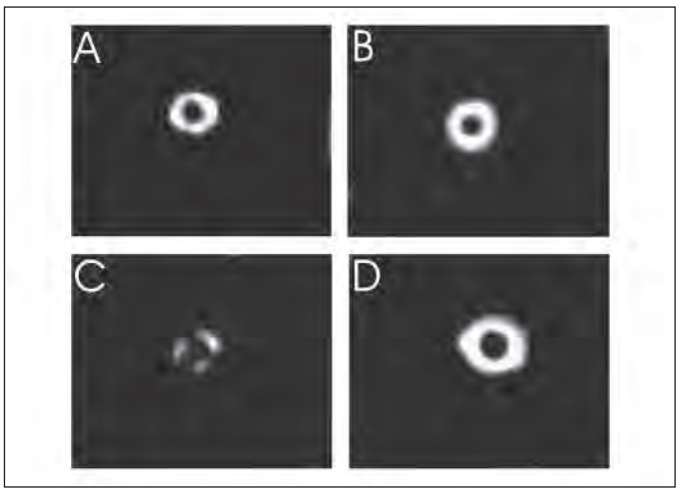

FIGURE 6. **Representative examples of nuclear envelope formation with 75% DAG/25% PtdCho LUVs substituted for MV1.** *A*, LUVs (75%DAG/25% PtdCho (w/w)) added to MV2 but no inducer added. *B*, LUVs (75% DAG/25% PtdCho) added to MV2, GTP, and wortmannin. *C*, LUVs (75% DAG/25% PtdCho) added to GTP but no MV2. *D*, LUVs (75% DAG/25% PtdCho) added to MV2, PI-PLC, and ET-18-OCH_3. All reactions contained decondensed sperm nuclei, cytosol (S150), and an ATP-generating system. (See TABLE FOUR.)

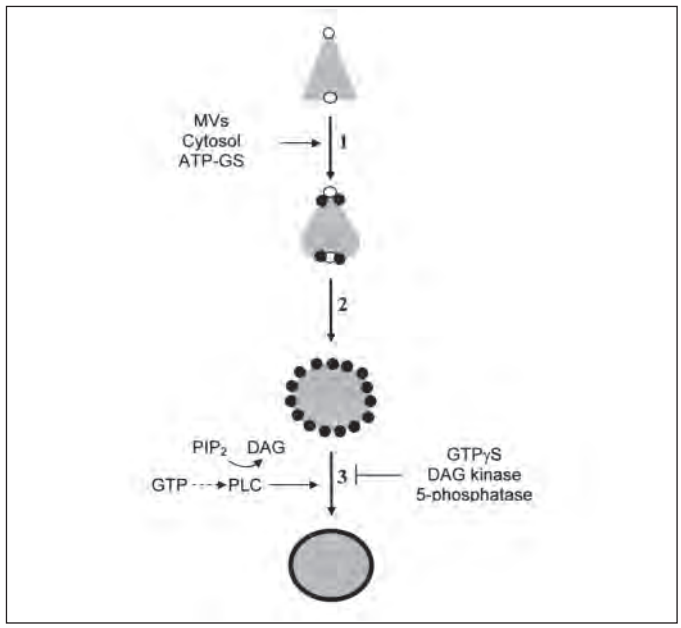

FIGURE 7. **Model of nuclear envelope assembly.** *Step 1*, condensed chromatin (*gray*) deconsenses in the presence of soluble cytosolic proteins (25). Simultaneously MVs (*black*) bind to the surface of the chromatin, initially around regions of the lipophilic structures (*white circles*), detergent-resistant membranes in the centriolar and acrosomal fossae (8). These steps require an ATP-generating system. *Step 2*, chromatin fully decondenses to a sphere ~4 μm in diameter (8), and MV binding is completed. *Step 3*, MVs fuse to form the double bilayer of the nuclear envelope. This process is initiated by GTP hydrolysis, which requires a PLC activity in the cytosol (14). Endogenous activated PLC hydrolyzes PtdIns(4,5)P_2 to DAG. Polyunsaturated DAG (mostly DAG 18:0/20:4) can be generated from MV0 (Fig. 2). DAG, by destabilizing membranes and inducing negative curvature (26, 27), and/or by recruiting non-PKC C1 domain containing proteins to the nuclear envelope, induces fusion of MVs. This final step also has a low (<50 nM) calcium dependence (4, 14). Fusion is subject to inhibition by GTPγS and BAPTA, exogenous DAG kinase, and the 5-phosphatases SopB and Syn1, which remove DAG and PtdIns(4,5)P_2, respectively, from the system (Ref. 14 and Fig. 1). In a further step, nuclei can be induced to swell in the presence of additional ATP (13), in a process requiring lamin B import to the nucleus (data not shown) (28).

binds via peripheral proteins to two specific regions of the sperm nucleus (9) containing that portion of the sperm nuclear membrane that does not break down following fertilization (23) and that is characterized by an unusual underlying osmiophilic "cup" (10). In contrast, the major MV2 subfraction binds all around the nuclear periphery through lamin B receptor, a chromatin-binding intrinsic inner nuclear membrane protein (24).

The high concentration of PtdIns in MV1 prompted us to hypothesize that its role was to generate, upon PI-PLC catalyzed hydrolysis, a local enrichment of membrane destabilizing DAG leading to initiation of fusion with the other membrane vesicles that make up the bulk of the

envelope precursors (10, 12). Several lines of evidence presented here support this hypothesis.

First, PI-PLC-mediated fusion requires that MV1 be present. Second, pretreatment of MV1 with bacterial PI-PLC renders it fusigenic. These observations are consistent with the notion that exogenous PI-PLC acts to induce fusion in the cell-free system by production of DAG in the MV1 membrane fraction. Use of synthetic membranes of known composition permitted a third test. LUVs of 75% PtdIns/25% PtdCho mimic MV1, binding to the same regions as MV1 and conferring both GTP and PI-PLC induced fusion in the presence of MV2. Moreover, similar model membranes containing 75% DAG are also fusigenic in the absence of inducers.

Although the relevant endogenous PI-PLC activities required for nuclear envelope formation (which are blocked by the inhibitor U73122 but not its inactive analog U73343 (14)) would act upon PtdIns(4,5)P_2, and the bacterial enzyme acts upon PtdIns, both produce membrane diglycerides (16). Their soluble products are $InsP_3$ or inositol 1-phosphate, respectively. To avoid the classic signaling pathway involving $InsP_3$ production and kinetic complications of kinase activities required for PtdIns(4,5)P_2 production, we chose to use the prokaryotic PI-PLC (16). Since prehydrolyzed and washed MV1 was capable of initiating nuclear envelope formation, the effects of soluble inositols were eliminated. Use of DAG-rich LUVs also ruled out a role for production of these soluble inositols from MV1 in nuclear envelope formation in the cell-free system.

Since MV2 hydrolyzed with PI-PLC was unable to form nuclear envelopes in the absence of MV1, despite containing (typically low levels of) PtdIns (11) and DAG, a requirement for MV1 may be understood on the basis of its lipid ratios. We propose that high levels of PtdIns, either in a membrane domain or in a separate set of vesicles, may provide sufficient DAG upon PtdIns hydrolysis to locally initiate membrane fusion. DAG, by virtue of its physical properties, facilitates the phase transition of the lipid bilayer from lamellar to hexagonal II. This type of phase transition induces a localized destabilization of the membrane structure, which in turn favors membrane fusion. That DAG can lead to membrane fusion is supported by several reports using synthetic membrane systems treated with PLCs (17, 19, 20).

The fatty acid chains of the PtdIns and DAG species may play an additional role. The new method for quantification of DAGs by LC-MS presented here permitted a detail of analysis and sensitivity previously unattained in a natural membrane fusion system. That the PI-PLC preferentially produces diglycerides of long chain, polyunsaturated fatty acid content is intriguing, since these are expected to have major effects on increased membrane fluidity and alteration of other structural properties that could facilitate or fine-tune membrane fusion processes.

Our current and previous work emphasizes a role for DAG that is distinct from the classical pathways of signaling utilizing protein kinase C or other C-1 domain containing receptors, which typically involve low levels of DAG (Ref. 14 and Fig. 7). Although DAG can be generated in several ways, such as through PC-PLC hydrolysis of PtdCho or PLD pathways starting with PtdCho, it is worth noting there are no known eukaryotic PC PLCs. Moreover, we have neither been able to induce NE formation with bacterial PC-PLC nor inhibit it with D609 (29), a compound that inhibits both PC-PLC and PLD activities (30). Furthermore, the molecular species composition of PtdIns changes during fusion, whereas PtdCho is identical before and after binding and hydrolysis (14). Since PI-PLC pretreatment of MV1 is sufficient to lead to NE formation in the absence of further inducers in the cell-free system, it is unlikely that other sources of DAG are necessary.

We additionally suggest an important role for lipid modification in biological membrane fusion reactions. We propose that at high DAG levels, alterations of structure of natural membranes in localized regions can affect fusion events.

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Membrane Transport, Structure, Function, and Biogenesis:

Diacylglycerol Induces Fusion of Nuclear Envelope Membrane Precursor Vesicles

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