Nuclear envelope assembly is promoted by phosphoinositide-specific phospholipase C with selective recruitment of phosphatidylinositol-enriched membranes

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Nuclear envelope (NE) formation in a cell-free egg extract proceeds by precursor membrane vesicle binding to chromatin in an ATP-dependent manner, followed by a GTP-induced NE assembly step. The requirement for GTP in the latter step of this process can be mimicked by addition of bacterial PI-PLC [phosphoinositide (PtdIns)-specific phospholipase C]. The NE assembly process is here dissected in relation to the requirement for endogenous phosphoinositide metabolism, employing recombinant eukaryotic PI-PLC, inhibitors and direct phospholipid analysis using ESI-MS (electrospray ionization mass spectrometry). PtdIns (phosphatidylinositol) species analysis by ESI-MS indicates that the chromatin-bound NE precursor vesicles are enriched for specific PtdIns species. Moreover, during GTP-induced precursor vesicle fusion, the membrane vesicles become partially depleted of the PtdIns 18:0/20:4 species. These data indicate that eukaryotic PI-PLC can support NE formation, and the sensitivity to exo-

genous recombinant PtdIns-5-phosphatases shows that the endogenous PLC hydrolyses a 5-phosphorylated species. It is shown further that the downstream target of this DAG (diacylglycerol) pathway does not involve PKC (protein kinase C) catalytic function, but is mimicked by phorbol esters, indicating a possible engagement of one of the non-PKC phorbol ester receptors. The results show that ESI-MS can be used as a sensitive means to measure the lipid composition of biological membranes and their changes during, for example, membrane fusogenic events. We have exploited this and the intervention studies to illustrate a pivotal role for PI-PLC and its product DAG in the formation of NEs.

Key words: diacylglycerol, electrospray ionization mass spectrometry, membrane fusion, nuclear envelope, phosphatidylinositol, phosphoinositide-specific phospholipase C.

INTRODUCTION

Membrane fusion and fission lie at the heart of membrane trafficking events essential for diverse cellular processes, including endocytosis, secretion, ER (endoplasmic reticulum) remodelling and NE (nuclear envelope) formation. Most attention on membrane fusion has been paid to the role of proteins, for example SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) and their regulators [1] in facilitating fusion, but lipid metabolism and composition may also play important roles [2–5]. For example, PtdIns(3)*P* helps recruit fusion machinery, including monomeric GTPases, during early endosome vesicle fusion [4]. Work with synthetic membranes shows that certain lipid classes, such as phosphatidylethanolamine or DAG (diacylglycerol), strongly promote fusion [6–8]; others inhibit [9]. Membrane structures called ‘stalks’ occur at the intermediate stages of fusion [10,11]. Rapid, asymmetric and localized generation of DAG by PLC (phospholipase C) is at the origin of the formation of the ‘stalks’ in synthetic membrane experiments. Although DAG alone may trigger synthetic membrane fusion, in biological membranes the fusion process also depends

upon the activity of proteins such as SNAREs and small GTPases, in addition to DAG and other lipids [12].

Previous results from modelling studies [10] have illustrated that the stalk energy is mostly independent of the distance between the fusing membranes, but dependent on the lipid composition of the membrane monolayers. Moreover, the elastic energy of the monolayers originates from the hydrocarbon chains, and the manner in which the fatty acid chains are stretched and tilted plays a key role in formation of the stalk intermediate. Recently, the importance of lipid components in alteration of membrane curvature leading to membrane fusion and fission in synthetic giant unilamellar vesicles has been demonstrated elegantly [13]. Although roles of individual membrane lipids are emerging in a wide range of signalling events within cells [2,5,14], the relevance of phase behaviours and thermotropic properties of individual phospholipid molecular species to membrane fusion has not been elucidated, in particular the contribution of PtdIns (phosphatidylinositol) to fusogenic processes.

NE disassembly and reassembly occur during each mitosis in typical eukaryotic somatic cells, and also occur in sperm nuclei following fertilization. Cell-free systems from eggs of several

Abbreviations used: ATP-GS, ATP-generating system; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N,N*-tetra-acetic acid; DAG, 1,2-diacylglycerol; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; PtdCho, phosphatidylcholine; DMPC, dimyristoyl-PtdCho; PtdIns, phosphatidylinositol; DPPI, dipalmitoyl-PtdIns; ER, endoplasmic reticulum; ESI-MS, electrospray ionization mass spectrometry; GAP, GTPase-activating protein; GTP[S], guanosine 5'-[γ-thio]triphosphate; LB, lysis buffer; MV, membrane vesicle; NE, nuclear envelope; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; SXN, nuclei preparation buffer; Syn1-5ptase, synaptojanin 1 phosphatase; TN, Tris/NaCl buffer.

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species have been used to dissect biochemically the dynamics of NE assembly [15–17]. In these systems, extracts containing cytosol and various MVs (membranous vesicles) are incubated with demembranated sperm nuclei. Sperm chromatin decondense, and MVs bind to the chromatin surface. The MVs subsequently fuse around the decondensed chromatin to form an NE with pores that is capable of protein import [28]. Fusion normally requires GTP hydrolysis. The subfractionation of sea urchin MVs has previously shown distinct subpopulations [18]. Two-dimensional NMR spectroscopy studies have shown that MV1 is biologically unique, comprising phosphoinositides (≈ 80 mole%) and PtdCho (phosphatidylcholine) only [2], whereas MV2 α and MV2 β are derived from the Golgi and ER respectively, and as such have lipid compositions and display protein markers consistent with these origins [2,18].

We have suggested previously a role for PtdIns in NE formation in the sea urchin cell-free system [2]. When PtdIns is hydrolysed by an exogenously added bacterial PI (phosphoinositide-specific)-PLC, NE assembly takes place in the absence of the GTP inducer, and we have suggested that formation of the membrane-destabilizing product of PtdIns hydrolysis, DAG, leads to membrane fusion [2]. Unlike bacterial PI-PLC, known eukaryotic PI-PLCs use PtdIns(4,5) P_2 , not PtdIns, as a substrate, but nonetheless generate DAG and inositol polyphosphates [19,20]. Sea urchin PLC δ and PLC γ isotypes have been identified, although to date, their possible roles in NE formation have not been investigated [21–23]. We have therefore set out to analyse by ESI-MS (electrospray ionization mass spectrometry) the PtdIns molecular species present before and after NE formation, and having established differences, investigated the routes and consequences of this metabolism.

MATERIALS AND METHODS

Buffers and reagents

Sample buffer was 20 mM Tris/HCl (pH 7.4)/150 mM NaCl/100 mM NaF/10 mM Na₄P₂O₇/10 mM EDTA/1 % Triton X-100. SXN (nuclei preparation buffer), TN (Tris/NaCl buffer) and LB (lysis buffer) were prepared as described previously [24]. The ATP-GS (ATP-generating system) comprised 1 mM ATP, 20 mM creatine phosphate and 1 mg/ml creatine kinase in LB. *Lytechinus pictus* sea urchins were purchased from Marinus (Long Beach, CA, U.S.A.), *Paracentrotus lividus* sea urchins were obtained from Universidade Lusófona, Lisbon, Portugal. The former species was used in experiments unless otherwise stated. Preparation of PLC γ 2 (PI-PLC), purified to apparent homogeneity with *in vitro* specific activity towards PtdIns(4,5) P_2 of 120–180 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, was made using a baculovirus system [25]. The inositol 5-phosphatase SopB was obtained from Dr E. Galyov (Institute for Animal Health, Compton, Berks., U.K.), and Syn1-5ptase (synaptojanin 1 phosphatase) construct (S470-R962; lacking the Sac phosphatase and proline-rich domains) was from Dr R. Woscholski (Imperial College London, U.K.). Recombinant DAG kinase, BAPTA (bis-(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid), Gö6976, PMA, U73122 and U73343 were purchased from Calbiochem. BIM I was purchased from Alexis Corporation. Bacterial PtdIns-specific PI-PLC, ATP, GTP[S] (guanosine 5'-[γ -thio]triphosphate), creatine phosphate, creatine kinase and GTP were purchased from Sigma. The lipophilic membrane probe DiOC₆ (3,3'-dihexyloxycarbocyanine iodide) was obtained from Molecular Probes, and the lipid standards DPPI (dipalmitoyl-PtdIns) and DMPC (dimyristoyl-PtdCho) were from Echelon and Sigma respectively. Regarding antisera, anti-Ran-GTPase was purchased from BD

Transduction Labs, anti- α -tubulin was from Sigma and mA414 was supplied by Dr Sara Nakielnny (Cancer Research UK, London, U.K.).

Nuclei and egg extracts

Isolation and permeabilization of sperm nuclei were adapted from methods described previously [15,26], with a modified sonication step extended from 2 to 6 min. Nuclei were finally resuspended in freezing buffer [SXN supplemented with 0.16 % (w/v) BSA and 16.5 % (v/v) glycerol], frozen in liquid nitrogen and stored at -80°C . S10 cytoplasmic (G₁ phase) from eggs at 10 min post-fertilization and S100/S150 cytosolic egg extracts were prepared as described previously [2].

ESI-MS of PtdCho and PtdIns lipids

S10 (100 μl) was combined with 15 μl of demembranated nuclei and 6 μl of ATP-GS and incubated for 1 h at room temperature. Samples were underlain with 0.5 M sucrose in TN and centrifuged at 900 *g* at 4°C for 20 min to separate chromatin-bound (pellet) and unbound (supernatant) membranes. The supernatant (S1) was retained, and the pellet was resuspended in 100 μl of S150 cytosolic extract. This was divided, one-half was left untreated and the other was treated with 1 mM GTP for 2 h, after which both were centrifuged as above. The pellets (resuspended in 200 μl of TN) and S1 supernatant were added to 4 ml of ice-cold acidified chloroform/methanol (2.5:1), together with 5 nmol of DPPI and 16 nmol of DMPC as internal standards (Previously, DMPC has been used for calibration of PtdCho species [27]). Extracts were probe-sonicated for 10 s, and filtered through a 0.2 μm membrane. Phase separation was induced with 0.2 vol of K₄EDTA (0.2 M, pH 6.0), followed by centrifugation at 800 *g* at 4°C for 15 min [28]. The lower phase was removed and dried under nitrogen.

All ESI-MS analyses were performed on a triple-quadrupole tandem mass spectrometer (Quattro Ultima, Micromass, Manchester, U.K.) equipped with an ESI interface. Total phospholipid extracts were dissolved in methanol/chloroform/water/ammonia (7:2:0.8:0.2, by vol.) for single-stage and tandem-MS analysis of PtdCho and PtdIns. Samples were dissolved in 20 μl of solvent and analysed by nanospray ESI-MS. Dry argon was used as the collision gas (3.5×10^{-3} mbar; 1 bar $\approx 10^5$ Pa). All data were recorded at mass resolution as a cumulative ion count of 10–50 scans/collection, with a scan time of 12 s.

PtdCho and PtdIns species were detected by positive or negative ionization respectively. Following fragmentation with argon gas, PtdCho molecules produced a fragment with $m/z = +184$, corresponding to the protonated phosphocholine head-group, and precursor scans of the $m/z = 184$ moiety provided the diagnostic determination of PtdCho. Collision gas-induced fragmentation of PtdIns species generated a common dehydrated inositol phosphate fragment, with $m/z = -241$, and precursor scans of this $m/z = -241$ moiety provided diagnostic determination of PtdIns. Data were acquired and processed using MassLynx NT software. The predominant PtdIns molecular species reported for the major ion peaks resolved were determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization. The relative intensities of these fatty acyl fragment ions permitted their gross assignment to either the *sn* – 1 or *sn* – 2 positions of the glycerol backbone of the individual phospholipid species.

GraphPad InStat software (version 3.0 for Mac-2001) was used for the statistical analysis. An unpaired *t* test with Welch correction was used to determine significances of variation in phospholipid, with *P* values determined at the 95 % confidence interval.

Binding and fusion assays

For S10 MV binding assays, 20 μ l of S10, 1.2 μ l of ATP-GS and 3 μ l of demembrated sperm nuclei resuspended in TN (1.2×10^6 nuclei/20 μ l) were combined and incubated for 1 h at room temperature. Subsequently, the fusion of bound MVs was induced with 1 mM GTP, 25 nM PMA or 100 ng/ml eukaryotic PI-PLC over 2 h. Fusion requires a concentration of ≤ 100 nM Ca^{2+} in the reaction mixture [29]. A dose-dependent assay with PMA and PI-PLC was performed to determine the optimum concentration for the fusion assays. To visualize the bound/fused MVs, reactions were labelled with 0.013 mg/ml DiOC₆ and viewed under a 100 $\times$ oil-immersion objective. DiOC₆ was excited with the 488 nm line of an Argon/Krypton laser, and the resulting fluorescence was separated using a combination of a dichroic beam-splitter (Q495LP; Chroma) and a HQ510/20 nm emission filter. Alternatively, decondensed nuclei were observed using phase contrast. Images were captured with a Hamamatsu Orca camera and processed in OpenLab. Twenty-five nuclei were scored on three independent occasions on the basis of them having a continuous fluorescent rim, and the means and S.E.M. of these results were calculated.

In inhibitor studies utilizing U73122 or U73343 (30 μ M), GTP[S] (2 mM), BAPTA (5 mM), Gö6976 (1 μ M), BIM I (5 μ M), DAG kinase (1 μ g/ml) and SopB (1 μ g/ml), these agents were applied to reactions for 15 min before the addition of the fusion-triggering agent. The PKC (protein kinase C) inhibitors were used at concentrations at which their inhibitory activity had already been verified (P. J. Parker, personal communication). Alternatively, U73122 was applied 15 min after GTP (referred to as GTP + U73122 in the text), or SopB was added simultaneously to ATP (referred to as SopB + GTP). Syn1-5ptase was added simultaneously to ATP-GS. U73122 doses applied in the present study did not exceed 30 μ M (IC_{50} values of 5–10 μ M are routinely reported), to preserve the specificity of this compound.

Detection of proteins in NEs

NEs were assembled on demembrated nuclei sequentially with ATP-GS and 50 nM PMA. Nuclei were induced to swell by the addition of 2 mM ATP-GS for a further 30 min [2]. Swollen nuclei were visualized by phase contrast microscopy, as described above. To collect swollen nuclei, the reaction mixture was centrifuged through 1 vol of 0.5 M sucrose in TN (supplemented with 5 mM MgCl_2) for 12 min (500 g, 4°C). Nuclei were washed once in LB and lysed in sample buffer. Protein content was determined using the Bradford assay, and samples were boiled at 95°C for 5 min. Of the mixture, 2.5 μ g of S10, swollen nuclei and unbound material were separated by SDS/PAGE, along with demembrated nuclei (normalized to the amount present in swollen nuclei sample), transferred to PVDF, and α -tubulin (1:2500 dilution), Ran GTPase (1:7500 dilution) and Nup62 (1:10 000 dilution) proteins were probed with monoclonal antisera. Chemiluminescence detection took place after incubation with horseradish-peroxidase-linked antisera and ECLTM (Amersham).

RESULTS

Nuclear-bound membranes are enriched in PtdIns and qualitatively different from non-nuclear membrane lipids

Non-nuclear cellular membrane lipids from the cell-free egg extracts were analysed by ESI-MS. The major phospholipids quantified in all membranes were PtdCho and PtdIns; other acidic phospholipids and phosphatidylethanolamine were only minor

Figure 1 Cytoplasmic MV lipid analysis by ESI-MS

Total MVs of cytoplasmic extracts (MV0) were analysed by ESI-MS. Molecular species of PtdIns were detected by diagnostic precursor scan of the dehydrated inositol phosphate fragment ion ($m/z = -241$), while molecular species of PtdCho were detected by equivalent scan of the PtdCho fragment ion ($m/z = +184$).

components (results not shown). Figure 1 illustrates analysis by ESI-MS of PtdIns and PtdCho spectra. Comparison of the spectra shows that molecular species from both phospholipid classes virtually all contained polyunsaturated fatty acids at their $sn - 2$ positions. The molecular composition of MV PtdIns was qualitatively comparable with that reported for most mammalian cells *in vivo*, and was dominated by the arachidonoyl-containing species PI-18:0/20:4 and PI-18:1/20:4. [Phospholipid molecular species are designated as PXA: n /B: n , where A and B are the number of carbon atoms in the fatty acyl groups esterified at the $sn - 1$ and $sn - 2$ position of the glycerol backbone respectively, and n is the number of unsaturated double bonds in each fatty acid chain. X is the lipid head-group, choline (PC) or inositol (PI)]. In striking contrast, PtdCho was composed almost exclusively of plasmalogen species, where the $sn - 1$ fatty acid is attached to the glycerol backbone by an ether instead of an ester bond (e.g. PC16:0a/20:5, where 'a' denotes an ether as opposed to an ester bond), with diacyl-PtdCho species as only minor components.

In order to quantify alterations to phospholipid concentrations and molecular compositions that might occur upon NE formation, nuclei were incubated in cytoplasmic extracts under 'binding' conditions (+ATP) and recovered by centrifugation. Control pellets from nuclei in TN buffer, total membranes (MV0) and cytosolic extracts (S100) with no nuclei added were analysed in parallel to determine the potential source of the phospholipids that contribute to NE formation. After addition of known amounts of phospholipid standards, fractions were quantified by ESI-MS. The demembrated nuclei in TN buffer used in these experiments contained only 0.11 nmol of PtdCho and no detectable amounts of PtdIns. After removal of membranes from S10 by centrifugation, the S100 fraction contained only 4.1% and 5.8% respectively of the PtdCho and PtdIns present in the initial cytoplasmic extract. These results indicate that the main phospholipid source was from the MVs derived from the fertilized cytoplasmic extracts, rather than demembrated nuclei themselves or the cytosol.

Under binding conditions with ATP, the pellet and supernatant (S1)-containing unbound membranes were analysed. The

Table 1 PtdCho and PtdIns quantification of unbound MVs (S1), bound MVs (ATP pellet) and assembled NEs (GTP pellet)

Cytoplasmic MVs from S10 were bound to isolated sperm nuclei *in vitro* with ATP. Unbound material (S1) was separated from the nuclei by centrifugation. Half of the resuspended pellet was treated with GTP to induce NE formation (GTP-bound membranes). Lipids were extracted from 100 % of S1, and from total ATP and GTP pellets and analysed by ESI-MS. PtdIns and PtdCho were quantified against standards. Data are expressed as mean lipid amounts calculated from three independent measurements.

	S1 unbound membrane	ATP bound membrane	GTP bound membrane
PtdIns (nmol)	22.76	0.98	1.08
PtdCho (nmol)	244	3.96	5.64
PtdIns/PtdCho	0.08	0.20	0.16

amounts of PtdIns and PtdCho and the PtdIns/PtdCho ratios were calculated. The amount of PtdIns and PtdCho in the S1 was 11-fold and 25-fold greater respectively than in the total pellet (ATP and GTP pellet combined; Table 1), consistent with previous results indicating that only a fraction of cytoplasmic MVs binds to the nuclei [18]. The ratio of PtdIns/PtdCho in the bound membranes was 2.5-fold higher than in S1 (0.20 to 0.08; Table 1). In addition to this PtdIns enrichment, the bound membranes were significantly different from the unbound in PtdIns molecular species composition (Figure 2A). For example, PI-18:0/20:4 was relatively depleted in bound membranes, whereas PI-18:0a/20:5 and PI-18:0a/22:6 were enriched. No variation in PtdCho molecular species between unbound and bound membranes was observed (Figure 2B).

The PtdIns composition alters upon NE formation

Having demonstrated selectivity for binding of PtdIns, but not PtdCho, to nuclei in response to ATP, we next investigated whether there were any further specific changes to either PtdIns or PtdCho in response to GTP-induced NE assembly. Comparison of PtdIns molecular species (Figure 2A) shows further alterations to the PtdIns composition following addition of 1 mM GTP. The fractional content of PI-18:0/20:4 was significantly lower compared with both S1 and ATP-dependent bound membranes, but that of the other major PtdIns species PI18:1/20:4 remained unchanged. Again, there was a selective increased content of the same two alkyl PtdIns species (PI-18:0a/20:5 and PI-18:0a/22:6) after GTP treatment. In comparison with the selective response observed for PtdIns, GTP caused no changes to PtdCho composition. The final PtdCho composition of assembled NEs after GTP treatment was identical both to ATP-dependent bound vesicles (Figure 2B) and to unbound MVs.

During the process of GTP-induced NE formation, the most striking change was the decrease of PI-18:0/20:4, the precursor to PtdIns(4,5) P_2 -18:0/20:4, the predominant physiological substrate for endogenous PI-PLC *in vivo* (B. Larijani, unpublished work). This finding, together with the recent identification of sea urchin PLCs [21,23], led us to investigate whether PLC was contributing to NE assembly.

Recombinant PI-PLC induces NE formation in extracts in the absence of added GTP

In cell-free extracts from fertilized sea urchin eggs, NE formation involves two steps. In the first, at least three MV populations from the egg extracts bind to exogenously added demembrated sperm nuclei in an interaction that is ATP-dependent [29]. Formation of NEs results from fusion of bound vesicles upon addition and hydrolysis of GTP (fusion reaction; Figure 3A and Table 2) [30].

Figure 2 The molecular species composition of PtdIns changes upon binding of MVs to chromatin and after NE assembly

The quantities of PtdIns (A) and PtdCho (B) species in the S1 (black bars), ATP pellet (grey striped bars) and GTP pellet (grey solid bars) were determined as described in Table 1, and expressed as the mole% composition of total PtdIns or PtdCho. Data are expressed as means $\pm$ S.D. from three independent experiments; * $P < 0.05$ at the 95 % confidence interval.

In addition to GTP, NE formation can also be induced by addition of a bacterial PtdIns-specific PI-PLC [2], which hydrolyses unphosphorylated PtdIns to DAG and inositol 1-phosphate. Such an activity is unknown in eukaryotic cells, whose PI-PLCs greatly prefer PtdIns phosphorylated at the D-4 and D-5 positions [PtdIns(4,5) P_2], yielding DAG and Ins(1,4,5) P_3 [31]. The latter two products classically lead to membrane-associated PKC activation and Ca^{2+} release from intracellular stores respectively. To see whether a eukaryotic enzyme could also promote NE formation, we added PI-PLC to the cell-free system in the absence of GTP at a concentration selective for PtdIns(4,5) P_2 hydrolysis (100 ng/ml). This enzyme was almost as effective as the bacterial enzyme [2]: both induced NE formation well above the ATP control (87 % and 98 % of nuclei respectively compared with 7 %; Figure 3A and Table 2).

Given that bacterial PLC and eukaryotic PI-PLC only share DAG as a common product, it seems highly likely that the formation of DAG rather than the different inositol products is the important branch of the hydrolytic pathway leading to NE formation. Indeed, ESI-MS analysis of PtdIns species in bacterial PI-PLC-treated (0.16 unit/ml) MV0 shows a significant hydrolysis of

Figure 3 PI-PLC and PMA induce NE formation *in vitro* via the PtdIns(4,5) P_2 -DAG pathway

(A) MVs from fertilized cytoplasmic extracts of sea urchin eggs were bound *in vitro* to sperm nuclei with ATP-GS (ATP). NE formation was induced by either GTP, eukaryotic PI-PLC (eukPI-PLC) or PMA. DiOC₆-labelled nuclei, after centrifugation through sucrose, were visualized by phase and fluorescence microscopy at 100 $\times$ magnification. Images are representative of three independent experiments. 'NE assembly' is defined by a smooth fluorescent rim as opposed to patchy fluorescence of 'bound' membranes. Scale bar = 5 μ m. ATP and PMA images taken are of *P. lividus* nuclei. (B) Total lipid extracts from untreated MVO and bacterial PI-PLC-treated MVO were analysed by ESI-MS. Molecular species of PtdIns were detected by diagnostic precursor scan of the dehydrated inositol phosphate fragment ion (m/z = 241). Note the hydrolysis of several PtdIns species by bacterial PLC, as indicated by the arrows.

Table 2 Quantification of NE formation induced by GTP, phosphoinositide-specific PI-PLC and PMA

NEs were assembled on sperm nuclei with ATP-GS (ATP) *in vitro*. NE formation was induced with GTP, phosphoinositide-specific PI-PLC (eukPI-PLC) or PMA. Alternatively, before the addition of inducers, reactions were incubated for 15 min with the inhibitors DAG kinase, U73122/U73343, SopB, BIM I, Gö6976 or BAPTA. Syn1 type II catalytic domain (Syn1) was added simultaneously to ATP-GS. After 2 h incubation, nuclei were visualized as described in Figure 3, and 25 nuclei scored for complete NEs. Data are expressed as the means $\pm$ S.E.M. from three independent experiments.

Treatment	Nuclei with complete NE formation (%)
ATP	7 $\pm$ 5
GTP	97 $\pm$ 1
Bacterial PLC	98 $\pm$ 2
EukPI-PLC	87 $\pm$ 4
DAG kinase + eukPI-PLC	48 $\pm$ 2
U73122 + eukPI-PLC	63 $\pm$ 6
U73343 + eukPI-PLC	93 $\pm$ 4
SopB + eukPI-PLC	48 $\pm$ 4
Syn1 + eukPI-PLC	45 $\pm$ 7
PMA	88 $\pm$ 4
PMA + BIM I	96 $\pm$ 0
PMA + Gö6976	95 $\pm$ 3
BAPTA + eukPI-PLC	4 $\pm$ 4
BAPTA + PMA	18 $\pm$ 7

PtdIns species (predominantly PI-18:1/20:4, PI-18:0/20:4 and PI-18:0/22:5) to the fusogenic lipid DAG (Figure 3B). The inhibition of NE formation by 1 μ g/ml DAG kinase (87% to 48%; Table 2) in the presence of eukaryotic PI-PLC confirms this hypothesis (DAG kinase converts DAG into phosphatidic acid).

The PI-PLC activity could be partially inhibited with 30 μ M U73122, a specific inhibitor (IC₅₀ 5 μ M) of PLC-mediated hydrolysis of PtdIns(4,5) P_2 , but not the inactive analogue U73343. As described above, eukaryotic PI-PLCs strongly prefer PtdIns(4,5) P_2 as substrate. We therefore hypothesized that if PI-PLC was inducing NE formation via DAG generation from PtdIns(4,5) P_2 hydrolysis, the depletion of PtdIns(4,5) P_2 from NE precursor vesicles would inhibit NE formation. To test this hypothesis, we utilized the inositol 5-phosphatases SopB and Syn1-5ptase. SopB has previously been described as a PtdIns(4,5) P_2 -depleting enzyme [32]. The Syn1-5ptase type II phosphatase domain (Ser⁴⁷⁰–Arg⁹⁶²) has a preference towards 'natural' PtdIns(4,5) P_2 -18:0/20:4 over other substrates [33]. Thus both these enzymes have an activity against the D-5 position of the inositol ring, which generates PtdIns4P. We used SopB and Syn1-5ptase (1 μ g/ml) as tools to deplete NE precursor vesicles of PtdIns(4,5) P_2 . Under these conditions, the ability of PI-PLC to promote NE formation decreased with SopB or Syn1-5ptase by approx. 50% in both cases (Table 2).

Induction of NE formation by DAG is not mediated by PKC

Given that the common product of bacterial PtdIns-specific PI-PLC and eukaryotic PI-PLC is DAG, we hypothesized DAG was responsible for the membrane fusion. PMA, a phorbol ester analogue of DAG, can be used to mimic the effects of DAG. PMA (25 nM) added to our system in the absence of GTP was fully effective at inducing NE formation (Figure 3A and Table 2). Classically, DAG exerts its effects through recruitment and activation of PKC activity. However, non-PKC receptors of DAG/PMA have been described [34]. Moreover, DAG can exert direct effects on membrane structure that result in localized membrane fusion [6–8]. To investigate whether NE formation was mediated by PKC activity, we used two PKC inhibitors: BIM I (5 μ M), a pan-specific inhibitor of PKC, and Gö6976 (1 μ M), an inhibitor with a

Table 3 Inhibition of NE formation by GTP[S], the PLC inhibitor U73122 and the PtdIns(4,5) P_2 phosphatase SopB

NEs were assembled on sperm nuclei *in vitro* with ATP-GS (ATP). MV fusion was induced with GTP, phosphoinositide-specific PI-PLC (eukPI-PLC) or PMA. Prior to the addition of the inducers, reactions were also incubated for 15 min with U73122 (U73122 + GTP) or GTP[S] (GTP[S] + eukPI-PLC; GTP[S] + PMA). Alternatively, U73122 was added 15 min after GTP (GTP + U73122). SopB was added simultaneously with the ATP-GS. After 2 h of incubation, nuclei were visualised as described in Figure 3, and 25 nuclei were scored for the complete NEs. Data are expressed as the means $\pm$ S.E.M. from three independent experiments.

Treatment	Nuclei with complete NE formation (%)
ATP	7 $\pm$ 3
GTP	97 $\pm$ 1
GTP[S] + eukPI-PLC	23 $\pm$ 13
GTP[S] + PMA	33 $\pm$ 5
U73122 + GTP	33 $\pm$ 7
GTP + U73122	49 $\pm$ 6
SopB + GTP	17 $\pm$ 4

strong preference for PKC α and PKC β [35,36]. Neither inhibitor added to the system in the presence of PMA had a significant effect in reducing PMA-induced NE formation, indicating that PMA was not acting through PKC activation (Table 2).

Although Ins(1,4,5) P_3 generation and consequent Ca^{2+} release triggered by PI-PLC is not critical for NE formation, free Ca^{2+} might still be required for NE assembly. In the presence of the calcium chelator BAPTA (5 mM), PI-PLC induction of NE formation was reduced to background levels (Table 2). This might reflect a requirement for PI-PLC itself. However, PMA induction was also almost completely inhibited, suggesting that free Ca^{2+} is required for NE formation in this system.

Relationship of GTP and exogenous PI-PLC induction of NE formation

The relationship between exogenous PLC action and the GTP inducer was investigated by use of GTP[S], a non-hydrolysable analogue of GTP. Exogenous PI-PLC induction was strongly affected by inclusion of 2 mM GTP[S] (Table 3). This indicates that the induction of fusion by PtdIns(4,5) P_2 hydrolysis via an exogenous source is blocked when GTP hydrolysis is inhibited. Certain proteins that contain GTPase-binding domains, such as Rac-GAP (GTPase-activating protein) and Ras-GAP [37,38], as well as regulators of SNAREs (Munc13) [34], can bind to DAG and phorbol esters. Thus NE formation may be dependent on association of such receptors with DAG. We therefore postulated that DAG promotes the GTPase activity needed for NE assembly, so inhibiting GTP hydrolysis should impair NE formation, even if sufficient DAG is present. This was consistent with the inhibition of PMA-induced fusion by GTP[S]. Additionally, in the presence of SopB, GTP did not induce fusion (Table 3). These data indicate that DAG, as well as GTPase, activity is required for the regulation of NE assembly.

Inhibition of endogenous PLC activity in extracts

To determine whether GTP induction of NE formation in egg extracts requires endogenous PLC activity acting on PtdIns(4,5) P_2 , induction was carried out in the presence of U73122. This reagent inhibited NE formation by two-thirds and one-half when added 15 min before or after GTP respectively (Table 3). These results indicate that GTP induction requires endogenous PLC activity. In further support of this interpretation, SopB phosphatase treatment of membranes inhibited NE formation to 17%, suggesting that the substrate of the endogenous enzyme is PtdIns(4,5) P_2 .

Figure 4 PMA induction results in recruitment of RanGTPase and a nuclear pore protein

NEs were assembled in the presence of ATP-GS and 50 nM PMA as described in the text (Decondensed). Nuclei were treated for an additional 30 min with 2 mM ATP-GS to induce nuclei swelling. (A) Decondensed and swollen nuclei were viewed by phase microscopy at 100 $\times$ magnification. Scale bar = 5 μ m. (B) Swollen nuclei were collected by centrifugation, washed and resuspended in SDS sample buffer. The protein content of S10, 0.1% nuclei, swollen nuclei and unbound material was assessed by Western analysis with antibodies specific for RanGTPase, the nuclear pore complex protein Nup62 (mAb414) and α -tubulin. Data are representative of two independent experiments.

Functionality of PMA-induced NEs

NEs induced by GTP have both protein and lipid content [29,39], and only nuclei with completely formed NEs undergo swelling from 4 to 8 μ m with additional ATP-GS [2,29,39]. Thus, if PMA-induced NEs were fully formed, they should contain nuclear membrane-associated proteins and swell. We therefore induced NE formation with ATP-GS and 50 nM PMA sequentially, and swelled nuclei with an additional 2 mM ATP-GS for 30 min. Phase-contrast microscopy (Figure 4A) indicated nuclei had swollen to 8 μ m compared with decondensed nuclei (4 μ m) [2,29,39]. In addition, protein extracts normalized for protein concentration were separated and RanGTPase (a component of NE formation machinery and nuclear import/export [40]) and the nuclear pore complex protein Nup62 [41] were detected by Western analysis. Nuclear pore complexes are soluble, and insert only into an intact NE formed by the fusion of MVs [42]. Thus the presence of pore proteins and the normally soluble RanGTPase [43] would be indicative of a functional NE. The data in Figure 4(B) show that demembrated nuclei contained neither. After NE assembly triggered by PMA, both RanGTPase and Nup62 were detected in extracts from swollen nuclei. We were additionally able to show the specificity of protein recruitment to the NE. α -Tubulin, a predominantly cytoplasmic marker was present in demembrated nuclei and S10 egg cytoplasmic extract, but no enrichment of α -tubulin was observed in swollen nuclei (α -tubulin is observed in nuclei due to remnants of the axoneme in demembrated nuclei preparations; M. Garnier and B. Larijani, unpublished work from electron microscopy experiments). This is in accordance with the

observations of others, showing that α -tubulin displays a cytoplasmic distribution in female gametes *in vitro* after fertilization [44]. We therefore concluded that PMA-induced NEs are fully functional and capable of protein import, like those formed by GTP, as described previously [29].

DISCUSSION

We have shown that specific PtdIns components are involved in the assembly of the NE. The use of ESI-MS to monitor specific lipids after binding of MV precursors to chromatin provides a unique insight into the nature of the recruited vesicles. ESI-MS has the advantage of being highly precise and sensitive, thus enabling us to detect the molecular species of PtdIns that are enriched in binding of NE precursor vesicles to chromatin. By two-dimensional NMR spectroscopy, we have previously described an MV subpopulation (MV1) enriched in phosphoinositides; in particular, PtdIns [2]. It is probable that PtdIns enrichment in the vesicle population bound to chromatin is a reflection of a critical involvement of MV1 in NE formation [18]. ESI-MS has also revealed alterations of PtdIns species following GTP-induced NE assembly. To date, the correlation of phospholipid molecular species selectivity in NE formation has not been addressed.

We note a significant difference between recruitment of PtdCho and PtdIns molecular species precursors to the nuclear membrane. Whereas the PtdCho species composition remained unchanged between unbound, bound and assembled NE membrane populations, there was a progressive modification of specific PtdIns components from the cytoplasmic membranes to the assembled nuclear membranes. The most prominent change was the decreased fractional content of PI-18:0/20:4 once the NE had formed in the presence of GTP. This reduction is significant, since PI-18:0/20:4 is the precursor of PtdIns(4,5) P_2 -18:0/20:4, the predominant physiological substrate for endogenous PI-PLC *in vivo* (B. Larijani, unpublished work). *In vitro*, the decreased PI-18:0/20:4 may reflect an increased flux to PtdIns(4,5) P_2 to replenish PtdIns(4,5) P_2 -18:0/20:4 hydrolysed by the endogenous PLC accompanying fusion. It was not possible to reproducibly verify the PtdIns(4,5) P_2 species composition during NE assembly, because the concentration of this lipid was too low to be readily detected. Alternatively, the altered PtdIns composition of fused NEs may reflect preferential selection of 1-alkyl-2-acyl species (PtdIns-18:0a/20:5 and PtdIns-18:0a/22:6). Such ether-linked PtdIns molecular species are uncommon in mammalian cell membranes and thus no functional consequences of their enrichment up to 25–30% of total PtdIns (Figure 2A) have been investigated.

We have also shown that exogenous PI-PLC induces NE formation in a cell-free system. This enzyme is expected to mimic the endogenous PLC activities of the sea urchin egg more closely than the prokaryotic enzyme used previously [2]. The exogenous enzyme applied would have only basal activity, but would display nonetheless, under such conditions, a substrate preference for PtdIns(4,5) P_2 over PtdIns4P [19,31]. Moreover, such basal activity was sufficient to induce NE formation in our cell-free system. However, PI-PLCs have a number of catalytic activators [19,20], and it is possible that some of these were present in our G_1 -phase egg extracts modifying the recombinant PI-PLC, rendering it fully active, conditions under which PtdIns(4,5) P_2 is still the favoured substrate. The exogenous eukaryotic PI-PLC activity, is sensitive to the SopB and Syn 1 (S470-R962) phosphatases, which indicates that the PI-PLC substrate is PtdIns(4,5) P_2 . Moreover, the endogenous PI-PLC activity is also sensitive to SopB activity, again indicating PtdIns(4,5) P_2 as the substrate. SopB and Syn 1 will also dephosphorylate the D-5 position of other substrates. Nonetheless, natural PtdIns(4,5) P_2 is a substrate of SopB [32] and the preferred

substrate of Syn 1 (S470-R962) [33], and it is therefore likely that the dephosphorylation of natural PtdIns(4,5) P_2 as opposed to alternative substrates is the activity responsible for the effects of these enzymes on NE assembly. NE formation by endogenous and exogenous PLCs, as well as the apparent *in vivo* activity (results not shown), is sensitive to the PLC inhibitor U73122. Thus for the first time we show that PI-PLC has a role in NE formation.

Ca^{2+} was required for NE formation, in agreement with previous studies [42]. However, such Ca^{2+} dependence may be satisfied by basal calcium levels, as opposed to Ins(1,4,5) P_3 -triggered Ca^{2+} release, since bacterial PI-PLC, which does not generate Ins(1,4,5) P_3 , also induces NE assembly [2]. Thus the only product common to both the bacterial and eukaryotic PI-PLCs is DAG, and one could therefore conclude that it is DAG generation that is responsible for the formation of the functional NE from the chromatin-bound precursor membranes. Supporting this proposal, we have first demonstrated in MV0 the hydrolysis of PtdIns to DAG by the bacterial PI-PLC. Secondly, we observed that the DAG analogue, PMA, is a potent inducer of NE formation, and thirdly, we observed an inhibition of NE formation in the presence of DAG kinase. Moreover, the functionality of PMA-induced NEs was confirmed by their content of proteins associated with pores and protein transport and their ability to swell in ATP-GS. A common downstream effector of DAG signalling is PKC, but we showed that PMA-induced fusion is independent of PKC activation and thus is likely to operate directly through alterations of membrane structure/curvature, through non-PKC receptors, or both. PMA-induced NE formation was greatly inhibited by inclusion of non-hydrolysable GTP[S], indicating a requirement for GTP hydrolysis in line with a previous study [42]. These data may be linked to previous studies, where it has been shown that DAG and phorbol esters bind to non-PKC receptors such as Munc13, a regulator of SNARE proteins in the fusion machinery, and Ras-GAP/Rac-GAP-containing proteins that bind to Rab5, a monomeric GTPase involved in homotypic membrane fusion [1,34,37]. The identification of DAG effectors is currently hampered by the incomplete status of the Pacific purple sea urchin (*Strongylocentrotus purpuratus*) genome sequence [45]. Its anticipated completion should facilitate the search for non-PKC DAG receptors, and PLC isotypes other than those already described [21,23], which are involved in NE formation. Although the specific endogenous PLC isotype involved in NE formation is yet to be identified, its activity would nonetheless be accompanied by a change in lipid species composition, as is demonstrated here.

In summary, we show how ESI-MS can be utilized as a tool to monitor in detail phospholipid species changes accompanying NE assembly. Here, ESI-MS reveals changes in relative proportions of different classes of phospholipids such as PtdIns and PtdCho, changes in a subset of species of PtdIns, and invariance of the subset of PtdCho species accompanying membrane binding and fusion. We hypothesize that the hydrolysis of PtdIns(4,5) P_2 by an endogenous PLC generates DAG, which may bind to non-PKC receptors, such as Ras-GAP/Rac-GAP-associated proteins and/or Munc13. Such proteins are downstream of PLC, and promote the GDP/GTP exchange on GTPases or facilitate SNARE assembly, thus activating and initiating pathways leading to fusion. In addition, DAG may also contribute directly to local membrane fusion events. Thus the pathway would be sensitive to SopB, a PtdIns(4,5) P_2 -depleting phosphatase, the PLC inhibitor U73122 and the non-hydrolysable GTP analogue GTP[S], consistent with our results. The endogenous PLC isoform responsible for NE assembly in this system requires further investigation upon completion of the sea urchin genome. The robust ability of PMA to induce NE formation indicates a need for detailed studies on the role of DAG in fusion of NE precursors.

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