CDP-diacylglycerol phospholipid synthesis in detergent-soluble, non-raft, membrane microdomains of the endoplasmic reticulum.

Mark G. Waugh*, Shane Minogue*, Emma L. Clayton* and J. Justin Hsuan*

*Centre for Molecular Cell Biology
Division of Medicine
University College London
Royal Free Campus
Rowland Hill Street
London NW3 2PF
United Kingdom

Corresponding author: Dr. M.G. Waugh
Telephone: 020 7433 2822
FAX: 020 7433 2817
e-mail: m.waugh@ucl.ac.uk

Running title: membrane lipid microdomains of the endoplasmic reticulum.
Summary

Phosphatidylinositol is essential for numerous cell functions and is generated by consecutive reactions catalyzed by CDP-diacylglycerol synthase (CDS) and phosphatidylinositol synthase. In this study we investigated the membrane organization of CDP-diacylglycerol synthesis. Separation of mildly disrupted A431 cell membranes on sucrose density gradients revealed co-fractionation of CDS and phosphatidylinositol synthase activities with cholesterol-poor, endoplasmic reticulum membranes and partial overlap with plasma membrane caveolae. Co-fractionation of CDS activity with caveolae was also observed when low-buoyant density, caveolin-enriched membranes were prepared using a carbonate-based method. However, immunosolation studies determined that CDS activity localized to endoplasmic reticulum membranes fragments containing calnexin and type III inositol (1,4,5)-trisphosphate receptors but not to caveolae. Membrane fragmentation in neutral pH buffer established that CDP-diacylglycerol and PI syntheses were restricted to a sub-fraction of the calnexin-positive endoplasmic reticulum. In contrast to lipid rafts enriched for caveolin, cholesterol and GM1 glycosphingolipids, the CDS-containing ER membranes were detergent soluble. In cell imaging studies, CDS and calnexin co-localized in microdomain-sized patches of the endoplasmic reticulum and also unexpectedly at the plasma membrane. These results demonstrate that key components of the phosphatidylinositol pathway localize to non-raft, phospholipid-synthesizing microdomains of the endoplasmic reticulum that are also enriched for calnexin.
Introduction

There is a large body of literature supporting the idea of lateral lipid segregation at the plasma membrane, trans-Golgi network and endosomes, with a particular focus on the characterization and functions of cholesterol- and glycosphingolipid-enriched membrane microdomains often referred to as lipid rafts (1, 2). Much less is known about possible membrane domain organization at the endoplasmic reticulum (ER) which is the largest cellular membrane structure and also the principal site for cellular phospholipid synthesis (3). Particular regions of the ER that form contacts with mitochondria (reviewed in (4)), usually referred to as mitochondria-associated membranes (MAM) are known to be major sites for cellular phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine production (5). There are reports that MAMs contain detergent-insoluble membrane microdomains (6, 7) analogous to plasma membrane rafts, and that MAMs are enriched for molecular chaperones such as calnexin (8) as well as signaling molecules such as type III inositol (1,4,5)-trisphosphate receptors (IP3R) (9, 10). This has lead to the proposal that MAMs represent important sites for the compartmentation and regulation of intracellular Ca2+ release (10), a key event in phosphatidylinositol (PI) -dependent, phospholipase C (PLC) signaling. However, the intramembrane distributions of enzymes involved in the synthesis of PI and its precursor CDP-diacylglycerol (CDP-DAG) at the ER have not yet been characterized in detail.

The cellular localization and possible compartmentation of processes involved in the provision of PI for receptor-stimulated PLC signaling have been widely debated (11-14). In a classical, simplified model for phosphoinositide signaling, agonist activation of G protein-coupled and tyrosine kinase receptors occurs at the plasma membrane and leads to the rapid PLC-catalyzed hydrolysis of PI 4,5-bisphosphate (PI(4,5)P2) into the second messenger molecules diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3). The IP3 then diffuses and is free to bind IP3R. These are large transmembrane channel proteins which in the tetrameric, ligand-activated state facilitate the release of Ca2+ sequestered in the ER. In order to sustain high levels of signaling through PLC, PI(4,5)P2 must be re-synthesized via PI and PI 4-phosphate,
and radiolabelling studies have pointed towards the existence of agonist-sensitive and insensitive metabolic pools of PI, and possibly a closed cycle of intermediates (11, 15).

In contrast to the large body of literature on receptor-dependent phosphoinositide signaling much less information is available on the metabolic pathways and mechanisms involved in PI synthesis. In ER membranes (16), phosphatidic acid (PA), formed following the phosphorylation of DAG by DAG kinase, is condensed with CTP to produce CDP-DAG in a reaction catalyzed by CDP-DAG synthase (CDS) (17). The CDP-DAG liponucleotide can then undergo PI synthase-catalyzed headgroup exchange with inositol to form PI (18). Although PI synthesis has been reported to occur in the plasma membrane (19, 20) and to a lesser extent in the Golgi (21), it is generally accepted that similar to IP$_3$Rs, the ER is the primary site for the synthesis of both PI and CDP-DAG (reviewed in (17, 18)). In concordance with this view, the single human PI synthase enzyme (22) and the major CDS isozyme have been demonstrated to be ER proteins (23-26). Interestingly, despite large fluxes in intracellular Ca$^{2+}$ concentrations during PLC signaling, there are demonstrations that both CDS and PI synthase are inhibited by Ca$^{2+}$ in the μM to mM range (27-29). Moreover, increasing the levels of either CDS or PI synthase by overexpression of the recombinant enzymes induces disproportionately small increases in the cellular levels of their phospholipid products (22). Hence it is not clear how the ER localization and Ca$^{2+}$ sensitivity of CDS and PI synthase are compatible with the repletion of their lipid products following receptor activation.

While both PI and IP$_3$R localize primarily to the ER, there are independent studies showing that these molecules can also be found in plasma membrane caveolae (30, 31). One possible inference from such studies is that plasma membrane rafts contain a pool of PI and IP$_3$R important for phosphoinositide signaling. However, the impurity of caveolae raft preparations (32, 33) and the recent identification of detergent-insoluble, raft-like ER membrane domains (7, 34) suggest that a more thorough characterization of the compartmentation of these important signaling molecules is necessary.
This study utilizes different subcellular fractionation techniques combined with immunoisolation of ER membrane domains and confocal immunofluorescence microscopy, to establish that a pool of calnexin and IP$_3$R localize to non-caveolar, buoyant ER subdomains which are also hotspots for PI and CDP-DAG synthesis. These membrane domains have biochemical properties consistent with the phospholipid-rich, non-raft, ER membrane domains predicted by Shaikh and Edidin (35).
Methods

Materials

Secondary antibodies, [$\alpha^{32}$P]CTP (20mCi/ml), [$^3$H]Inositol with PT6–271 stabilizer (17.1Ci/mmol), prestained molecular weight markers, Protein G Sepharose 4 Fast Flow and the ECL western blotting detection system were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Anti-caveolin, anti-p115 and anti-IP$_3$R (type III) antisera were obtained from BD Biosciences (Oxford, UK). Anti-CDS1 was bought from AbD Serotec. Anti-calnexin antiserum was purchased from Assay Designs and Stressgen (Michigan, USA). Cell culture reagents and the Amplex Red cholesterol Assay kit were from Life Technologies (Paisley, UK). Protease inhibitor cocktail tablets (Complete™, without EDTA) were from Roche Diagnostics (Burgess Hill, West Sussex, UK). All other reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

Cell culture, radiolabelling and analysis of phosphoinositide lipids.

A431 cells were maintained at 37°C in a humidified incubator at 10% CO$_2$. Cells were cultured in DMEM supplemented with Glutamax, 10 % fetal calf serum, 50 i.u./ml penicillin and 50 μg/ml streptomycin. Standard methods were used for the analysis of [$^3$H]inositol-labeled phosphoinositides (32). A431 cells were cultured in inositol-free medium containing 1% fetal calf serum in the presence of 2 μCi/ml [$^3$H]inositol for 48 h. Following subcellular fractionation, membrane fractions (100 μl) were extracted with an equal volume of chloroform:methanol:1M HCl (60:36:4). Samples were vortexed and centrifuged for 10 s in a microcentrifuge at 10,000rpm. Organic phases were collected and re-extracted with methanol:1M HCl (1:1). Samples were vortexed and centrifuged as before and the organic phase from each tube collected. Lipids were resolved by TLC on Silica-60 plates (Merck) which had been pretreated with 1% potassium oxalate, 2 mM EDTA in 50% methanol and developed using an acid solvent system composed of propan-1-ol:2 M acetic acid (65:35) containing 1% 5M H$_3$PO$_4$. TLC plates were then imaged using a Typhoon 9400 phosphorimager (GE Healthcare).
Subcellular fractionation of A431 cells
For these experiments 2 - 4 confluent 15 cm dishes of A431 cells were used. Protein loading of the gradients was measured as 6.6 ± 2.3 mg (mean ± S.E.M., n = 3) and lipid loading was 188.3 ± 33.6 (n = 3) moles cholesterol/mg protein. The method used for subcellular fractionation was essentially the same as that described by Sillence and Downes (36) with some minor modifications. A431 cells were harvested on ice in 0.25M sucrose, 10mM EGTA, 10mM EDTA, 20mM Tris-HCl, pH 7.4 and homogenized by 15 strokes using a hand-held Dounce homogenizer. A post-nuclear supernatant (PNS) was obtained by centrifugation at 2000rpm for 5min in a bench-top centrifuge. All subsequent steps were carried out on ice or at 4°C. The resulting nuclear pellet was re-homogenized and another PNS obtained. The PNSs from both clearing steps were combined and loaded onto a 10ml continuous 24-50% sucrose gradient formed in an ultracentrifuge tube using the pump and gradient mixing system from an SMART separation unit GE Healthcare (Little Chalfont, Buckinghamshire, UK). Following centrifugation for 16–18h at 35000rpm (151000 x g) in a Beckman SW41 Ti rotor, 1ml samples were collected starting from the top of the tube for further analyses. Refractive index measurements of individual gradient fractions were determined with a Leica AR200 refractometer and these values were converted to sucrose densities using linear regression and standard conversion tables (37).

Isolation of caveolin-rich buoyant membrane domains from A431 cells
Caveolin-rich buoyant membrane domains were prepared using a detergent-free method as previously described (32). All procedures were carried out at 4°C. A431 cell monolayers grown to confluence in 15 cm dishes were washed twice with phosphate-buffered saline and scraped into 2ml of 100 mM Na₂CO₃, pH 11.0 containing COMPLETE protease inhibitors, 10mM EGTA and 10mM EDTA. Cells were disrupted by sonication (6 x 10s bursts) using a Soniprep 150 sonicator (MSE) on setting 10 –12. Sonicated cells (2ml) were mixed with 2ml of 90% (w/v) sucrose in MBS buffer (25mM Mes, 150mM NaCl, pH6.5) and placed in a 12ml ultracentrifuge tube. A 5-35% (w/v) discontinuous sucrose gradient was formed above the sample by layering on 4ml of 35% (w/v) sucrose solution followed by 4ml of 5% (w/v) sucrose solution. The sample was then centrifuged at 39,000rpm (175,000 x g) for 16-18h in a Beckman SW41 Ti rotor. A thin band of membrane was identified at the 5-35%
(w/v) sucrose interface that was enriched in caveolin but excluded the bulk of the cellular protein (38). 1 ml fractions were collected from the top of each gradient.

In some experiments, as an assay for resistance to detergent solubilization, the detergents 10 mM β-octylglucoside and 4 mM deoxycholate were added to the sodium carbonate sonication buffer (39).

**Fractionation of A431 sonicated cell membranes in neutral pH buffer**

A431 cells were harvested by scraping into neutral pH buffer consisting of 0.25M sucrose, 10mM EGTA, 10mM EDTA, 20mM Tris-HCl, pH 7.4 containing COMPLETE protease inhibitors, and probe sonicated by 3 x 5 sec bursts with a Vibra-cell probe (Sonics) sonicator at amplitude setting 40 operated in pulsed mode. This sonicated fraction was transferred to a 12 ml polycarbonate ultracentrifuge tube (Beckman) and adjusted to 45% (w/v) sucrose by the addition of an equal volume of 90% (w/v) sucrose in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1mM EGTA to give a final volume of 4 ml. The sample was overlaid with 2 ml of 35% (w/v) sucrose, 2 ml of 20% (w/v) sucrose followed by 4 ml of 5% (w/v) sucrose, and subjected to discontinuous equilibrium sucrose density gradient centrifugation overnight at 4°C at 180,000 g using a swing-out SW41 Beckman rotor. 1 ml gradient fractions were then harvested beginning from the top of the tube and stored at -20°C.

**Immunoblotting of A431 cellular fractions**

Samples from sucrose gradient fractions were mixed with an equal volume of 2x sample buffer and 40 µl samples separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and probed with various antibodies. Bound antibody was detected using the ECL system. Western blots were quantified by densitometric analyses using image analysis software in Adobe Photoshop CS4.

**Immunoprecipitation of caveolae and calnexin-rich domains**

Caveolae were immunoisolated as described previously [40]. For the isolation of calnexin-containing membranes, samples were pre-cleared with 20 µl Protein G Sepharose beads for 1h at 4°C. Cleared samples were incubated for 2h at 4°C with anti-caveolin antiserum (10 µl/ml), which had been prebound to Protein G Sepharose.
Immunocomplexes were collected by centrifugation and washed four times with 20mM Tris-HCl, pH 7.4.

CDS assays
CDS activity was assessed by measuring the incorporation of radiolabelled CTP into PA to form the liponucleotide $^{32}$P[CDP-DAG (40). 50µl samples from each gradient fraction were mixed with an equal volume of assay buffer composed of 20mM Tris-HCl pH 7.4, 40mM MgCl$_2$, 2mM EGTA and 20μCi/ml $[^{32}$P]CTP. Where indicated, exogenous PA was added as a substrate for CDS to a final concentration of 0.5mM. Samples were incubated at room temperature for 30min. Reactions were terminated and organic phases were extracted by the addition of an equal volume of chloroform:methanol:1M HCl (60:36:4). Samples were vortexed and centrifuged for 10s in a bench-top centrifuge at 10,000rpm. Organic phases were collected and re-extracted with methanol:1M HCl (1:1), vortexed and centrifuged as before, and the organic phase from each tube collected. Lipids were resolved by TLC on Silica-60 plates (Merck), which had been pretreated with 1% w/v potassium oxalate, 2mM EDTA in 50% methanol and developed using an acid solvent system composed of propan-1-ol:2M acetic acid (65:35) containing 1% 5M H$_3$PO$_4$. TLC plates were then exposed to X-ray film to visualize radiolabelled CDP-DAG. The amount of radioactivity associated with each spot identified by TLC was determined by counting Cerenkov radiation.

PI synthase assays
PI synthase activity was assayed by mixing equal volumes of sample (50µl) with assay buffer to give a final composition of 10mM Tris-HCl pH7.4, 10mM MgCl$_2$, 1mM EGTA, 1mM CTP and $[^3$H]inositol (1–2µCi/tube). Note that 1 mM CTP was included to enable substrate CDP-DAG to be produced from endogenous PA. Samples were incubated for 1h at 37°C and reactions were terminated by the addition of an equal volume of chloroform:methanol:1M HCl (60:36:4). Lipids were then extracted and analyzed by TLC as described for the analysis of CDP-DAG synthesis. TLC plates were then sprayed with ENHANCE™ to allow the visualization of radiolabelled phospholipids. Following fluorography the identity of radiolabelled lipid spots was determined by comparison with non-radiolabelled lipid standards visualized by iodine staining. The amount of radioactivity associated with each spot
was determined by scraping spots off the TLC plates and liquid scintillation counting. Alternatively, each organic phase was counted directly by liquid scintillation counting where TLC analysis revealed that [\(^3\)H]PI was the only radiolabelled lipid produced in the assay.

**Cholesterol mass assays**

The cholesterol content of equal volume membrane fractions (50 µl) was assayed using the Amplex red cholesterol assay kit from Molecular Probes (Life Technologies, Paisley, UK).

**Detection of GM1 glycosphingolipid.**

A dot blotting method was employed to establish the gradient distribution of GM1 glycosphingolipid (41). Equal volume 1 µl samples of each subcellular fraction were dotted onto nitrocellulose membrane and probed with HRP-conjugated cholera toxin B subunit (1:10,000) to detect GM1 glycosphingolipid using ECL reagents.

**Confocal immunofluorescence microscopy.**

COS-7 cells were grown on poly(L)lysine-coated glass coverslips for 24 h, fixed with 4% (v/v) formaldehyde for 10 min on ice, permeabilized by treatment with -25°C methanol for 2 min (42), then immunostained with anti-calnexin and anti-CDS. After counterstaining with Hoechst 33342 (Life Technologies), the coverslips were mounted in ProLongGold anti-fade reagent (Life Technologies). For plasma membrane and EGFR imaging experiments the methanol step was not included. Cells were imaged as described previously (42) using a Zeiss LSM 510 Meta laser-scanning confocal microscope system.
Results

Co-fractionation of CDS and PI synthesis with IP$_3$R and calnexin and to a lesser degree with caveolin.

CDS and IP$_3$R function at different ends of the phosphoinositide dependent signaling cascade but are both known to localize primarily at the ER. However, it is not known if both CDS and IP$_3$R localize to the same ER membrane regions. To address this issue we determined the subcellular distribution of CDS activity and IP$_3$R in A431 cells using a subcellular fractionation scheme that involves disruption of cells by Dounce homogenization in neutral pH buffer (36). Post-nuclear supernatants prepared from such cell homogenates were centrifuged in a continuous 24-50% (w/v) sucrose density gradient (Figure 1). This procedure separates different organelles on the basis of their equilibrium buoyant density, a physical property which is largely determined by organelle-specific protein:lipid ratios. Equal volume samples from each gradient fraction were subsequently assayed for CDS activity (Figure 1A) using endogenous membrane PA as the lipid substrate. In separate experiments using the same subcellular fractions, the synthesis of PI was measured using endogenous membrane associated CDP-DAG and exogenous [³H]inositol as substrates for PI synthase (Figure 1B). Note that a high concentration of CTP (1mM) was included in the PI synthase assays in order to maintain membrane levels of CDP-DAG. Using these methodologies both CDS and PI synthase activity manifested as broad peaks in fractions 7 and 8 of the sucrose gradient corresponding to a sucrose density of 1.2 – 1.23 g/ml (Figure 1c). Since these assays utilize membrane associated lipids and enzymes, it is possible to infer that both the CDS and PI synthase enzymes can access PA and CDP-DAG phospholipids in these membrane fractions. The gradient distribution of CDS activity closely paralleled that of CDS protein as determined by anti-CDS immunoblots (Figure 1A) thus demonstrating that CDP-DAG synthesis reported the gradient distribution of the CDS enzyme.

Comparison of the distribution of CDS activity with western blots directed against different subcellular membrane markers (Figure 1D) demonstrated that CDS activity was well resolved from the Golgi-associated protein p115 but partially overlapped with immunoreactivity for caveolin. Caveolin is a protein marker for cholesterol-enriched plasma membrane caveolae (43, 44), and is also present at the trans-Golgi
network (45) and to a lesser degree on ER membranes (46). Additionally we investigated the gradient distributions of the established ER-resident calcium binding lectin calnexin and type III IP$_3$R calcium release channels (8-10). Immunoreactivity for both proteins substantially co-fractionated in fractions 6 to 10 (Figure 1A) which is also the region of the gradient where both CDP-DAG and PI synthesis were localized to.

Measuring the distribution of the lipid raft-associated lipids cholesterol (Figure 1E) and GM1 glycosphingolipid (Figure 1F) revealed that the peak distributions for both molecules were in the buoyant fractions 4 and 5 (1.17 – 1.18 g/ml sucrose) corresponding to the Golgi and plasma membrane–enriched region of the gradient. Moreover, and particularly in the case of cholesterol, there was very little overlap with the gradient distributions of either CDS or PI synthase activity. Hence, subcellular fractionation following mild homogenization demonstrated that both PI synthase and CDS activities co-fractionated with cholesterol-poor, dense, ER membranes but also raised the possibility that a minor pool of both enzymes could be localized at least partly to more buoyant caveolae membranes.

**CDS activity in non-caveolar, buoyant membrane domains of the ER.**

Our initial density gradient analyses revealed partial overlap between caveolin, CDS activity and IP$_3$R, leading to the possibility that subpopulations of these primarily ER proteins may also co-localize in caveolae. Alternatively, as some caveolin protein has been found associated with ER membranes (46) there was a possibility that CDP-DAG synthesis and IP$_3$R localized to an ER fraction that also contained caveolin. We investigated these scenarios using an established detergent-free method to isolate a caveolin-enriched low buoyant density membranes (38) followed by the immunoisolation of intact caveolae from this membrane preparation (32, 47).

As a first step to isolate caveolae membranes, A431 cells were scraped into a sodium carbonate buffer, sonicated to disrupt membranes, and fractionated on a 45-35-5% (w/v) sucrose step gradient (38). In common with other groups and in concordance with our previous work (38, 48) we found that the low buoyant density membranes (1.01 -1.06 g/ml sucrose) found in fractions 4, 5 and 6 of the step gradient were enriched in caveolin protein (Figures 2A and 2B). Furthermore, these buoyant
membranes contained little of the total cellular protein (Figure 2A). We have previously shown (49) that carbonate addition leads to the isolation of membranes with a high lipid:protein ratio due to removal of peripheral membrane protein but not phospholipids (32, 39, 50, 51) by alkaline pH during the cell disruption step (51). When CDS activity was assayed across the gradient fractions, the distribution was found to closely parallel that of caveolin, with activity concentrating in the caveolin-enriched fractions (Figure 2D). When the distributions of IP$_3$R and calnexin were determined by western blotting subpopulations of both proteins were detected in the caveolin-rich buoyant membrane fraction (Figures 2B and 2C). However, quantitative analysis of the immunoblots revealed that a substantial pool of IP$_3$R also localized to fractions 10 - 12 in the dense region of the gradient (Figure 2C), thereby indicating a degree of heterogeneity in terms of ER membrane biophysical properties. This heterogeneity apparent in the distribution of membrane regions with different buoyant densities was particularly clear when comparing the gradient distributions of CDS activity and IP$_3$R (Figure 2D). Typically 80-90 % of the total cellular [$^{32}$P]CDP-DAG generation was associated with the buoyant fractions 4-6 compared with just 40-60 % of the cellular IP$_3$R or calnexin contents.

Given that CDP-DAG synthesis was targeted to the buoyant fraction and that this phospholipid is a metabolic precursor to PI, we extended our analyses to investigate the distribution of PI synthase activity. Assaying for [$^3$H]PI generation in the membrane fractions revealed that the distribution of PI synthase activity did indeed closely parallel that of [$^{32}$P]CDP-DAG production (Figure 2E) suggesting that the enzymes and lipid substrates required for phosphatidylinositol synthesis are present within the same buoyant-membrane fraction that also contains caveolae lipid rafts.

It remained possible that sonication in sodium carbonate had caused substantial fusion of ER and plasma membranes or alternatively, that calnexin and CDS localized to caveolae rafts. However, both of these scenarios were discounted by the finding that under conditions known to clear 70-100% of the cellular caveolae (32), we did not detect substantial co-immunoprecipitation of either CDS activity or calnexin with immuno-purified caveolae (Figure 2F). These analyses also showed that the CDS and calnexin were targeted to non-caveolar membrane domains. This finding prompted us to consider the possibility of immunoisolating the buoyant ER membranes present in
the caveolin-rich buoyant membrane fraction. We found that using a polyclonal antiserum directed against the carboxy-terminus of calnexin it was possible to isolate membranes containing the target antigen calnexin along with both IP$_3$R and CDS activity (Figure 3). Taken together, these results established that calnexin, CDS and IP$_3$R proteins were co-localized within the same ER membrane domain and also that a widely used technique to isolate caveolin-enriched membranes also contains buoyant raft-like ER membranes.

**CDS and PI synthase activities localize to a sub-fraction of the calnexin containing ER membrane.**

Due to the membrane stripping effect of carbonate which is known to remove peripherally associated proteins (51) and it was not possible to infer from the carbonate gradient results that PI and CDP-DAG synthesis are compartmentalized within the ER. This lead us to modify our membrane fractionation scheme and to carry out the experiment in the absence of carbonate at neutral pH while retaining the probe sonication step which we have shown previously to result in the formation of membrane vesicles in the 50-250 nm diameter size range (32, 48, 49). Using this approach we observed almost identical gradient distributions of CDP-DAG and PI generation (Figure 4A and 4B). However, there were substantial differences in the gradient distributions of PI and CDP-DAG synthesis versus both calnexin and IP$_3$R (Figure 4C and 4D). Under these conditions, a peak of CDS and PI synthase activity co-fractionated with a buoyant fraction of calnexin and IP$_3$R. Conversely, very little CDP-DAG and PI synthesis was measured in fractions 9-12 corresponding to the 45% sucrose layer and which contained 60-70% of the total ER membrane associated calnexin and IP$_3$R. From these results we deduce that most of the cellular CDS and PI synthase activities localize to a sub-fraction of the calnexin containing ER membrane.

**CDS localizes to detergent-soluble membrane domains distinct from cholesterol-rich lipid rafts.**

We next investigated if the CDS-enriched ER membrane fractions shared properties other than buoyancy with plasma membrane rafts. Resistance to detergent solubilization is a characteristic property of membrane rafts, hence we investigated the gradient distribution of CDS activity in membranes prepared in the presence of β-
octylglucoside (10 mM) and deoxycholate (4 mM); a detergent mix which unlike Triton X-100 does not result in significant solubilization of acidic phospholipids (52) or inhibition of CDS activity (53). In these gradients a significant proportion of the cellular caveolin content was detected in the low buoyant density (1.06 – 1.1 g/ml sucrose), detergent resistant membranes fractions 4 - 6 (Figures 5A and 5B), and was well resolved from calnexin which localized to the dense (1.19 -1.2 g/ml sucrose) region of the gradient. These buoyant fractions contained little of the total cellular protein content (Figure 5C) but were particularly enriched in the raft lipids cholesterol and GM1 glycosphingolipid (Figure 5 D and 5G). In contrast to the raft lipids and caveolin, the CDS enzyme in common with calnexin was found to localize to the dense region of the gradient (Figure 5E). This dense region of the gradient also contained a pool of PI, PI4P and PI(4,5)P₂ phospholipids (Figure 5F) but was practically devoid of either cholesterol or GM1 lipids which are known to be enriched in lipid rafts. This lead us to infer that the CDS-containing ER membrane microdomains described here are biochemically different in terms of their lipid content and detergent solubility to plasma membrane lipid rafts.

Imaging CDS distribution in the ER.

We employed confocal immunofluorescence microscopy to investigate if the CDS and calnexin-rich ER domains could be distinguished from the bulk of the ER. The intracellular localizations of calnexin and CDS were imaged by immunofluorescence staining of cells which had been permeabilised in -20°C methanol in order to preserve ER membrane architecture (Figure 6). These experiments revealed extensive staining of a reticular network of intracellular membranes which is characteristic of localization to the ER (Figure 6, detail 1). Most strikingly, CDS and calnexin were unevenly distributed throughout the ER with co-localization in 1 - 2 μm patches, often adjacent to membrane domains staining for only one or other of these proteins (Figure 6, detail 1). There were also distinct patches of co-localization at the plasma membrane although this was less extensive than at the ER (Figure 6, detail 2). This patchwork of separate and overlapping localization could be seen more clearly in higher resolution images (Figure 6). Our confocal microscope is routinely calibrated with dye-labeled Tetraspeck beads (Life Technologies) using the same objective and acquisition settings. This gives an x-y resolution of at least 200-250 nm at these emission wavelengths; figures which closely correspond to calculations of theoretical
diffraction-limited resolution. Given this level of imaging resolution it is accurate to imply that calnexin and CDS co-localize in membrane microdomains. These results are consistent with the membrane immunoisolation and cell fractionation data demonstrating that cellular CDS activity is concentrated in calnexin-rich membrane microdomains.
Discussion

Through the use of a variety of cell fractionation, immunoisolation and immunofluorescence imaging techniques we demonstrate that a pool of CDS activity localizes to calnexin-containing, ER membrane domains that co-fractionate with low-buoyant density caveolae, but with biochemical properties that are different to those ascribed to plasma membrane rafts. Specifically, the novel phospholipid-synthesizing membrane domains we describe here are detergent-soluble and are not enriched for either cholesterol or glycosphingolipids. This sets them apart from the detergent resistant ER domains implicated in the synthesis of mammalian glycosylphosphatidylinositol anchor intermediates (54), the organization of export to the Golgi (55) and also from the detergent-insoluble membrane domains to which erlin-1/2 (7) and sigma-1 chaperones (6) are targeted.

Another novel finding from our co-immunoprecipitation studies is the origin of the CDP-DAG-synthesizing domains in the calnexin and type III IP$_3$R-containing region of ER. We have shown on a number of previous occasions that the sonication procedure we use to disrupt the ER membranes gives rise to membrane vesicles in the size range of 60–200 nm diameter (32, 48, 49); hence the ability to co-immunoprecipitate calnexin, IP$_3$R and CDS on intact vesicles demonstrates that these proteins localize in the same microdomain of the ER. The differential localizations of CDP-DAG synthesis, calnexin and type III IP$_3$R do not become apparent in subcellular fractionation experiments until these ER membranes are sonicated and this is most pronounced when membranes are sonicated using neutral pH buffer. Moreover, the differing equilibrium densities and partial overlaps of CDS, calnexin and IP$_3$R in the density gradient fractions following sonication indicate a substantial degree of membrane domain heterogeneity within the ER.

The low buoyancy of phospholipid-rich membrane domains in neutral pH buffer is indicative of a high protein to lipid ratio under physiological conditions. The density shift of these membranes apparent following carbonate treatment indicates that peripherally associated proteins account for the lack of buoyancy in neutral pH buffer (51). This differs from cholesterol-rich membranes at other cellular sites such as the
trans-Golgi network which tend to be intrinsically buoyant due to their relatively low protein to lipid ratio (56). The low abundance of cholesterol at the ER, which is a tightly regulated process (57), and the exclusion of cholesterol from model membranes containing unsaturated lipid side chains (58, 59), further argues against a predominant role for classical lipid raft domains in the organization of phospholipid synthesis. The membrane microdomains that we describe here appear to have the properties expected of the so-called “non-raft” phospholipid-rich membrane domains, the existence of which was first postulated by Shaikh and Edidin (35). To the best of our knowledge this is the first experimental evidence that such membrane domains exist in cells and furthermore, our results indicate that these domains are an important site for cellular phospholipid synthesis.

Our analyses of the biochemical and membrane fraction data is supported by confocal microscopy which demonstrates that CDS and calnexin co-localize in micron-sized patches of membrane but that there are also portions of the ER where there is no overlap between these proteins. This is somewhat unexpected as ER membranes are generally thought to mainly exist in a liquid disordered state due to a lipid composition that is conducive to neither phase separation nor membrane microdomain formation. However, our visualization of a small pool of CDS–calnexin very close to, or on the plasma membrane may indicate that inter-membrane and inter-protein contacts could have a role in the formation and/or stabilization of these ER microdomains. The presence of a small pool of calnexin at the plasma membrane has been reported in several previous studies (8, 60, 61) and is dependent on CK2 phosphorylation and inhibition of PACS-2 binding to calnexin (8). This implies that the subcellular localization of the CDS-calnexin domain may not be static and opens the possibility of a trafficking route whereby CDS and IP$_3$R can co-traffic with phosphorylated calnexin to the plasma membrane (8). Localization of CDS extremely close to, or definitively on the plasma membrane has ramifications for the long-running puzzle concerning possible spatial restrictions to phosphoinositide substrate supply which would arise if CDP-DAG synthesis was exclusively compartmentalized at the ER. Our observations may indicate that local rates of CDP-DAG synthesis and the abundance of CDS in a particular membrane environment may be key factors in regulating the supply of phosphatidylinositol at the plasma membrane.
In conclusion, our results demonstrate that a pool of IP$_3$R associates with membrane microdomains that are highly-enriched for calnexin and CDP-DAG synthesis. Future work will determine the functional consequences of CDS targeting to these novel membrane regions, including the pool of the enzyme we have imaged at the plasma membrane. Since IP$_3$R and CDS and are key proteins in receptor-dependent phosphoinositide signaling, it is possible that the membrane domains we have identified here are important for the spatial integration of phospholipid synthesis with the propagation of signaling from phosphoinositide-derived second messengers. It is noteworthy that calnexin is a Ca$^{2+}$ binding protein that can function as a low-affinity, high capacity Ca$^{2+}$ buffer (62-64) and also that CDP-DAG generation is inhibited by mM Ca$^{2+}$ concentrations (27). Therefore, we speculate that the close proximity of calnexin, IP$_3$R and CDS may facilitate localized coupling of Ca$^{2+}$ release through IP$_3$R with compensatory PI resynthesis via CDS through calnexin-mediated buffering of localized Ca$^{2+}$ increases in close proximity to the ER membrane.

**Acknowledgements**

M.G.W. and S.M. acknowledge support from the BBSRC (grant BB/G021163/1) and Royal Free Hospital NHS Trust support for the advanced microscopy unit and the Centre for Biomedical Sciences (J.J.H.). E.L.C acknowledges support from the Peter Samuel Royal Free fund. We thank Samrina Aslam for her assistance with the imaging studies.

**Abbreviations**

The abbreviations used are: CDP-DAG, CDP-diacylglycerol; CDS, CDP-diacylglycerol synthase; CLM, caveolin-rich light membranes, ER, endoplasmic reticulum; IP$_3$R, inositol 1,4,5-trisphosphate receptor; MAM, mitochondria-associated membrane; PI, phosphatidylinositol; PI(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; PLC, phospholipase C; PA, phosphatidic acid; TLC, thin layer chromatography.
References

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triton-insoluble membranes in subcellular compartments that include the endoplasmic reticulum. Biochem J 343 Pt 3: 627-635.
Figure Legends

**Figure 1. CDS activity co-fractionates with type III IP$_3$R, calnexin and PI synthase.**

A431 cells were homogenized in neutral pH buffer and the PNSs subjected to ultracentrifugation on a 10ml, 20-50% continuous sucrose gradient. Fractions 1 and 2 correspond to the loading layer, and fraction 12 corresponds to the densest fraction. 1ml fractions were collected beginning at the top of the gradient for analyses. (A) Gradient distribution of CDS enzyme determined by immunoblotting and CDS-catalyzed $[^{32}P]$CDP-DAG (total 129311 c.p.m) generation using endogenous PA and added [$\alpha^{32}$P]CTP as substrates. (B) Distribution of [$^3$H]PI generation (total of 51444 dpm) catalyzed by PI synthase using endogenous membrane–associated CDP-DAG and added [$^3$H]inositol as substrates. (C) Protein content of the gradient fractions as determined by the Bradford assay and sucrose density of the gradient fractions as measured by refractometry. (D) Immunoblots showing the gradient distributions of p115, a marker protein for the Golgi apparatus; caveolin, a marker protein for plasma membrane caveolae, the ER proteins IP$_3$R and also CDS. (E) Distribution of cholesterol in the gradient fractions as measured by the Amplex Red cholesterol assay. (F) Distribution of GM1 glycosphingolipid in the gradient fractions as detected by dot-blotting with HRP-conjugated cholera toxin B subunit. Data are representative of at least two independent determinations from three separate subcellular fractionation experiments.

**Figure 2. CDP-DAG and PI generation in a caveolin-rich buoyant membrane fraction but not in immunoisolated caveolae.**

A431 cells were sonicated in the presence of 0.1M sodium carbonate, pH 11.0 and the homogenate centrifuged in a discontinuous sucrose density gradient. Fractions 5 and 6 correspond to the interface of the 5 and 35% sucrose layers. (A) Protein content of the gradient fractions as determined by the Bradford assay and sucrose density of the gradient fractions as measured by refractometry. (B) Immunoblots demonstrating the gradient distributions of caveolin, calnexin and IP$_3$R. (C) Densitometric analysis comparing the distributions of caveolin, calnexin and IP$_3$R in the gradient fractions. (D) Distribution of CDS activity (total cpm 32517) as
measured using α[32P]CTP and endogenous PA as substrates for the enzyme compared with the distribution of IP₃R. (E) Distribution of [3H]PI synthesis (total dpm 450094) in the gradient (F) Caveolae were immunoprecipitated from the caveolin-enriched buoyant fractions 5 and 6 using established methods and equal volumes of the caveolae depleted supernatants (+ lanes) or control (- lanes) analyzed by immunoblotting for caveolin and calnexin content, or endogenous CDS activity. The results presented here are representative of three independent experiments.

Figure 3. Co-immunoprecipitation of CDS activity, calnexin and IP₃R on intact membranes from the buoyant fraction.
Anti-calnexin immunocomplexes (+) and control samples (-) isolated from caveolin-rich buoyant membrane fractions were bound to Protein G Sepharose beads and analyzed by immunoblotting for the presence of calnexin and IP₃Rs. [32P]CDP-DAG generation was detected by CDS enzyme assays carried out on intact membrane domains immunoisolated on beads using endogenous PA as substrate. Figure shows an image obtained from a TLC autoradiograph used to detect [32P]CDP-DAG. Data are representative of three independent experiments.

Figure 4. Fragmentation of ER membranes by probe sonication and density gradient separation at neutral pH reveals differences in the distributions of CDP-DAG and PI synthesis compared with calnexin and IP₃R.
A431 cells were harvested by scraping into neutral pH buffer, probe sonicated and membranes separated on a 45-35-20-5% (w/v) sucrose discontinuous density gradient. 1 ml gradient fractions were then harvested beginning from the top of the tube for further analysis. Distributions of (A) [32P]CDP-DAG (total cpm 29228) and (B) [3H]PI synthesis (total dpm 205590) generation in the gradient fractions. Immunoblots and densiometric analyses of (c) calnexin and (D) IP₃R distributions in the gradient fractions. Data are representative of two independent experiments.
**Figure 5.** Unlike caveolae lipid rafts, calnexin and CDP-DAG synthesizing membrane domains are soluble in detergent.

Cells were harvested and sonicated in the presence of 0.1M sodium carbonate, and the detergents β-octylglucoside (10 mM) and deoxycholate (4 mM) and the homogenate centrifuged in a 45-35-5% (w/v) sucrose discontinuous density gradient. Fractions 5 and 6 correspond to the interface of the 5 and 35% sucrose layer. (A) Densitometric analysis comparing the distributions of caveolin and calnexin in the gradient as determined by immunoblotting. (B) Sucrose density profile of the gradient fractions as measured by refractometry. (C) Protein content of the gradient fractions measured using the Bradford assay. (D) Distribution of cholesterol in the gradient fractions determined by the Amplex Red cholesterol mass assay. (E) Immunoblot showing the distribution of CDS protein in the gradient fractions. (F) TLC separation of \(^{3}H\)inositol labeled phospholipids in the gradient fractions (G) Dot-blot showing distribution of GM1 glycosphingolipid in the gradient fractions. Each data panel shown is representative of at least two independent measurements from three subcellular fractionation experiments.

**Figure 6.** ER and plasma membrane microdomains enriched for calnexin and CDS.

(A) Cells grown on coverslips were fixed and permeabilized in methanol and immunostained with anti-calnexin (green) and anti-CDS (red) antibodies. (B) Zoomed image showing overlap between calnexin and CDS on membrane patches on reticular membranes. (C) Detail of calnexin and CDS localization at the plasma membrane. Scale bar is 10 μM. Images are representative of three independent experiments.
Figure 2

A. Graph showing protein and density distribution across fractions.

B. Western blot images of Caveolin, Calnexin, and IP3R.

C. Bar graph showing the percentage of total Caveolin, Calnexin, and IP3R across fractions.

D. Graph showing CDS activity and IP3R across fractions.

E. Graph showing PI synthase activity across fractions.

F. Western blot images for Caveolin, Calnexin, and [32P]CDP-DAG.
Figure 3

[32P] CDP-DAG
IP3R
Calnexin

+ −
Figure 4

A) CDS Activity % of Total

B) PI Synthase Activity % of Total

C) Calnexin % of Total

D) IP$_3$R % of Total
Figure 5

A. Caveolin and Calnexin levels in different fractions.

B. Sucrose density gradient profile.

C. Protein concentration profile.

D. Cholesterol levels as a percentage of total.

E. Western blot of CDS protein.

F. Gel showing PI, PI(4,5)P_2, and PI4P.

G. Western blot of GM1 raft lipid.
Figure 6

(A)

(B) Detail 1

(C) Detail 2