Signalling and non-caveolar rafts
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Abstract
Rafts are small membrane domains containing discrete subsets of lipids and proteins. Although microscopic raft structures termed 'caveolae' were described nearly 50 years ago, the importance of rafts, particularly signalling within rafts, is only beginning to be understood. Our studies focus on receptor-dependent phosphoinositide signalling. Using their characteristic buoyancy in density gradients, we and others found that the epidermal growth factor (EGF) receptor, phosphatidylinositol 4-kinase and phosphoinositides are localized within a caveolin-rich fraction of A431 carcinoma cells. We subsequently found that membrane fragments containing the EGF receptor and most cellular phosphoinositides can be separated from caveolae. Consequently, components of EGF-dependent phosphoinositide signalling localize to one or more novel types of raft, the composition of which we are currently determining. A key component is the type I phosphatidylinositol 4-kinase, which, for many years, has proven difficult to purify and clone. We describe our recent purification from rafts and cloning of this elusive enzyme, and discuss how the structure sheds light on the rafting of this enzyme.

Introduction
Rafts are small membrane domains containing characteristic subsets of lipids and proteins [1–3]. A synthesis of the available physicochemical and biochemical data led to the proposal that rafts exist as cholesterol- and sphingolipid-rich membrane microdomains that spontaneously adopt a liquid-ordered phase as opposed to the more disordered, fluid-phase properties of the general plasma membrane (reviewed in [4,5]). This apparent phase separation [6] presumably underlies an as-yet- poorly-defined mechanism for molecular segregation within the plasma membrane, whereby certain molecules, such as glycosylphosphatidylinositol-anchored and acylated proteins, dynamically and preferentially associate with the exoplasmic and cytoplasmic leaflets of rafts respectively, as opposed to other domains of the membrane bilayer. The propensity for signalling molecules to interact functionally within a raft environment has implications for agonist-dependent phosphoinositide signalling. First, phosphoinositide substrates and agonist receptors are both integral components of the plasma membrane. Secondly, compartmentation of phosphoinositides into agonist-sensitive and -insensitive pools has been inferred from some, though not all, metabolic-labelling studies [7,8].

A specialized subset of rafts termed 'caveolae' can be at least partially purified on the basis of their insolubility in the non-ionic detergent Triton X-100 and their low buoyant density. Co-purification with agonist-sensitive phosphoinositides led Pike and Casey [9] to suggest that agonist-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] in A431 human epidermoid carcinoma cells occurs in caveolae. Caveolae are 50–100 nm invaginations of the plasma membrane that are enriched in a 21 kDa coat protein called caveolin. The suggestion that agonist-dependent PtdIns(4,5)P2 biosynthesis is localized exclusively within caveolar rafts was striking, since it physically defined for the first time a subcompartment of the plasma membrane in which an agonist-sensitive PtdIns(4,5)P2 pool was localized.

Are caveolae sites of agonist-dependent PtdIns(4,5)P2 biosynthesis?
Because no mechanism exists for the rapid translocation of PtdIns(4,5)P2 in cells, we initially hypothesized that caveolae should also be important sites of PtdIns(4,5)P2 synthesis via PtdIns and PtdIns4P in A431 cells. To test this hypothesis, a caveolin-rich, low-buoyant-density
membrane (CLM) fraction was isolated by disrupting A431 cells using sonication in the presence of high-pH carbonate buffer, followed by discontinuous density-gradient centrifugation. This method, originally developed by Song et al. [10], was employed because it allowed caveolae to be purified in the absence of detergents, the addition of which has been shown to affect the composition of rafts [11]. Using this approach, we demonstrated that PtdIns, PtdIns(4,5)P_2 and the type II PtdIns 4-kinase co-localized with caveolin in the CLM fraction [12]. The type II PtdIns 4-kinase had previously been shown to be the major isoenzyme at the plasma membrane of A431 cells [13,14] and to be regulated by epidermal growth factor (EGF) [14,15]. These initial findings supported the hypothesis of caveolar PtdIns(4,5)P_2 synthesis. However, when a polyclonal anti-caveolin antiserum was employed as an additional purification step, more than 70% of the caveolae, but less than 1% of the total cellular phosphoinositides and PtdIns 4-kinase activity, were found in the immunopurified caveolae [12]. These results suggest that cellular PtdIns4P metabolism is predominantly restricted to non-caveolar rafts present in the CLM fraction. Heterogeneity of the CLM preparation was confirmed using electron microscopy, which revealed that vesicles in this fraction exhibited a broad size range from 50-500 nm, whereas immunopurified caveolae were 50-100 nm in diameter.

The revised hypothesis, namely that PtdIns(4,5)P_2 synthesis is overwhelmingly confined to non-caveolar rafts, is arguably more generally applicable because some cells, including lymphocytes and neuronal cells [16,17], have no discernible plasma membrane caveolae. In contrast, both PtdIns4P synthesis and non-caveolar rafts have been found in all mammalian cell types. As EGF elicits a modest increase in type II PtdIns 4-kinase activity in A431 cells [14,15], we investigated whether EGF receptors were in caveolae or non-caveolar rafts. Using the methods described above for raft purification, EGF receptors were found to be localized and activated within non-caveolar rafts [18]. In addition, neither the bulk of the cellular EGF receptors nor type II PtdIns 4-kinase activity co-fractionated with rafts prepared in the presence of detergent [9]. In summary, these results indicated that EGF receptors, PtdIns and PtdIns 4-kinase localize to one or more novel types of raft, which have different properties to caveolae and detergent-insoluble rafts.

**Characterization of a raft phosphoinositide kinase: the type II PtdIns 4-kinase**

In order to characterize the PtdIns4P-producing rafts, a molecular definition of the components was required. Notably absent, however, were reagents with which to study the type II PtdIns 4-kinase, which had not been cloned. Consequently, a strategy was devised to purify and clone the type II PtdIns 4-kinase from A431 cells [19]. Along with its role as a key constituent of the PtdIns4P-producing rafts, the type II PtdIns 4-kinase is the predominant PtdIns kinase activity in many mammalian cells [20]. However, despite years of study, there had been little real progress in purifying the enzyme sufficiently to allow sequencing and cDNA cloning. The technical problems that had rendered previous attempts to purify the protein unsuccessful arose primarily from the poor solubility of the enzyme in non-ionic detergents and its tendency to form aggregates. Our observation that the type II PtdIns 4-kinase was localized to membrane rafts, which are thought to be cholesterol- and sphingolipid-rich entities, provided the impetus for the design of a more rational, microscale approach to its purification [19]. The first step in this approach was to prepare the PtdIns 4-kinase-rich CLM fraction, which provided a highly purified starting material. Further purification was effected by carrying out sonication and subsequent chromatography steps in the presence of different concentrations of the detergents β-octyl glucoside and deoxycholate. This detergent pair had previously been shown to be effective in solubilizing rafts [21,22] and the type II PtdIns 4-kinase [23]. The inclusion of 10 mM octyl glucoside and 4 mM deoxycholate during sonication removed several raft proteins from the CLM fraction, whereas the proportion of the cellular PtdIns 4-kinase activity in the buoyant fraction was unchanged. Buoyant rafts could then be harvested by ultracentrifugation at 190000 g. Subsequent treatment of the pelletcd rafts with an increased detergent concentration of 100 mM β-octyl glucoside and 40 mM deoxycholate led to efficient solubilization of the type II PtdIns 4-kinase activity. Hence, an appreciation of the lipid environment of the enzyme was crucial, both for the generation of a highly purified starting material and for efficient solubilization of the type II PtdIns 4-kinase. Sequential chromatographic separations using MonoQ and MiniS columns were subsequently employed to further purify the enzyme.
PtdIns 4-kinase activity was found to co-fractionate with a 52 kDa protein, which is consistent with numerous previous reports of the size of this isoenzyme. The candidate 52 kDa protein band was excised from a silver-stained SDS/PAGE separation of the most active MinIS fractions, digested with trypsin, and then subjected to analysis by MS (performed by Dr R. Cramer and Mr S. Corless of the Ludwig Institute for Cancer Research, University College London). Mass mapping led to the identification of a 1437 bp open reading frame (ORF), which accounted for all the peptide masses. MS also produced sequence information from four peptides, all of which verified the ORF assignment.

The corresponding full-length cDNA was cloned from A431 cell mRNA and expressed in bacteria as a glutathione S-transferase fusion protein. The recombinant protein possessed PtdIns 4-kinase activity, which was stimulated by low concentrations of Triton X-100 and inhibited by adenosine and the monoclonal antibody 4C5G. broccoli as a glutathione S-transferase fusion protein. The recombinant protein possessed PtdIns 4-kinase activity, which was stimulated by low concentrations of Triton X-100 and inhibited by adenosine and the monoclonal antibody 4C5G. MS also produced sequence information from four peptides, all of which verified the ORF assignment.

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Surprisingly, database searches revealed that the amino acid sequence has no homology with other PtdIns 4-kinase isoenzymes, or with any other known phosphoinositide or protein kinase. These searches did, however, identify a second human isoform [19], as well as homologues in a variety of organisms, including yeast and plants, thereby identifying the type II PtdIns 4-kinase as the first member of a novel phosphoinositide kinase family. We have termed the two human isoforms alpha and beta. The absence of sequence homology with previously cloned PtdIns 4-kinases is particularly intriguing, because it suggests that two PtdIns 4-kinase families have evolved independently.

Discussion

Despite its strong association with membranes, the type II PtdIns 4-kinase sequence has no obvious transmembrane domains. A palmitoylation site has been identified [24], and this modification might contribute to the membrane localization of the enzyme. Palmitoylation is known to be required for raft localization and signalling by several proteins, including CD8β [25], lck [26] and the linker for activation of T-cells ('LAT') protein [27]. However, not all palmitoylated proteins occur in rafts [28]. Consequently, the specific mechanisms or modifications that target the type II PtdIns 4-kinase to its raft-associated substrate remain to be elucidated. Nevertheless, the cloning of the type II PtdIns 4-kinase provides a useful step forward towards a better understanding of the compartmentation of PtdIns4P and PtdIns(4,5)P2 biosynthesis.

The mechanism by which phosphoinositides form rafts is an intriguing conundrum. The prevailing model of raft formation involves the coalescence of glycosphingolipids and cholesterol to form a liquid-ordered phase [4]. According to this model, saturated acyl groups partition into this phase far more favourably than unsaturated groups, because more linear structures allow the necessary close packing. However, phosphoinositides contain predominantly stearoyl (C18.0) and arachidonyl (C20.4) groups, and would therefore be expected to be excluded from glycosphingolipid- and cholesterol-rich rafts. On the other hand, the palmitoylated (C16:0) type II PtdIns 4-kinase would be expected to be included. Consequently, several fundamental questions arise. For example, do phosphoinositides form novel types of raft with novel structural requirements? If so, is the type II PtdIns 4-kinase included or excluded from these rafts? If the latter is true, what are the mechanisms by which the type II enzyme gains access to its substrate? Our ongoing identification of additional raft components will help elucidate not only the molecular organization of phosphoinositide and PtdIns 4-kinase rafting, but also the organization of agonist-dependent phosphoinositide signalling.

References


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