

Ras effectors: Buying shares in Ras plc

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The debate over whether activated Ras can regulate phosphoinositide-specific phospholipase C (PLC) has been contentious and at times heated. The argument may be resolved by the recent identification of a novel Ras-regulated PLC, but some unexpected properties of this protein are sure to stimulate further controversy.

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The Ras proteins are small GTP-binding proteins that have achieved notoriety as products of genes that are found mutated in approximately 15% of all human tumours [1]. In cells harbouring a Ras oncogene, the Ras oncoprotein is locked in the GTP-bound active conformation as a result of a defective GTPase activity. In this state, Ras is constitutively active and can transmit signals for proliferation, differentiation and oncogenesis in the absence of cellular stimulation. To fully appreciate how Ras signals downstream, there is a need to identify the proteins that interact directly with the active GTP-bound form of Ras.

Among the pathways that signal downstream from activated Ras, the one that starts with the serine-threonine kinase Raf and follows with activation of the ERK mitogen-activated protein (MAP) kinases, remains of key importance for many facets of Raf biology. However, it is now well appreciated that Ras uses a multitude of downstream effectors, in addition to Raf, to cause its diverse biological actions. The biochemically and genetically best-characterised of these are the phosphoinositide 3-kinases (PI 3-kinases) and members of the Ral guanine nucleotide exchange factor (RalGEF) family (reviewed in [2]). Results reported recently suggest that this select group has now been joined by phosphoinositide-specific phospholipase C epsilon (PLCε) — a novel PLC that binds to, and is regulated by, activated Ras [3–6].

Phosphoinositide-specific PLCs are a family of enzymes that regulate the hydrolysis of the inner plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) [7]. This reaction yields two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), which induce, respectively, activation of protein kinase C and the release of internally stored Ca²⁺. Prior to the discovery of PLCε, ten mammalian PLC isoforms had been identified: PLCβ1–4, PLCδ1–4 and PLCγ1–2, all of which contain a

δ-like core, composed of two regions of high sequence similarity (X and Y) which together constitute the PLC catalytic domain, a pleckstrin homology (PH) domain, an EF-hand domain, and a C2 domain (Figure 1) [7]. Besides this δ-like core, the β isozymes have carboxy-terminal extensions of around 400 residues, and the γ isozymes have an insertion of about 500 residues between the two halves of the catalytic domain. These structural differences are linked to the distinct mode of regulation for each isozyme: association with heterotrimeric G proteins of the Gq family stimulates the β isozymes, whereas the γ isozymes, which contains Src homology (SH2 and SH3) domains, are activated by receptor or cytosolic tyrosine kinases. The mechanism of regulation of PLCδ is less clear, but changes in intracellular Ca²⁺ may be involved [7].

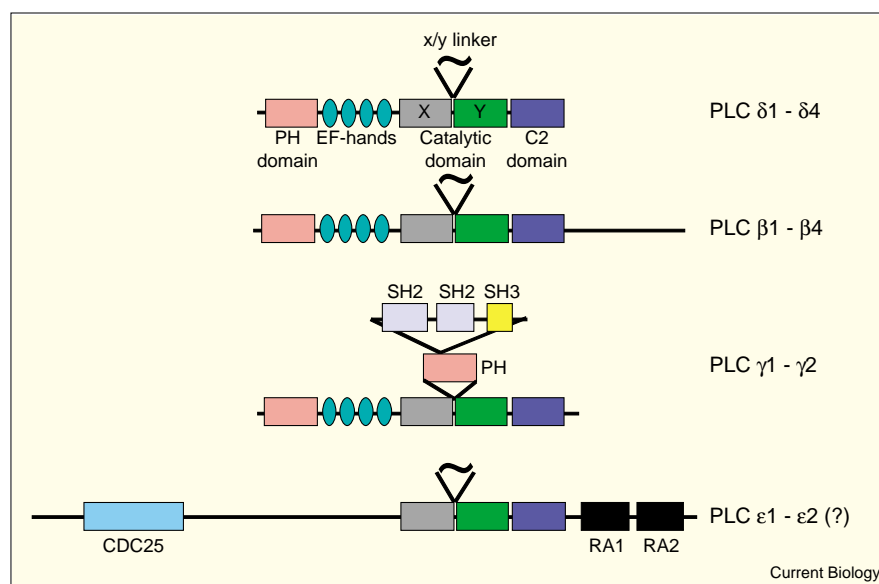
Sequence analysis of human [4,5], rat [6] and *Caenorhabditis elegans* [3] forms of PLCε showed that, whereas the centrally located X and Y domains and the C2 domain are highly conserved in this isozyme, the PH and EF-hand domains are absent. At the molecular level, however, the diagnostic features of PLCε are the presence of an amino-terminal Ras GTP exchange factor domain and two carboxy-terminal Ras-association domains, RA1 and RA2. This unique structural organisation clearly suggests that PLCε represents a novel fourth class of mammalian PLC isoforms. Interestingly, the human PLCε described by Lopez *et al.* [4] has an amino-terminal deletion of approximately 304 amino acids compared with the one cloned by Song *et al.* [5]. Although this deletion does not remove any of the functional domains, it does suggest the existence of at least two splice variants of human PLCε.

From the degree of conservation with other Ras effectors, one would predict that the RA domains of PLCε should bind activated Ras. Indeed, the RA2 domain of PLCε was found to bind Ras in a GTP-dependent manner, with an affinity comparable to the binding of Ras to Raf-1 [6]. Furthermore, the RA2 domain is typical of other Ras-binding domains in that it interacts with Ras through residues predicted by structural and functional analysis to be important in other Ras effectors [8]. For instance, mutation of a single lysine residue critical for Ras binding to other RA domains [8] — K2150 in rat PLCε — inhibits Ras binding to the RA2 domain [6]. Finally, like its binding to other Ras effectors, Ras binds to the RA2 domain of PLCε through its effector domain, as mutations in this region — such as T35S and Y40C — inhibit binding [6].

So clearly the RA2 domain of PLCε binds to activated Ras. But what is the significance of this interaction in terms of

Figure 1

A diagrammatic representation of the newly expanded family of mammalian PLC isozymes. The various molecular domains, and the possibility of two human PLC ϵ splice variants (tentatively termed PLC ϵ 1 and PLC ϵ 2) are discussed further in the text.



the ability of PLC ϵ to hydrolyse PIP₂? To address this question, Kelley *et al.* [6] examined the activity of PLC ϵ in COS-7 cells in the presence or absence of a constitutively active mutant form of H-Ras. They not only found that the mutant Ras markedly stimulated the PLC activity of PLC ϵ , but observed a complete correlation between the mutational effects on Ras binding to the RA2 domain and the resulting ability of Ras to stimulate the enzymatic activity of PLC ϵ [6]. Such an elegant analysis provides strong evidence that the binding of activated Ras to the RA2 domain results in activation of PLC ϵ . The criteria for being a genuine Ras effector — namely the ability to bind to and be regulated by activated Ras — are thus fulfilled by PLC ϵ . (Note, for those researchers using the various Ras effector mutants to specifically activate a given Ras effector, it is important to consider that the E37G mutant stimulates PLC ϵ and should therefore not be considered as specifically activating RalGDS; furthermore, the D38N mutant — up to now considered a universal inhibitor — significantly activates PLC ϵ [6].)

The precise mechanism by which Ras activates its effectors is currently unclear. For instance, the mechanism of Raf-1 activation is complex, requiring the Ras-induced translocation of Raf-1 to the plasma membrane, induction of a conformational change and modification by phosphorylation (see [9] for a current model). For the purposes of this discussion, however, it is notable that evidence is accumulating to suggest that Ras activates Raf-1 by binding to two Ras-binding domains [10,11]. In addition to the high-affinity RA domain, Raf-1 also has an adjacent cysteine-rich domain (CRD) that binds Ras in a

GTP-independent manner. It has been proposed that Ras interaction with this domain is critical for Ras activation of Raf-1 — a similar requirement for dual Ras binding sites appears necessary for Ras activation of yeast adenylyl cyclase [12].

Given that PLC ϵ has two RA domains, can a similar model account for the Ras-mediated activation of PLC ϵ ? A number of observations are consistent with this. First, for Ras to activate PLC ϵ it must be post-translationally modified [6]. Secondly, activated Ras can induce the plasma membrane translocation of cytosolic PLC ϵ [5]. Finally, the RA1 domain of PLC ϵ is not only similar to the CRD of Raf-1, in that it binds Ras with a low affinity and in a GTP-independent manner [6], but it is also required for Ras-mediated activation of PLC ϵ [6]. Thus, the mechanism for Ras activation of PLC ϵ is certainly complex, involving translocation and/or activation via the tandem RA domains.

Activated Ras may not, however, be the sole regulator of PLC ϵ . As with a number of other Ras effectors, the RA2 domain of PLC ϵ also binds the GTP-bound form of the Ras-related protein Rap1A — this results from Rap1A having an effector domain virtually identical to that of Ras [5,6]. Although this interaction may target PLC ϵ to a perinuclear localisation, rather than the plasma membrane [5], its physiological relevance remains to be established. Lopez *et al.* [4] also suggest that PLC ϵ is one of the few effectors for the G_{12/13} family of heterotrimeric G-proteins. These highly oncogenic G proteins — a GTPase deficient mutant of G α_{12} can fully transform NIH3T3

cells [13] — are currently poorly characterised. $G\alpha_{12}$ is known to stimulate c-Jun via activation of Ras, but the mechanism by which $G\alpha_{12}$ activates Ras is unknown [14]. Given that PLC ϵ contains an amino-terminal Ras GTP exchange factor domain, it is tempting to speculate that PLC ϵ may act as a direct link between $G\alpha_{12}$ and Ras. Indeed, expression of PLC ϵ promotes Ras activation by the exchange of GTP for bound GDP [4], but it is unclear whether $G\alpha_{12}$ can directly stimulate this activity.

The ability of PLC ϵ to activate Ras raises the possibility that PLC ϵ may positively regulate its own PLC activity. If this is the case, how can such a feedforward cycle be regulated? Although there are a number of possible mechanisms, it is tempting to speculate about the involvement of GAP1^m and GAP1^{IP4BP}. These GTPase-activating proteins (GAPs) switch-off Ras signalling by stimulating the intrinsic Ras GTPase activity, thereby converting Ras from the active GTP-bound to the inactive GDP-bound conformation [15]. As the GAP activities of GAP1^m and GAP1^{IP4BP} are stimulated by $G\alpha_{12}$ and inositol 1,3,4,5-tetrakisphosphate (IP₄), respectively [16,17], it is an intriguing possibility that they may be involved in negatively regulating PLC ϵ . (Note that IP₄ is a second messenger generated by the phosphorylation of IP₃, one of the products of PLC ϵ -stimulated PIP₂ hydrolysis.)

However, the really important question remains: how does PLC ϵ fit in with our understanding of Ras signalling? Although there is a clearly established link between Ras and PI 3-kinases [1,2] — the other major inositol-based signalling pathway in cells — relatively little information is available on a link between Ras and PLC. Back in 1986, the groups of Lewis Cantley [18] and Mike Wakelam [19] reported independent evidence that an increase in the level of inositol phosphates could be detected in cells expressing activated Ras. This result — indicative of an increase in PLC activity — led the authors to suggest that activated Ras lay upstream of PLC [18–20]. At the time, these data were viewed as extremely controversial, and they subsequently have been largely ignored (see for example [21,22]). The identification of PLC ϵ rekindles interest in these data, and may in fact validate the basic conclusion drawn by Cantley and Wakelam. Irrespective of this, the really important point is that the characterisation of PLC ϵ as a Ras effector has again placed PLC in the spotlight of Ras biology. As with other Ras effectors, the next crucial step is to establish the role of PLC ϵ in transmitting signals from activated Ras through to changes in proliferation, differentiation and oncogenesis. As Ras most likely uses distinct sets of effectors in a combinatorial fashion to effect its diverse biological actions, this will be an exciting, but challenging problem.

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