Minireview

# Regulation of proliferation and apoptosis by Ras and Rho GTPases through specific phospholipid-dependent signaling

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**Abstract Small GTPases are molecular switches that control signaling pathways critical for diverse cellular functions. Recent evidence indicates that multiple effector molecules can be activated by small GTPases. As a result, complex biological processes such as cell proliferation and apoptosis are turned on. Thus, rather than a single linear pathway from the membrane to the nucleus, the integration of complementary signals is required for these events to occur. In fact, the coordinated activation of small GTPases may constitute some of the critical modulators of those signals triggering either proliferation or cell death. In addition to the activation of specific kinases cascades, phospholipid-derived messengers are candidates to compose some of the most critical elements associated to regulation of signaling cascades capable of discerning among life and death. Both proliferation and apoptosis needs competence and progression signals. Phospholipase D and sphingomyelinase may be important players in this decision-maker step.**



*Key words:* Ras; Rho; GTPase; Phospholipid; Transformation; Apoptosis

## 1. Ras superfamily of proteins

The family of *ras* genes include more than 50 members grouped in at least three major subfamilies or branches according to their primary structures [1]. They all code for proteins with a molecular mass of 20–25 kDa and homologies ranging from 30 to 60% that bind and hydrolyze GTP allowing them to modulate the activity of a great diversity of effector molecules. Thus, when associated to GDP, these GTPases stay as resting, non-active complexes but become activated when bound GDP is exchanged for GTP by their interaction with specific exchange factors [1,2]. The three major branches comprise the Rab subfamily, involved in the regulation of secretion and vesicular trafficking, the Ras subfamily, involved in the regulation of cell growth and differentiation, and the Rho subfamily, involved in the regulation of cell growth and apoptosis. In addition, activation of Ras and Rho proteins affect cell morphology. Finally, an important member of this superfamily is the family of ADP-ribosylation factors (ARF) involved in the activation of cholera toxin, regulation of phospholipase D (PLD) and membrane traffic [3].

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## 2. Phospholipid metabolism in cell signaling: involvement in cell proliferation

Membrane phospholipids are essential structural components of eukaryotic cells, but also play an important role in the control of diverse cellular responses. One of the best characterized examples is phosphatidyl-inositol,1-4 bisphosphate (PIP2). Hydrolysis of this lipid by a phosphoinositide (PI)-specific phospholipase C (PI-PLC) results in the generation of diacylglycerol (DAG), and inositol-1,4,5-triphosphate (IP3). This pathway is activated by a large variety of mitogenic factors by either tyrosine phosphorylation or heterotrimeric GTPases in the regulation of PI-PLC [4]. Generation of DAG and IPs induces activation of PKC [5]. However, this pathway is dispensable for mitogenic stimulation by growth factors since normal mitogenicity is observed with mutated receptors unable to activate PI-PLC [6,7].

Mitogenic stimulation of RTKs induces a bimodal production of DAG. The first wave occurs immediately after stimulation and is maintained for a few minutes. The second wave takes place at later times of stimulation and is much more sustained in time. The early DAG peak is associated to PI hydrolysis by specific phospholipases, while no detectable release of IP3 is associated to the late DAG peak [4,5]. These results suggested the existence of a DAG source different from that of PI hydrolysis that was identified as phosphatidylcholine (PC) [8]. This hypothesis was supported further by the evidence that several growth factors induce PC hydrolysis in a more sustained manner that PI hydrolysis, coincident with the second wave of DAG. The finding that oncogenic *ras*-p21s induced the constitutive increased generation of phosphorylcholine (*P*Cho) and DAG, provided the evidence for a constitutive increase in the hydrolysis of PC [9].

*P*Cho is produced in fibroblasts as a result of treatment with mitogenic factors at both early (1 min) and late times (4–12 h) of induction, as a consequence of the consecutive activation of PLD and choline kinase [10,11]. Phosphorylcholine (*P*Cho) has been proposed as an essential lipid metabolite involved in the regulation of DNA synthesis in murine fibroblasts. This hypothesis is based on the finding that inhibition of *P*Cho production by hemicholinium-3 (HC-3), a choline kinase inhibitor, completely eliminates DNA-synthesis in cells stimulated with growth factors including PDGF, bFGF and EGF but not by serum, implying a non-toxic effect [10,11]. Furthermore, the inhibitory effect is cell-cycle dependent [11] and can be reverted by addition of insulin [10]. Consistent with these results, novel compounds derived from HC-3 with up to 2000-fold higher inhibitory activity towards ChoK, show also a similar effect on DNA synthesis stimula-


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tion by growth factors [Hernández-Alcoceba et al., submitted], providing further support to the role of *P*Cho in the regulation of the mitogenic response. Finally, tumor samples are frequently associated with an increase in *P*Cho compared to the normal cells [12,13], a strong support to the hypothesis of an important role for this metabolite in cell growth control.

Other lipid pathways have been implicated in the regulation of cell growth. PLD is activated by receptor Tyr kinases (RTK) or PKC activators [8]. Hydrolysis of PC by PLD generates phosphatidic acid (PA) and choline, and represents also a common response to a large variety of mitogens. PA is a weak mitogen for fibroblasts, while Lyso-PA (LPA), which results from phospholipase A2 action on PA, shows a more potent mitogenic activity [14,15]. Choline itself has no mitogenic activity, but it is required for the biosynthesis of PC [16]. A role in the regulation of cell growth has been reported for sphingosine-1-phosphate [17] or sphingosylphosphorylcholine [18]. These metabolites are related to $IP_3$-independent $Ca^{2+}$ mobilization, and PLD activation [19].

## 3. Regulation of PLD by small GTPases of the Ras superfamily

Generation of *P*Cho and DAG in both serum-treated cells and in *ras*-transformed cells requires a two-hits mechanism [20]. When choline kinase (ChoK) was inhibited by hemicholinium-3 (HC-3), *P*Cho levels did not increase after serum stimulation, while DAG increased as in HC-3 untreated cells, an indication that *P*Cho production was sensitive to ChoK blockage. By contrast, when cells were stimulated by serum in the presence of propranolol, DAG levels were those of non-stimulated cells, indicating that DAG production was sensitive to PA hydrolase. Moreover, while no significant activation of PC-PLC was detected, a clear activation of a PC-PLD was observed after serum addition [20]. Similar results were observed in the basal, constitutively increased levels of DAG and *P*Cho found in *ras*-transformed NIH 3T3 cells [20–23]. Thus, by using HC-3 and propranolol, generation of DAG and *P*Cho in normal cells stimulated by serum and in *ras*-transformed cells can be uncoupled. These results are similar to those found in fibroblast cells transformed by the *src* or *raf* oncogenes [23,24]. Activation of PLD by Ras is independent of PKC, but synergizes with phorbol esters [25]. In addition, *ras* transformation uncouples PLD activation by growth factors through specific interference with the activation of the PI-PLCγ pathway [25]. These results suggest the existence of both forward and feed-back mechanisms in the regulation of cell responses by phospholipid metabolites.

By contrast to the results obtained in the *ras*-transformed cells, in normal cells stimulated by serum, PLD activation follows a transient kinetics with a maximal activity detected at 30 min of stimulation, and a residual activation of around 20% increase from 1 to at least 12 h of stimulation [26]. As a consequence, PA levels rise in NIH 3T3 cells stimulated by serum and decreases to almost basal levels after 60 min. However, at late times of serum stimulation, both choline release, and *P*Cho and DAG levels are elevated, like in *ras*-transformed cells. The nature of this increase is still not clear, but a residual PLD activation could explain all these events as previously suggested [26]. DAG does participate in the activation of the conventional and novel PKCs with important consequences regarding the progression to cell proliferation or apoptosis [[5]; see next paragraph].

The comparative study from both *Xenopus* oocytes and mammalian fibroblasts indicates that *ras*-p21 proteins activate a PLD enzyme in both systems, generating PA, DAG and *P*Cho as active metabolites [10,11,27,28]. However, the biological function of each one of these metabolites depends on the specific cellular system. In fibroblasts, in addition to the increased PLD activity, other enzymes such as choline kinase and PA-phosphohydrolase may be also required. However, in *X. laevis*, PA seems to be sufficient to carry out the biological function through activation of the Ser/Thr kinase cascade [27,28]. These results could also be expanded to other cellular systems with different biological responses.

Other small GTPases and oncogenes are also capable of activating PLD. Basal levels of *src*- [24] and *rho*-transformed cells [29] have increased PLD activity with elevated PA, Cho and *P*Cho metabolites. These results are consistent with those reported using cell-free systems, where both Rho and ARF-1 proteins have been shown to activate PLD [30,31]. Finally, it has been reported recently that activation of PLD induced by *src* is mediated by a Ras- and Ral-dependent mechanism [32].

Regulation of PLD may be complex since it has been demonstrated that activation of PLD by growth factors follows a two-ways mechanism. Both a PI-PLC/DAG/PKC-dependent pathway, triggered by growth factors, and a Ras-dependent, PKC-independent pathway has been demonstrated [25]. Furthermore, while activation by growth factors and phorbol esters show a cooperative pattern which results in an additive mechanism, transformation by the *ras*, *sis*, *src* and *met* oncogenes results in a synergistic activation of PLD by phorbol esters treatment [25]. In addition, transformation by the *ras*, *src* and *met* oncogenes results in a complete abrogation of the PI-PLC-dependent activation of PLD by growth factors [25]. However, transformation by other oncogenes such as *fgr*, *fms* or overexpression of TGFα, does not affect PLD activation by phorbol esters or growth factors, an indication that each oncogene may or may not use the PLD pathway in a defined and specific manner dependent on endogenous signaling pathways. PLD has potent mitogenic activity by itself [15,20]. Thus, significant differences in PLD regulation by different oncogenes may contribute to the unrestricted cell growth in transformed cells.

## 4. Phospholipids and apoptosis

There are at least two important types of lipid derivatives which may affect regulation of apoptosis: ceramides and PI3K-derived metabolites PI(3,4)P3 and PI(3,4,5)P3. Ceramides are produced as a consequence of activation of sphingomyelinase, a phospholipase C specific for sphingomyeline, while PI(3,4)P3 and PI(3,4,5)P3 results from the activation of PI3K. Ceramides function as inhibitors of cell growth by a mechanism that may involve a protein phosphatase of the PP2A type, and at least two types of Ser/Thr protein kinases [33,34]. Ceramides have been described as natural PKC inhibitors, generated as a result of treatment with several agents such as TNFα, γINF, IL-1 and others. As a further support for the role of ceramides in the regulation of cell growth, it has been recently found that ceramides induce apoptosis and that this effect can be reverted by activation of PKC with phorbol esters. Ceramides have been shown to induce the

activation of both the Raf1 kinase pathway in HL60 [35] as well as the JNK/SAPK pathway in U937 cells [36]. Ceramides can be converted into sphingosine-1-phosphate (SPP) via sphingosine, and these two metabolites, ceramides and SPP, have been suggested to carry out opposite effects on cell growth and apoptosis [37]. A recent report suggest that SPP and *P*Cho synergize for mitogenic stimulation [38], suggesting a cross-talk connection of these two metabolites.

## 5. Small GTPases in apoptosis

Strong evidence that Ras-related proteins may be involved in the regulation of apoptosis mostly comes from Ras and Rho proteins. Both proteins have been shown to behave as oncogenes in murine fibroblasts [39–41]. Although the Ras proteins are more potent than Rho proteins, *ras*-dependent transformation requires Rho function [40,41]. In addition, Ras is capable of inhibiting apoptosis in murine fibroblasts and epithelial cells by activating PI3K [42–44]. The products of the PI3K can trigger survival signals through activation of the serine/threonine kinase PKB/Akt [42–44], but other pathways cannot be ruled out. Activation of PI3K is achieved by growth factors such as PDGF or IGF-I, and oncogenic Ras proteins. Rho proteins (*Aplysia* Rho, and the human Rho A, Rho C, and Rac1), on the other hand, enhances apoptosis in murine fibroblasts and the human K562 erythroleukemia cells after serum deprivation, by a p53-independent mechanism [[45]; Esteve et al., submitted]. Finally, apoptosis by Rho proteins is related to the generation of ceramides [29] and can be blocked by Bcl2 expression both in vitro and in vivo [Esteve et al., submitted].

It has been recently demonstrated that apoptosis induced by Rho proteins requires complementary signals (Fig. 1), similar to the requirement for competence and progression signals in cell proliferation [[29,45]; Esteve et al., submitted]. While the progression signal is associated to ceramides production [29], the competence signal has to be yet identified. However, some hints are already available that can help identifying this partner. Rho proteins can induce the activation of multiple signaling pathways, including lipid enzymes such as PLD [29–31], PI3K [46] and PI5K [47], several diverse serine/threonine kinases [48–54] and myosin phosphatase [54]. In addition, Rho proteins can induce the activation of transcription factors such as Jun [55,56], SRF [57] and NF-κB [58]. Among all these effectors and activated pathways, it is critical to establish which ones are relevant for proliferation and apoptosis. Evidence for a dual role of JNK/SAPK and NF-κB in cell transformation and apoptosis has been reported [59], as for Rho [29,39–41,45] suggesting that they could be involved in either process induced by Rho.

Therefore, Ras and Rho proteins have the ability to activate transforming signals as well as signals that regulate apoptosis, either positively or negatively. The final outcome depends upon the environmental conditions and cell type [29,39–45]. A critical component of the signaling pathways discerning between proliferation and apoptosis may be the

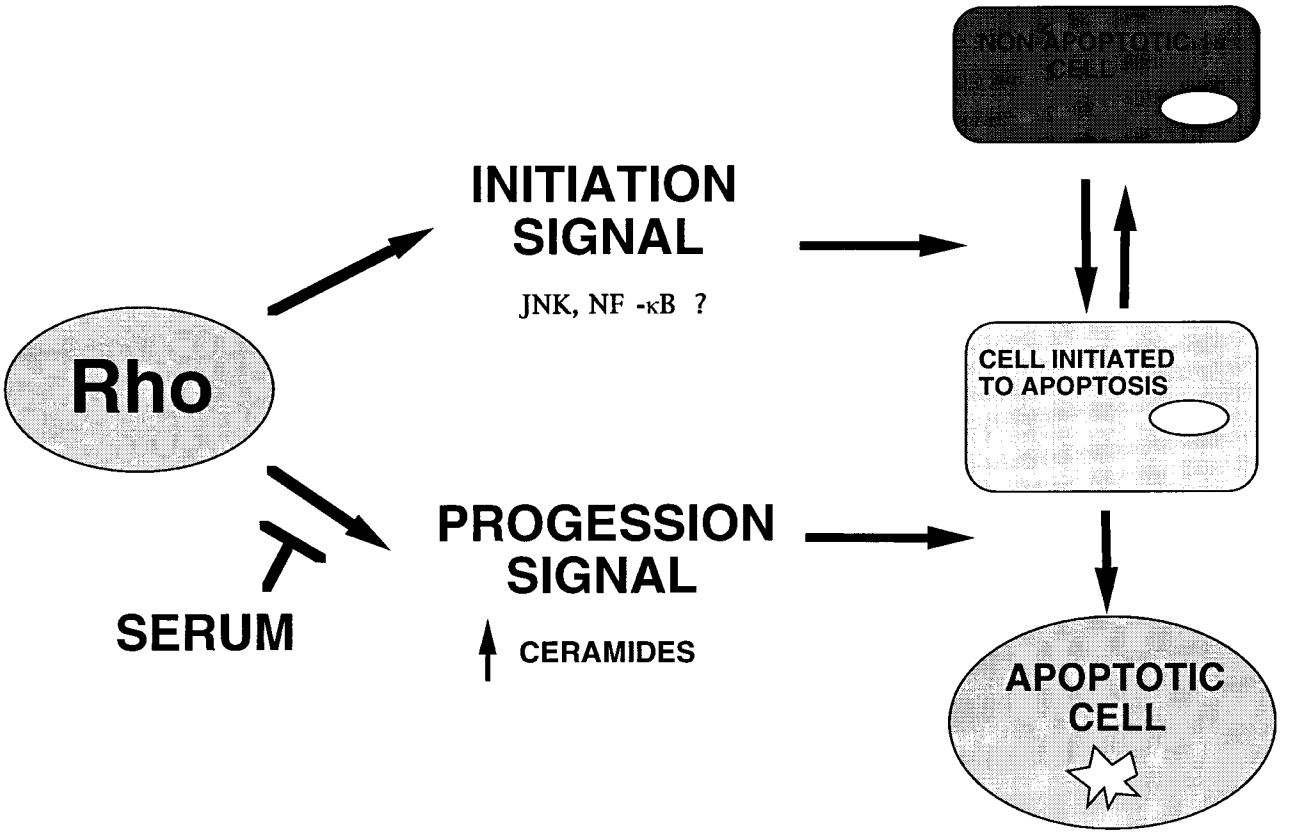


Fig. 1. Requirement of complementary signals for the induction of apoptosis. At least two complementary signals are required for the induction of apoptosis by Rho proteins in murine fibroblasts. While the progression signal has been identified as ceramides, the initiation signal could be any of the known signaling pathways activated by Rho, such as the JNK/SAPK kinase or the NF-κB transcription factors, two molecules involved in apoptosis in several cell systems.

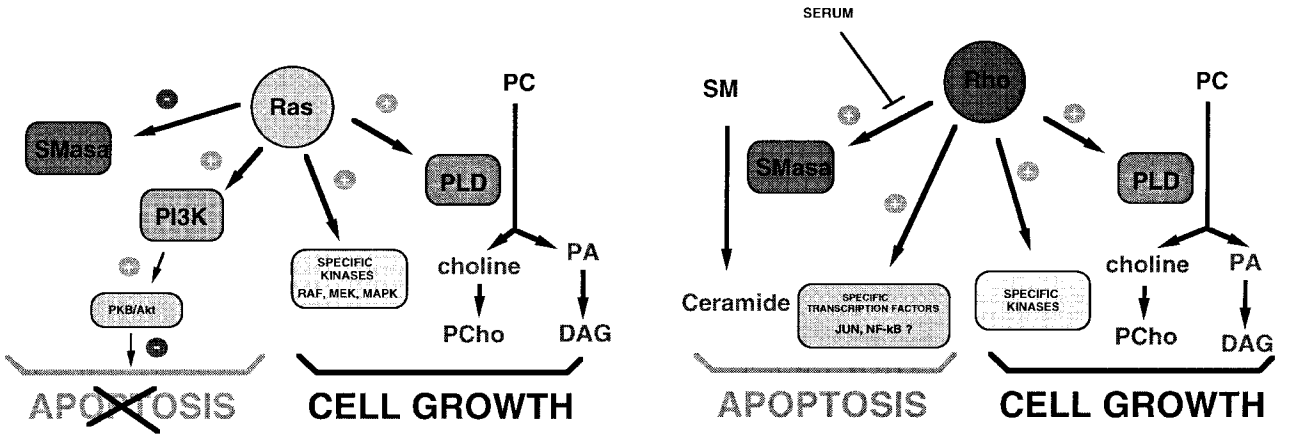


Fig. 2. Differential effects of Ras and Rho activation. Activation of specific signaling pathways involving lipid metabolism and diverse kinases would end in the generation of either cell growth or apoptosis. The final cellular response depends upon the integration of the complementary pathways activated. See text for details.

lipid component activated in each case. A major difference among these two proteins is that constitutively activated Ras protein can activate PLD and PI3K but not ceramides production, even in the absence of survival signals such as serum. These signals can turn on a survival response. Rho proteins activate PLD and PI-related kinases, but after serum removal they can activate also sphingomyelinase, resulting in the generation of ceramides (Fig. 2). Other components of both Ras- and Rho-dependent signaling such as the Raf/MAPK and the JNK/SAPK kinases and specific transcription factors may play a role in the integration response.

## 6. Perspectives and future

The Ras superfamily of proteins comprises small, but functionally complex, proteins which serves as critical switches for the regulation of multiple cellular functions. Both the Ras and Rho branches have been shown to be critical for regulation of cell proliferation and apoptosis. The mechanism of activation/inactivation of small GTPases has been elucidated concisely, and some of the specific molecular species involved identified, so we can now have a reasonably good understanding on their importance for diverse cell responses. However, a complete picture of the intricate and complex interactive networks for Ras and Rho proteins is still poorly understood. Thus, the finding that Ras proteins physically interact with at least Raf-1 kinase, PI3K and Ral-GDS activating all these pathways, may not be sufficient to explain all their known functions. Similarly, Rho proteins have shown to be even more complex than Ras, with a larger number of effector molecules identified so far. On top of that all, Ras-mediated transformation requires Rho functions, and both Ras and Rho play a dual role in transformation and apoptosis.

All the results discussed above suggest that phospholipid metabolites are important elements in the regulation of signaling cascades activated by GTPases of the Ras and Rho families. They function either in combination with complementary signals that activate specific kinases/phosphatases cascades ending in the upregulation of diverse transcription factors (AP-1, Fos, Jun, NF-κB, etc), or as an integral part of the kinases/phosphatases pathway. Simultaneous activation of both 'proliferation' as well as 'apoptosis' signals is a frequent event. The final result depends on the proper integration of signals by the target cell. Thus, understanding of the biochemical pathways activated by each effector for both Ras and Rho proteins will be critical for a better knowledge of the biological consequences and a more comprehensive consideration of the mechanism of regulation of both cell growth and apoptosis.

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