

Regulation of choline kinase activity by Ras proteins involves Ral–GDS and PI3K

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Ras proteins are molecular switches that control signaling pathways critical in the onset of a variety of human cancers. The signaling pathways activated by Ras proteins are those controlled by its direct effectors such as the serine-threonine protein kinase Raf-1, the exchange factor for other GTPases Ral–GDS, and the lipid kinase PI3K. As a consequence of Ras activation, a number of additional enzymes are affected, including several members of the serine-threonine intracellular proteins kinases as well as enzymes related to phospholipid metabolism regulation such as phospholipases A2 and D, and choline kinase. The precise mechanisms by which *ras* oncogenes impinge into these later molecules and their relevance to the onset of the carcinogenic process is still not fully understood. Here we have investigated the mechanism of regulation of choline kinase by Ras proteins and found no direct link between PLD and choline kinase activation. We provide evidence that Ras proteins regulate the activity of choline kinase through its direct effectors Ral–GDS and PI3K, while the Raf pathways seems to be not relevant in this process. The importance of Ras-dependent activation of choline kinase is discussed.

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Introduction

Ras GTPases constitute a central point in signal transduction pathways that lead to the regulation of cell growth (Malumbres and Pellicer, 1999; Hernández-Alcoceba *et al.*, 2000; Ramírez de Molina *et al.*, 2001a) and have been widely implicated in the carcinogenic process in humans (Barbacid, 1987; Bos, 1989; Rodenhuis, 1992; Bernhared *et al.*, 2000). The mechanisms by which Ras GTPases subvert the normal regulation in cells is still not completely understood. A great effort has been devoted to identify the signaling pathways governed by Ras proteins. As a result,

evidence has been provided that supports a role of lipid-derived second messengers in the transforming properties of Ras oncoproteins.

Phosphatidylcholine (PC), the major component of the plasma membrane, is hydrolyzed by phospholipase D (PLD) to yield phosphatidic acid (PA) and choline. PA is then either hydrolyzed to generate diacylglycerol (DAG), or deacylated to form lyso-PA (LPA). On the other hand, choline is phosphorylated by choline kinase (ChoK) to generate phosphorylcholine (PCho). Both PLD and ChoK have been demonstrated to play a role in malignant transformation. PLD has been implicated in the metastatic process (Reich *et al.*, 1995; Aguirre-Ghiso *et al.*, 1999), and increased levels of ChoK activity and PCho production in human cancers have been found (Bhakoo *et al.*, 1996; Nakagami *et al.*, 1999; Ruiz-Cabello and Cohen, 1992), keeping with previous reports using nuclear magnetic resonance techniques (NMR) (de Certaines *et al.*, 1993; Smith *et al.*, 1993). These observations have resulted in the development of different anti-tumoral strategies focused on PLD and ChoK (Rameh and Cantley, 1999; Gratas and Powis, 1993; Lucas *et al.*, 2001; Hernández-Alcoceba *et al.*, 1999; Lacal, 2001; Ramírez de Molina *et al.*, 2001a). Nevertheless, not much is known about the way these lipid-derived second messengers are mediating the process of transformation, and work aimed to understand this mechanism will help to improve the design of an efficient anticancer therapy.

In this sense, *ras* oncogenes have been shown to activate PLD and ChoK, resulting in an increase in PCho production (Lacal *et al.*, 1987; Lacal, 1990; Macara, 1989; Rameh and Cantley, 1999; Cuadrado *et al.*, 1993; Jiménez *et al.*, 1995; Lucas *et al.*, 2000; Bhakoo *et al.*, 1996; Ratnam and Kent, 1995). However, whether PLD and ChoK activation after transformation by *ras* takes place in a sequential or independent manner and the way it occurs, remains still unknown.

In its active form, bound to GTP, Ras interacts with several downstream molecules eliciting different signal transduction pathways whose alterations may lead to tumorigenesis. The most extensively studied effectors to Ras are the Raf serine/threonine kinase-1, the Ral–GDS exchange factor, and the lipid kinase PI3K (Malumbres and Pellicer, 1999; Katz and McCormick, 1997; Hernández-Alcoceba *et al.*, 2000; Crespo and

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León, 2000). The interaction of Ras with these effectors involves two switch domains known as effector domains I and II. The switch I region, comprises amino acids 32–40, and the switch II region amino acids 60–72 (Marshall, 1993; Akasaka *et al.*, 1996). Mutations in any of these two domains make Ras unable to interact with its downstream molecules decreasing Ras biological functions (Moodie *et al.*, 1995; Srivastava *et al.*, 1989; Sigal *et al.*, 1986). Furthermore, specific changes in residues of the effector domain I abrogate selectively Ras interaction with each of these effectors, allowing the activation of specific downstream signaling pathways elicited by this oncoprotein (White *et al.*, 1995). In addition, it has been demonstrated that a combination of at least two of these effector molecules is necessary for the transforming properties of Ras (White *et al.*, 1995).

The aim of this work was to study the mechanisms by which transformation by *ras* affects ChoK activity and the putative involvement of PLD and Ras effectors in this process. A model for ChoK regulation by oncogenic Ras is proposed.

Results

Phospholipase D overexpression has no effect on choline kinase activity

In order to investigate whether PLD and ChoK, two enzymes involved in the Kennedy pathway are regulated by Ras proteins by the same mechanism, we first tested whether Ras activation of ChoK was specific on the enzyme or a consequence of the activation of PLD. To that end, NIH3T3 cell lines stably over-expressing PLD1 and PLD2, the two known isoforms of this ubiquitous enzyme (Exton, 2000; Colley *et al.*, 1997) were generated. Over-expression of either PLD1 and PLD2 in the transfected cells was achieved (Figure 1a). *In vivo* PLD enzymatic activity was assayed by its well-characterized associated transphosphatidylating activity to butanol (Song *et al.*, 1991). Basal levels of PLD were greatly increased in these cell lines as a consequence of either PLD1 and PLD2 over-expression (Figure 1b).

Levels of ChoK activity were analysed in these cells and compared to the ones observed in NIH3T3 cells overexpressing oncogenic H-*ras*. Whereas *ras* over-expression results in a more than twofold increase in ChoK activity, PLD expression (either PLD1 or PLD2) has no effect on the activity of this enzyme (Figure 1c). In keeping with this observation, when cells were isotopically labeled with ^{14}C -Choline, a more than twofold increase in the intracellular basal levels of *PCho* was observed in *ras*-transformed cells, while the intracellular *PCho* levels were similar in both control and PLD-transfected cells (Figure 1d). These results demonstrate a regulation of ChoK independent of PLD activation, and suggest that ChoK is susceptible of specific regulation by oncogenic *ras*. Thus, ChoK

increase after transformation is not a consequence of an indirect effect due to Ras-mediated PLD activation.

Choline kinase is regulated upon ras transformation in a PLD independent manner

To further confirm this hypothesis, we transiently transfected the oncogenic version of H-*ras* and the two known isoforms of PLD in human embryonic Kidney HEK293T cells (Figure 2a). PLD1 is regulated by growth factors, protein kinase $\text{Ca}(\text{PKC}\alpha)$ and oncogenes like *ras*, whereas PLD2 seems to be constitutively active (Guillemin and Exton, 1998; Baldasare *et al.*, 1997; Muller *et al.*, 2000; Bourdoulous *et al.*, 1998). Thus, we also co-transfected PLD1 and *ras* in order to measure ChoK activity under conditions of PLD activation by this oncogene. A small but significant increase in PLD activity for cells over-expressing PLD1 and a sevenfold increase in PLD activity for PLD2 were observed (Figure 2b). Furthermore, co-expression of oncogenic Ras with PLD1 resulted in an enhanced PLD activity to a similar extent to that observed when overexpressing PLD2 (Figure 2b). Cells expressing oncogenic Ras display higher levels of both ChoK activity (Figure 3a) and intracellular *PCho* content (Figure 3b). However, these effects were not observed in cells overexpressing PLD (Figure 3). Finally, an almost sevenfold increase in PLD1 activity due to *ras* co-expression did not enhance ChoK activity nor increased basal intracellular levels of *PCho*, that are similar to the ones observed when expressing *ras* alone. All these results indicate that ChoK and PLD1 are regulated upon *ras* transformation in an independent manner. The strong activation of PLD1, but not PLD2, after Ras co-expression represents in itself an interesting observation that deserves further investigation and will be reported separately.

Ras proteins do not activate ChoK by direct interaction

Transformation by *ras* induces a constitutive increase of ChoK activity and consequently, of the basal levels of its product, *PCho* (Hernández-Alcoceba *et al.*, 1999; Lacal *et al.*, 1987; Lacal, 2001; Ramírez de Molina *et al.*, 2001b). As shown above, this is not a consequence of activation of PLD. We next investigated whether activation of ChoK by Ras was due to direct interaction of both proteins. To that end, a series of experiments were performed to address this issue. The Ras protein was purified as described under Materials and methods (Figure 4a). Addition of this recombinant Ras protein to NIH3T3 cell extracts resulted in a minor but significant increase in ChoK activity in a dose dependent manner (Figure 4b). As negative controls, addition of similar amounts of bovine serum albumin (BSA) or GST (purified as the Ras protein) had no significant effect in the activity of the enzyme. Next, precipitation of GST–Ras previously incubated with recombinant ChoK was carried out. No significant differences were observed when comparing ChoK

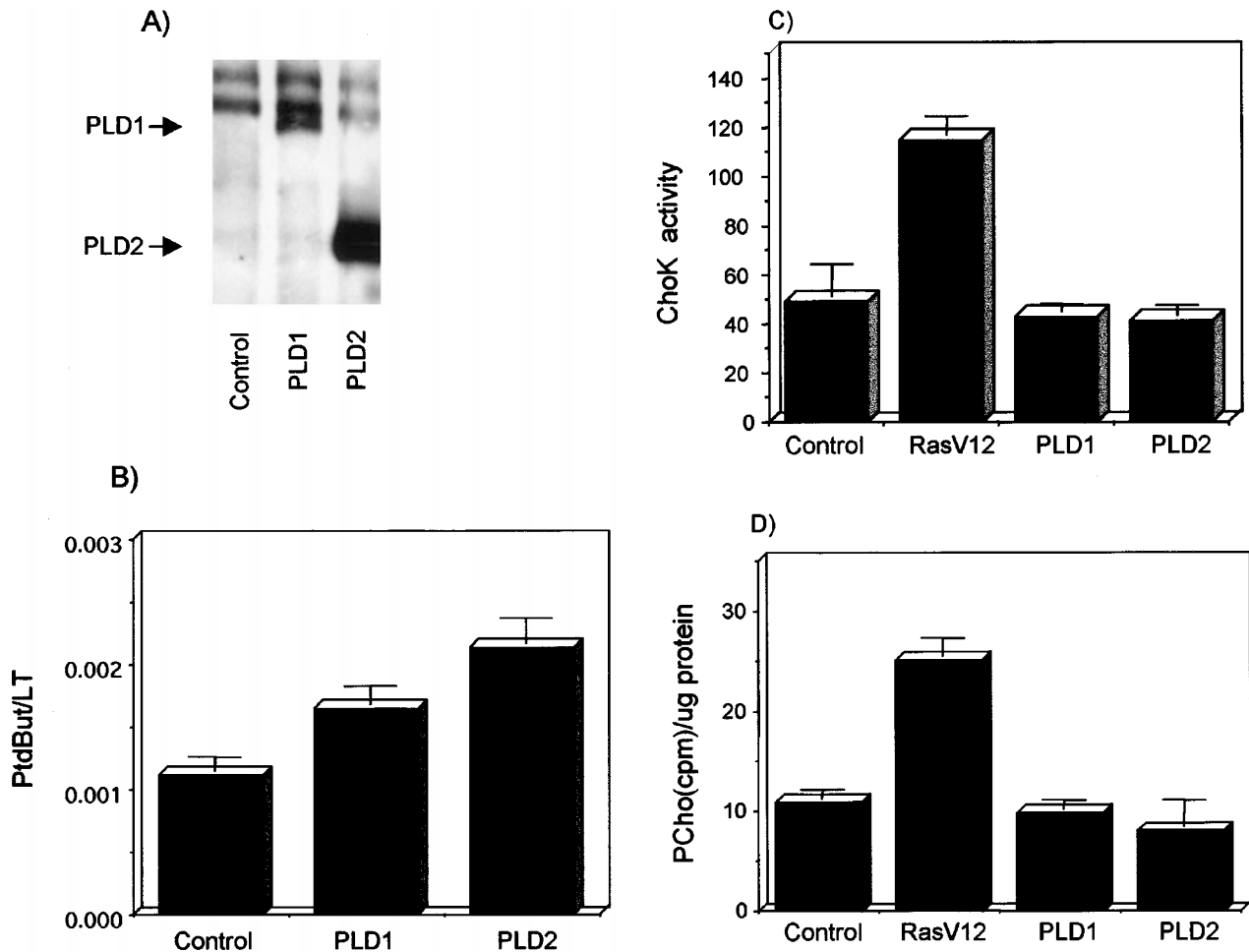


Figure 1 Overexpression of PLD1 and PLD2 results in constitutive increased basal PLD activity with no effect on both Choline Kinase activity and intracellular levels of *PCho*. (a) NIH3T3 cells were transfected with vector alone (control) or vector carrying each of the two known isoforms of phospholipase D, PLD1 and PLD2 carrying an HA-tagging by standard procedures, and selected with G418 as mass cultures. Expression of either PLD1 or PLD2 of these cell lines was tested by Western blot using a specific anti-HA antibody. Cells were grown to confluence, lysed and equivalent amounts of cell extracts were resolved by electrophoresis (SDS-PAGE), transferred to nitrocellulose paper and blotted against the correspondent antibodies as described under Materials and methods. NIH3T3 cells stably overexpressing RasV12 have been described previously (Lucas *et al.*, 2000). (b) Basal PLD activity of selected NIH cells expressing PLD proteins. Cells were grown and labeled for 72 h with 1 μ ci/ml [14 C]-glycerol in DMEM supplemented with 10% new born calf serum. Cells were then washed and incubated for 30 min in serum free DMEM supplemented with 0.5% 1-butanol. PLD activity was determined as indicated under Materials and methods. Results indicate the mean values \pm s.e.m. of three independent experiments each performed in duplicate. (c) Choline kinase assays of NIH3T3 cells stably overexpressing RasV12, PLD1 and PLD2 were performed as described in Materials and methods using 100 μ g cell extract per assay and 200 μ M of Cho as substrate. Data represents the mean \pm s.e.m. of four independent assays performed in duplicate. (d) NIH3T3 cells overexpressing RasV12, PLD1 and PLD2 were grown in DMEM supplemented with 10% new born calf serum and after a labeling period of 24 h in the presence of methyl[14 C]-choline, medium was discarded and cells were processed for determination of *PCho* generation as described in Materials and methods. Data are the mean \pm s.e.m. of four independent experiments in duplicate

activity of this precipitate with that of control, GST alone previously incubated with recombinant ChoK (Figure 5a). Similar results were obtained when measuring ChoK activity in a precipitate of GST-hChoK previously incubated with recombinant Ras (Figure 5b). Further controls indicated that Ras and ChoK were efficiently pulled down under these conditions, and that a lack of a physical interaction was the explanation for these negative results (Figure 5c). Thus, Ras effects on ChoK activation do not take place by a direct protein-protein interaction between

Ras and ChoK. However the relative small increase in ChoK activity suggest that the mechanism of regulation implies other components in Ras signaling.

Ral GDS and P13Kinase are the mayor contributors to ras-mediated activation of choline kinase

Ras proteins interact with several downstream molecules through two switch domains in order to exert their biological effects. The best characterized Ras effectors are Raf-1 kinase, P13 Kinase and the RalA

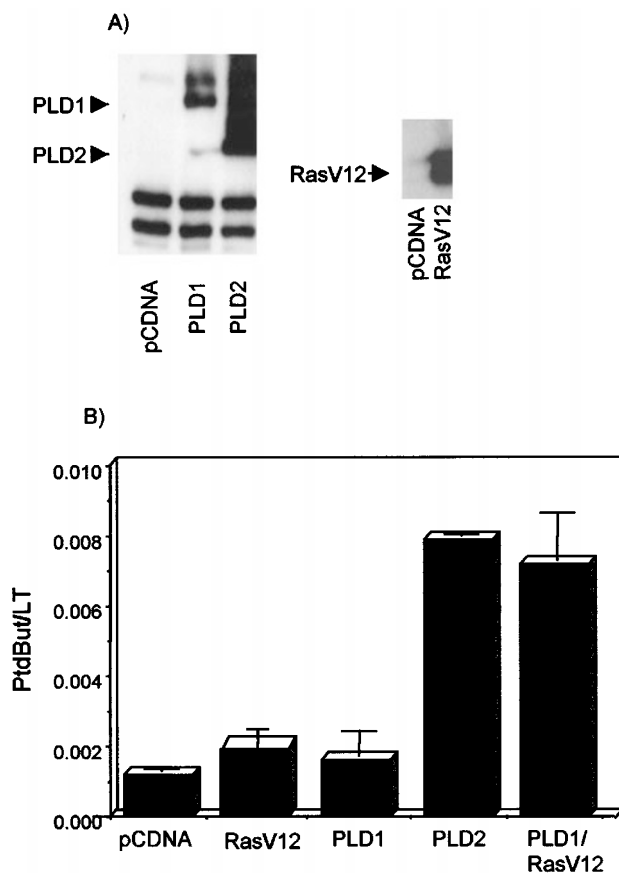


Figure 2 RasV12 stimulates PLD activity. (a) HEK 293T cells were transiently transfected with vector alone as control (pCDNA) or vector carrying PLD1, PLD2, RasV12 or both PLD1 and RasV12. Forty hours post transfection cells were harvested, lysed and overexpression of these proteins was tested by Western blot using an specific antibody as described in Materials and methods. (b) Basal PLD activity of HeK 293T cells expressing PLD, RasV12 and both PLD1 and RasV12. Cells were transiently transfected and after transfection cells were labeled for 24 h with 1 μ ci/ml [14 C]-glycerol in DMEM supplemented with 10% new born calf serum. After labeling period, cells were washed and incubated for 30 min in serum free DMEM supplemented with 0.5% 1-butanol. PLD activity was determined as indicated under Materials and methods. Results indicate the mean values \pm s.e.m. of four independent experiments each performed in duplicate

and B exchange factor Ral-GDS. Mutants for Ras proteins in these domains have been identified that selectively interact with one of these three effectors (White *et al.*, 1995; Rodríguez-Viciana *et al.*, 1997).

We next investigated whether any of these well-known effectors to Ras are involved in ChoK stimulation. To that end, NIH3T3 cells that stably overexpress RasV12 mutants that specifically interact with each of these molecules were generated. Two mutants were obtained with an oncogenic mutation at position 12 (Val12) that have additionally substituted residues at G37 and C40. Previous reports have demonstrated that mutation of residue 37 of Ras from a glutamine to glycine substitution (G37)

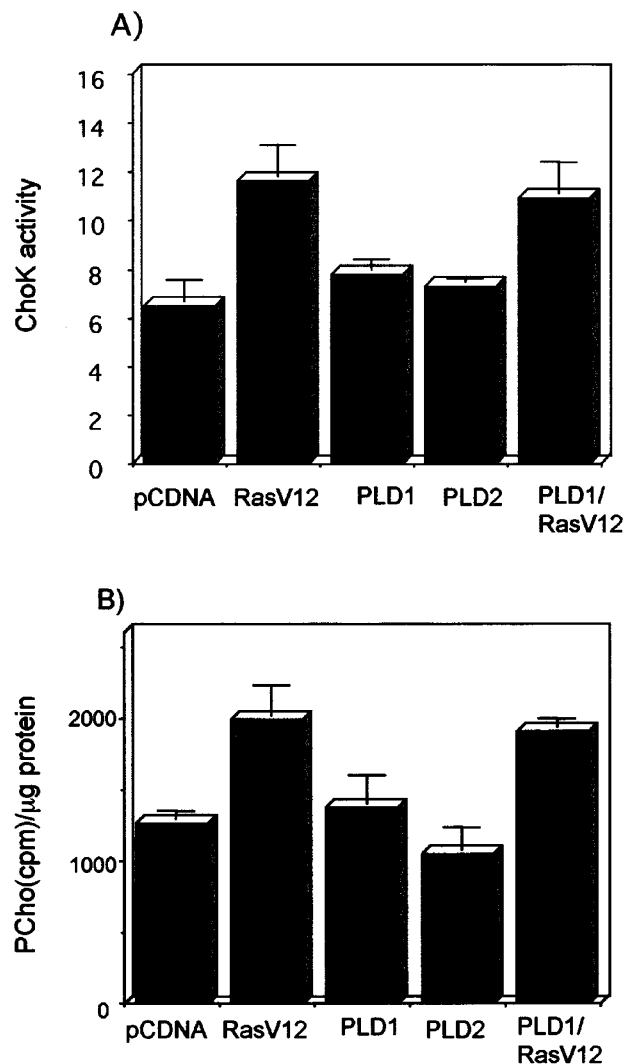


Figure 3 Ras-induced PLD activation has no effect on both choline kinase activity and intracellular levels of PCho. (a) Choline kinase assays of HEK293T transiently transfected with expression vectors of RasV12, PLD1, PLD2 and RasV12/PLD1 were performed as described in Materials and methods using 100 μ g cell extract per assay and 200 μ M of Cho as substrate. Data represents mean \pm s.e.m. of four independent assays performed in duplicate. (b) HEK293T cells overexpressing RasV12, PLD1, PLD2 and both, RasV12 and PLD1 were grown in DMEM supplemented with 10% fetal serum and after a labeling period of 24 h in the presence of methyl [14 C]-choline, medium was discarded and cells were processed for determination of PCho generation as described in Materials and methods. Data are the mean \pm s.e.m. of four independent experiments each performed in duplicate

restricts its binding solely to Ral GDS, whereas mutations at C40 restrict Ras interaction to PI3K (White *et al.*, 1995; Rodríguez-Viciana *et al.*, 1997). NIH3T3 cells overexpressing a constitutive active *raf-1* kinase were used to determine the involvement of this pathway in choline kinase activation. This cell line has been previously characterized (Hernández-Alcoceba *et al.*, 1997).

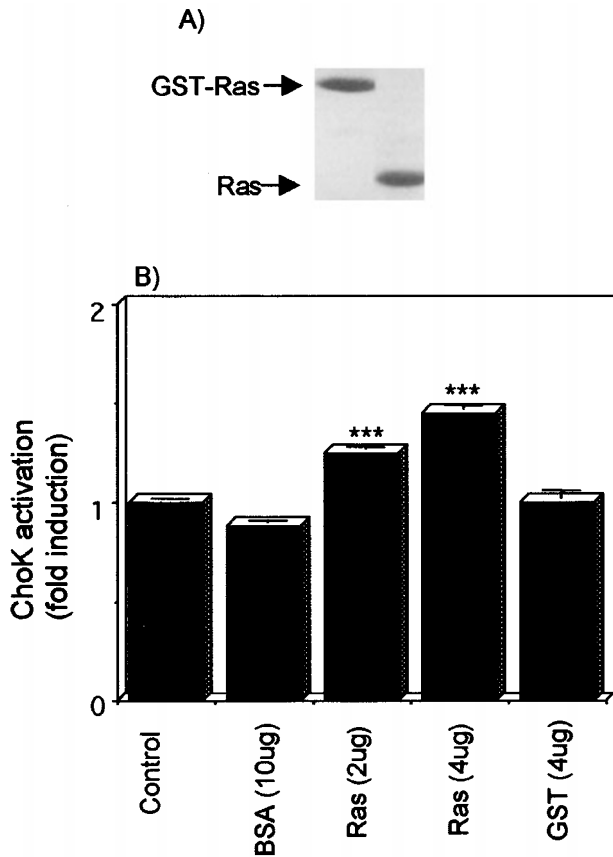


Figure 4 Addition of recombinant oncogenic Ras enhances ChoK activity in a dose-dependent manner. (a) RasV12 purification by the GST-system. A culture of *E. Coli* cells expressing GST-Ras, was grown, harvested and disrupted as described in Materials and methods. GST-Ras was purified using Glutathione Sepharose 4B and Ras was then liberated after thrombin cleavage as described. A Coomassie blue of GST-Ras and Ras purification is shown. (b) NIH3T3 cells were grown in DMEM supplemented with 10% new born calf serum to confluence, harvested and lysed. Then, the correspondent amounts of Ras, BSA and GST were added to equal amounts of cell extract, and choline kinase activity was assayed as described in Materials and methods. ***Indicates that the results were statistically significant ($P \leq 0.01$, $n = 11$) according to the Tukey test, GraphPad Instat v2.04

Basal ChoK activity was determined. Cells expressing the oncogenic RasV12 displayed a 2.4-fold increase in ChoK activity with respect to the control cell line (Figure 6a). Expression of RasV12G37 resulted in a 1.9-fold activation, and expression of RasV12C40 generated a 1.7-fold increase. By contrast expression of the constitutively active Raf-1 resulted in a small increase in ChoK activity (1.3-fold). Expression levels of mutated Ras proteins in all these cell lines were tested by Western blot using a specific antibody to Ras, and activation of MAPK pathway in cells overexpressing a constitutive active Raf-1 was determined by Western blot analysis using a specific antibody that recognizes phosphorylated-MAPKinase (Figure 6b).

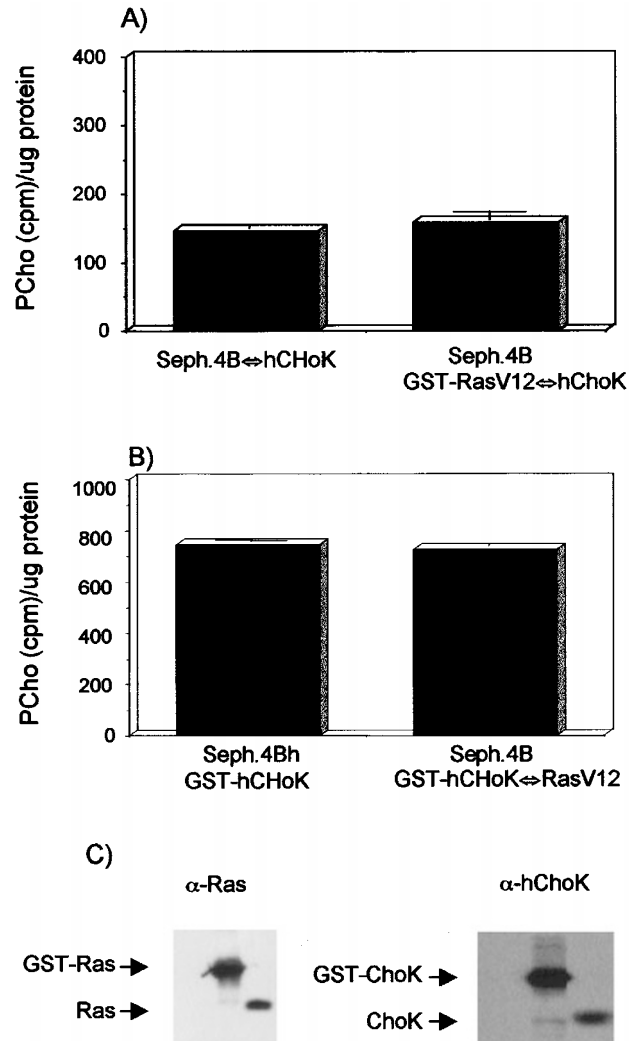


Figure 5 Choline kinase and Ras do not physically interact. (a) Cultures of *E. Coli* and BL21 cells expressing GST-Ras, GST-hChoK and an His-tagged hChoK were grown, harvested and disrupted as described in Materials and methods. GST-Ras was purified using glutathione sepharose 4B and incubated at different times with cell extracts containing His-hChoK or hChoK. After that, GST-Ras was precipitated and choline kinase activity was analysed as described under Materials and methods. Similar results were obtained at all times of incubation analysed (from 1 min to 2 h) and using His-hChoK or hChoK purified by the GST-system. Data shown is representative of two independent experiments each performed in duplicate. (b) GST-hChoK was purified using glutathione sepharose 4B and incubated at different times with cell extracts containing RasV12. After that, GST-hChoK was precipitated and choline kinase activity was analysed as described in Materials and methods. Similar results were obtained at all times of incubation assayed (from 1 min to 2 h). Data shown is representative of two independent experiments each performed in duplicate. (c) Expression of purified GST-hChoK and GST-Ras from cell lysates and expression of purified hChoK and Ras after thrombin cleavage. Western blot analysis was carried out using anti-hChoK serum and anti-Ras specific antibody as described in Materials and methods

The above results suggest that both Ral-GDS and P13K may contribute to the activation of ChoK, while Raf does not significantly affect this enzyme. In order

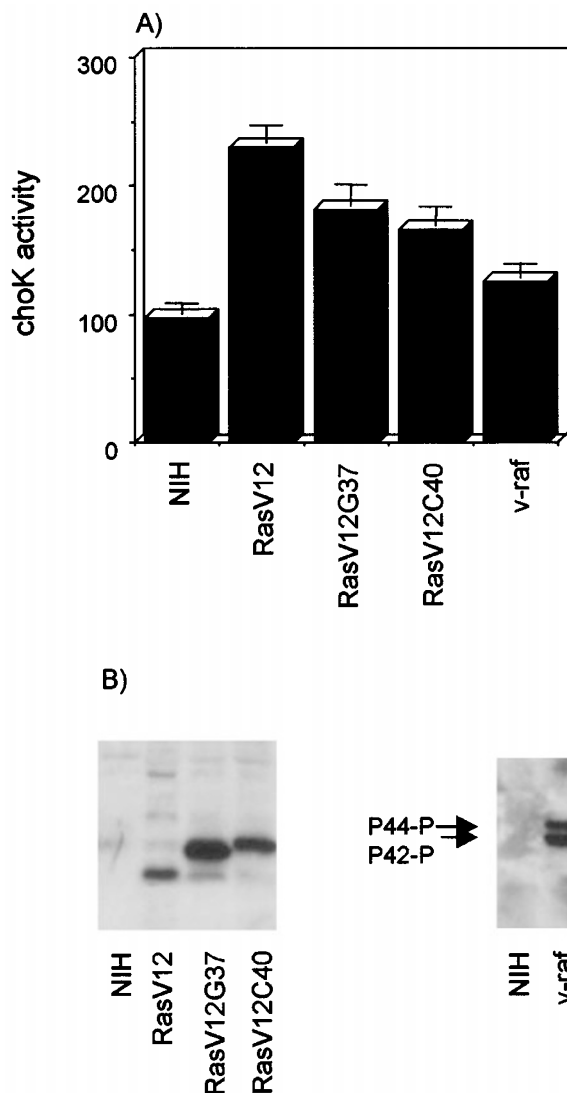


Figure 6 Constitutive ChoK activation is not mediated by a single Ras effector. (a) NIH3T3 cells stably overexpressing RasV12 mutants that specifically interact solely with RalGDS (RasV12G37) or P13Kinase (RasV12C40), and NIH3T3 cells stably overexpressing a constitutive active *raf-1* kinase were generated. Choline kinase activity of these cell lines with respect to control and *ras*-transfected cells was measured as described in Materials and methods. Data represents mean \pm s.e.m. of five independent experiments each performed in duplicate. (b) Expression levels of mutated Ras proteins in these cell lines were tested by Western blot using a specific Ras antibody, and activation of MAPK/ERK pathway in *raf-1*-transfected cells was determined using a specific phosphorylated-MAPKinase antibody, as described in Materials and methods

to further support this conclusion, the role of the Ras effectors was also investigated by alternative assays. Raf-1 cascade has been extensively implicated in Ras-induced effects (Gangarosa *et al.*, 1997; Kerkhoff and Rapp, 1997; Friedman *et al.*, 1994), although it may not be necessary for Ras-induced transformation in HEK293T cells (Kerkhoff *et al.*, 2000). To confirm the lack of an involvement of the Raf-1 pathway in Ras-

induced ChoK activation, the effects of PD98059, a specific inhibitor of MEK1 (Wang *et al.*, 1998), on ChoK activity was analysed. As expected from the previous results, ChoK activation was not significantly affected by PD98059 (Figure 7a). Under similar conditions, a drastic reduction of RasV12-induced MAPK/ERK activation was observed, indicating that this inhibitor efficiently interferes with this signaling pathway (Figure 7b).

We next analysed ChoK activity after transient transfection and co-transfection of Ras mutants that preferentially activate one specific effector (White *et al.*, 1995; Rodríguez-Viciano *et al.*, 1997). Under these conditions, oncogenic Ras-V12 induced a 3.7-fold activation of ChoK (Figure 8a). Consistent with the results obtained in stably transfected cells, a 2.4-fold increase in ChoK activity was observed after co-transfection of hChoK and RasV12G37, and a 2.1-fold increase was observed when expressing RasV12C40. Similar results, although at a lower rate, were obtained

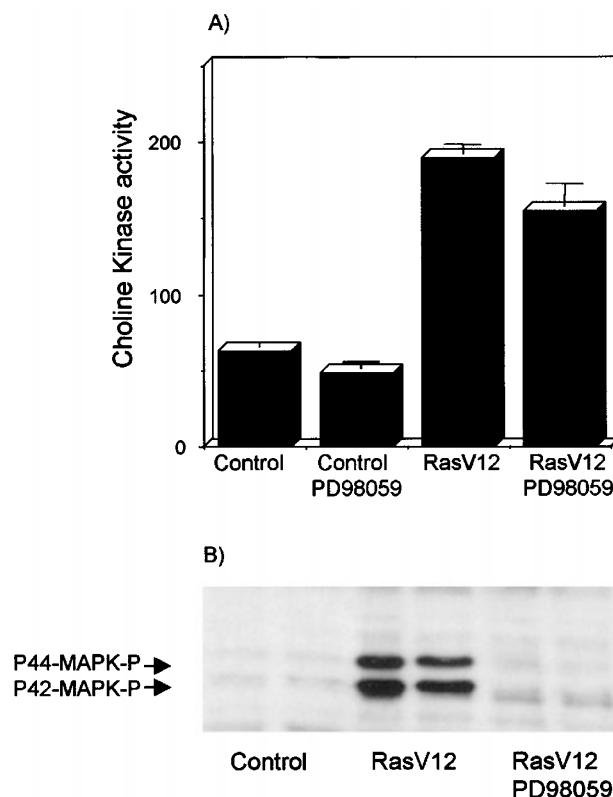


Figure 7 Inhibition of Raf-1/MEK/MAPK pathway has no significant effect on *ras*-mediated ChoK activation. (a) NIH3T3 cells stably expressing RasV12 or cells transfected with vector alone (control), were grown in DMEM supplemented with 0.5% new born calf serum for 40 h and then left untreated or treated 6 h with 50 μ M of PD98059. Choline kinase activity was assayed as described in Materials and methods. Data represents mean \pm s.e.m. of three independent experiments each performed in triplicate. (b) PD98059 interferes efficiently with MAPK signaling pathway. Inhibition of *ras*-induced MAPK activation after PD98059 treatment was determined by Western blot analysis using a specific phosphorylated-MAPKinase antibody as described in Materials and methods

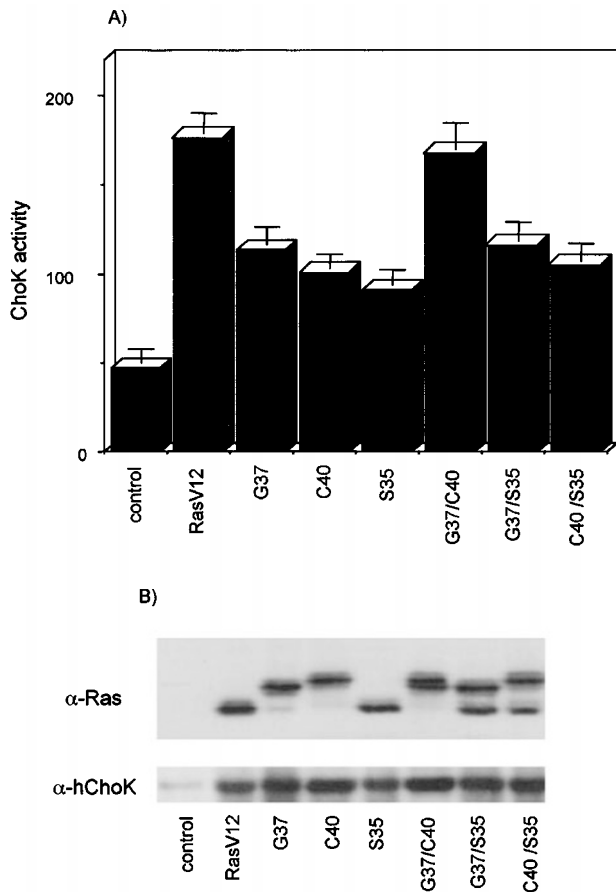


Figure 8 Ras-induced choline kinase activation is mediated by RalGDS and PI3Kinase. (a) HEK293T cells were transiently transfected with hChoK and RasV12 or RasV12 mutants that specifically interact with each of the three effectors alone, RalGDS (RasV12G37), PI3Kinase (RasV12C40), and Raf-1 (RasV12S35), and choline kinase activity was measured as described in Materials and methods. Data represents mean \pm s.e.m. of four independent experiments each performed in duplicate. (b) Expression levels of the mutated Ras proteins and hChoK in these cell lines were tested by Western blot using a specific anti-Ras antibody or serum anti-hChoK respectively

when using the endogenous choline kinase present in HEK 293T cells (data not shown). Surprisingly, expression of RasV12S35, that activates the Raf-1 pathway, displayed some effect on ChoK under these conditions, suggesting that this mutant may impinge into additional Ras effectors that participate in ChoK activation. In keeping with the results shown in Figure 6, Ras mutants that activate RalGDS and PI3K stimulate ChoK more efficiently than that of Raf-1, but none reached the full activation level of the oncogenic Ras-V12 (3.7-fold) although all mutants were expressed to a similar extend (Figure 8b). Thus, there was the possibility of the requirement of more than one signaling pathway for full activation of ChoK. To further investigate this possibility, co-transfections experiments were carried out. As shown in Figure 8a, co-expression of RasV12G37 and RasV12C40 resulted

in a 3.6-fold increase of ChoK activity, that is similar to the one obtained with Ras V12. By contrast, co-transfection of RasV12G37 or RasV12C40 with RasV12S35 only activated ChoK at a similar extent to that observed with RasV12G37 or RasV12C40 alone. These results suggest that both Ral-GDS and PI3K are mediating ChoK regulation by Ras proteins.

Discussion

A role of lipid-derived second messengers in the transforming properties of Ras oncoproteins has been previously reported. In this sense, *ras*, one of the most intensively studied oncogene to date, has been shown to activate PLD and ChoK and to increase the levels of several lipid derived metabolites such as DAG, PA and *PCho* (Lacal *et al.*, 1987; Lacal, 1990; Macara, 1989; Rameh and Cantley, 1999; Cuadrado *et al.*, 1993; Jiménez *et al.*, 1995; Lucas *et al.*, 2000; Bhakoo *et al.*, 1996; Ratnam and Kent, 1995). Moreover, the increased levels of *PCho* in *ras*-transformed cells have been demonstrated to be a consequence of ChoK activation (Lacal, 1990; Macara, 1989; Rameh and Cantley, 1999; Cuadrado *et al.*, 1993; Jiménez *et al.*, 1995; Lucas *et al.*, 2000; Bhakoo *et al.*, 1996; Ratnam and Kent, 1995; Hernández-Alcoceba *et al.*, 1997). PLD and ChoK are two enzymes whose activity belongs to the Kennedy pathway, responsible for the synthesis of phosphatidylcholine (PC). In addition, ChoK may be also involved in the generation of *PCho* that may serve as a second messenger (Lacal, 2001).

Some previous studies have postulated that ChoK may be regulated by the availability of its substrate, choline (Lacal, 2001). Thus ChoK could be activated by Ras proteins as a result of PLD activation, or follow alternative pathways involving the Ras protein itself, through direct interaction with ChoK or its effectors. We have addressed this issue here. Our results indicate that in mammalian cells ChoK is susceptible of a regulation by Ras independently of PLD activation and therefore, choline availability. Furthermore, we provide evidence that ChoK is activated by Ras proteins through a complex signaling pathway that involves two of its best known effectors, Ral-GDS and PI3K. Finally, our results suggest that ChoK itself may be relevant upon *ras*-induced transformation since it is up-regulated in a PLD independent manner.

In keeping with the relevance of lipid-derived second messengers in oncogenes-driven signaling and in human tumorigenesis, several human tumoral cell lines display elevated levels of *PCho*, an event that has been considered as a marker of tumorigenesis (Reich *et al.*, 1995; Nakagami *et al.*, 1999). Additional evidence gives support for a role of ChoK in human transformation and carcinogenesis. Thus, inhibition of ChoK has been demonstrated to be a novel efficient antitumor strategy in both oncogene transformed cells (Hernández-Alcoceba *et al.*, 1997; Cuadrado *et al.*, 1993; Jiménez *et al.*, 1995) and against *in vivo* xenografts of human

tumors (Hernández-Alcoceba *et al.*, 1999). These results have driven us to the development of a novel antitumoral strategy focused on inhibition of ChoK. Thus, further studies aiming at a better understanding of the mechanisms of regulation of this enzyme upon transformation might help us to improve the design of novel efficient antitumoral therapies.

Ras GTPases are activated in human cancers, being involved in up to 30% of all human tumors. They have been found overexpressed or mutated in a large variety of tumors such as pancreatic cancers, colorectal cancers, leukemias and lung adenocarcinomas (Bos, 1989; Rodenhuis, 1992). Great effort focused on understanding the specific signaling pathways downstream of Ras oncoproteins has been done. The most extensively studied molecules downstream of Ras are the Raf serine/threonine kinase-1, the Ral-GDS exchange factor, and the lipid kinase PI3K (Malumbres and Pellicer, 1999; Katz and McCormick, 1997; Hernández-Alcoceba *et al.*, 2000; Crespo and León, 2000). Raf-1, through the p42/p44 ERK/MAPK cascade has been implicated in Ras-induced transformation (Gangarosa *et al.*, 1997; Kerkhoff and Rapp, 1997; Friedman *et al.*, 1994). However, previous studies suggest that more than one effector may be necessary for a full *ras*-induced transformation (White *et al.*, 1995). Our results indicate that although Raf-1 can partially stimulate ChoK in acute transient experiments, it is not a key molecule in the constitutive activation of ChoK mediated by Ras, nor cooperates with other Ras effectors.

Ral-GDS, a nucleotide exchange factor for Ral GTPases is another well characterized Ras effector that induces transformation of NIH3T3 fibroblasts (Bos, 1997). Finally, PI3K and its products are involved in the control of cell proliferation and survival as well as human tumorigenesis (Moscatello *et al.*, 1998; Moore *et al.*, 1998; Barbadelli *et al.*, 1999; Ma *et al.*, 2000). These two Ras effectors, Ral-GDS and PI3K were able to induce partial ChoK activation both in transient as well as in stable transfections. Furthermore, combination of Ral-GDS and PI3K restored full activation of ChoK by Ras, suggesting that both signaling pathways cooperate. These results indicate that Ral-GDS and PI3K are necessary and sufficient for this effect. Thus, we provide here the basis for understanding the molecular signaling pathways derived from oncogenic Ras proteins that impinge into ChoK regulation.

Materials and methods

Cell cultures and transfections

NIH3T3 fibroblasts and HEK293T cells were grown in DMEM supplemented with 10% new born calf serum (Gibco) or 10% fetal calf serum (Gibco) respectively, under standard conditions of temperature (37°C) humidity (95%) and carbon dioxide. Transfection of NIH3T3 cell lines with 1–2 μ g of the indicated genes was carried out using the Lipofectamine Plus Reagent from GIBCO BRL as described

by the manufacturers. HEK293T cells were transfected with 0.5–1.5 μ g of the indicated genes by the calcium phosphate method as described (Montaner *et al.*, 1999). PDCR-Hras V12G37 and pDCR-H ras V12C40 were a generous gift from Dr J Downward, pCFEL-H ras V12 and pCFEL-H ras V12S35 were kindly given by Dr P Crespo, pCGN, HA-hPLD1 and pCGN HA-mPLD2 are a generous gift from MA Frohman, and a cDNA encoding mammalian choline kinase was generously given by Dr S Yamashita.

Analysis of phosphorylcholine production in cells

Cells were seeded on 6-well plates and grown to confluence in the presence of 1 μ Ci/ml methyl[¹⁴C]-choline chloride (50–60 Ci/mmol, Amersham International). Cells were rinsed with ice-cold TD buffer (137 mM NaCl, 5 mM KCl, and 20 mM Tris, pH 7.4) and fixed with 16% ice-cold trichloroacetic acid (TCA). TCA-soluble material containing PCho was washed three times with four volumes of diethylether, dried under vacuum, and resuspended in water. Samples were resolved in thin layer chromatography (TLC) plates (60 A silica gel, Whatman, Clifton, NJ, USA) using as liquid phase 0.9% NaCl: methanol: ammonium hydroxide (50:70:5; V:V:V). Radioactivity corresponding to PCho was automatically quantified by an electronic radiography system (InstantImager; Packard, Meriden, CT, USA). TCA insoluble material containing hydrophobic lipids was dissolved in 0.25 N sodium hydroxide and total lipids were resolved by scintillation counting.

Choline kinase assays

Choline kinase assays were performed essentially as described (Hernández-Alcoceba *et al.*, 1999), with cell extracts in buffer containing 100 mM Tris-HCl pH 8.0, 100 mM MgCl₂ and 10 mM ATP, using as substrate the physiological choline concentration (200 μ M) in presence of methyl[¹⁴C]-choline chloride (50–60 μ Ci/mmol, Amersham International). Reactions were performed at 37°C for 30 min and stopped with ice-cold trichloroacetic acid (TCA) at a final concentration of 16%. After washing with water-saturated ethylether, samples were lyophilized. Hydrophilic derivatives of choline were resolved in TLC plates as described above.

Anti-choline kinase serum

hCholine kinase (hChoK) was expressed in BL21 cells using the expression vector pGEX-4T2 and purified by the GST-system with Glutathione Sepharose 4B from Amersham Pharmacia Biotech as described by the manufacturers. Purified hChoK was mixed with an equal volume of Freund's complete adjuvant (from Sigma) and then injected into white rabbits (60–70 μ g/rabbit). Four weeks later, rabbits were given a booster injection of the antigen (60 μ g/rabbit) emulsified with Freund's incomplete adjuvant (Sigma). Booster injections were repeated every 2 weeks, and after four booster injections, serum was obtained from the rabbits. The antiserum obtained readily recognizes pGEX4T2 hChoK expressed in BL21 cells and pCDNA hChoK expressed in HEK293T cells. As well, this antiserum immunoprecipitates 85% of choline kinase activity from different human and mice cell extracts.

Analysis of protein levels by Western blot

Cells were grown under standard conditions in 100 mm dishes. Culture media was removed, cells washed with TD

buffer and 300 μ l of ice-cold lysis buffer (50 mM Tris, pH 7.4, 0.25% NP-40, 0.25% SDS, 150 mM NaCl, 15 mM β -Glycerophosphate, 10 mM Na PPI, 50 mM Na F, 10 mg/ml aprotinin, 1 mM PMSF) was added. Nuclei and detergent-insoluble material were removed by centrifugation at 13 000 r.p.m. for 20 min at 4°C. The resulting supernatants were assayed for estimation of total cell protein (Bio-Rad), and equal amounts of cell lysates (30 μ g) were boiled at 95°C for 5 min in SDS-PAGE sample buffer. For Western blot analysis, proteins were electrophoresed onto 10% SDS-PAGE gels poured in 20 \times 20 cm glass plates. Resolved proteins were transferred to nitro-cellulose, and blots were blocked for 2 h in 5% non-fat dried milk in T-TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). Blots were washed in T-TBS, and incubated for 1 h with each correspondent antibody and then developed by ECL as described by the manufacturers (Amersham). Determination of Ras over-expression was carried out using a polyclonal antibody against H-Ras (sc-520, Santa Cruz Biotechnology). PLD over-expression was determined using the monoclonal anti-HA antibody (Boehringer Mannheim). Determination of hCholine Kinase was carried out using the anti-ChoK antiserum obtained as described above.

Assays of PLD activity

Activation of PLD can be measured accurately by its transphosphatidylating activity on butanol to generate phosphatidyl-butanol (Song *et al.*, 1991). Shortly: cells were grown in 6-well plates and labeled for 72 h in the presence of 1 microCi/ml [$U-^{14}C$]-Glycerol. After this period, label media was discarded, cells washed with TD buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 20 mM Tris, pH 7.4) and incubated 30 min in serum-free DMEM supplemented with 0.5% 1-butanol. Cells were then scrapped in 1 ml methanol, and plates washed once with 0.5 ml of methanol. The two methanol samples were collected and mixed to 2 ml chloroform and 1 ml of water. The organic phases were dried in a 37°C heating block under a nitrogen stream and the lipids separated by thin layer chromatography (TLC) using Silica Gel 60A plates (Whatman, LK6D). The plates were developed with the upper phase of a mixture of ethyl acetate/iso-octane/glacial acetic acid/water (90:50:20:100) plus 1 ml of acetic acid. Plates were visualized and quantified using an electronic auto-radiography system (InstantImager,

Packard). Counts in phosphatidylbutanol (PtdBut) were normalized for the radioactivity incorporated into total lipids.

Assays of protein-protein interaction

Twenty ml of an overnight culture of *E. Coli* cells expressing GST-Ras, GST-hChoK and an His-tagged hChoK were added to 200 ml of LB medium containing 100 μ g/ml ampicillin and then cultured at 37°C. After 2 h, IPTG was added to a final concentration of 2 mM and cultivation was continued for 3 h. Then, cells were harvested by centrifugation and disrupted by sonication for 20 s followed by 1 min cooling on ice each time, four times in 3 ml of ice-cold lysis buffer (50 mM Tris. HCL pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, PMSF, leupeptin, aprotinin and pepstatine A as proteases inhibitors were added). Each cell supernatant was obtained by centrifugation at 14 000 r.p.m. for 20 min. GST-Ras and GST-hChoK from each cell supernatant were purified by the GST-system with glutathione sepharose 4B from Amersham Pharmacia Biotech. When necessary, Ras or hChoK were released after thrombin cleavage using thrombin protease from Amersham Pharmacia Biotech as indicated by the manufacturers. His-hChoK was purified using the ProBond resin from Invitrogen as described by the manufacturers. ChoK was measured after GST-precipitation of GST-Ras previously incubated with His-hChoK or hChoK obtained after thrombin cleavage. As well, ChoK activity after GST-precipitation of GST-hChoK previously incubated with purified Ras was measured as described above.

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