Choline Kinase Is a Novel Oncogene that Potentiates RhoA-Induced Carcinogenesis

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Abstract

Choline kinase is overexpressed in human breast, lung, colorectal, and prostate tumors, a finding that suggests the involvement of this enzyme in carcinogenesis. Here we show that overexpression of choline kinase induces oncogenic transformation of human embryo kidney fibroblasts and canine epithelial Madin-Darby canine kidney cells. Choline kinase lays downstream of RhoA signaling and is activated through ROCK kinase, one of the best-characterized RhoA effectors. In keeping with this, coexpression of RhoA and choline kinase potentiates both anchorage independent growth and tumorigenesis. Finally, choline kinase–mediated transformation is sensitive to MN58b, a well-characterized specific choline kinase inhibitor. These results provide the definitive evidence that choline kinase has oncogenic properties and that choline kinase inhibition constitutes a novel valid antitumor strategy. (Cancer Res 2005; 65(13): 5647-53)

Introduction

Phosphatidylcholine, the major class of glycerophospholipid present in all mammalian cells, is hydrolyzed by phospholipase D to yield phosphatidic acid and choline. This latter metabolite is phosphorylated by choline kinase to generate phosphorylcholine and represents the first step in the Kennedy pathway of biosynthesis of phosphatidylcholine. Recently, choline kinase has been proposed to play a relevant role in the onset of human cancer, because it is overexpressed in breast, lung, colon, and prostate tumors (1, 2). Furthermore, choline kinase activation is associated with variable indicators of greater malignancy in breast tumors (2). Consistent with these findings, nuclear magnetic resonance techniques have shown elevated levels of phosphorylcholine in human tumoral tissues with respect to their normal counterparts (3, 4), whereas choline kinase is activated by several growth factors (5, 6) and oncogenes (7–10). All these observations constitute the basis of the design of a new antitumoral strategy focused on specifically interfering with choline kinase activity. Choline kinase inhibitors with efficient antitumoral activity both *in vitro* and *in vivo* have been generated (10–15).

Besides its implications in malignancy, little is known on the mechanism of regulation of choline kinase. A potential candidate for upstream regulation of choline kinase is the family of small Rho GTPases (Ras-homologous GTPases). These GTPases are involved in the regulation of a wide variety of key cellular processes, including microtubule and actin cytoskeleton, migration, cell adhesion, phagocytosis, membrane transport, regulation of transcription factors, control of the cell cycle, and gene expression (16, 17). A consequence of the relevance of RhoGTPases in these critical cellular functions is that members of this family are widely involved in malignant transformation and human tumorigenesis (18–21). In this sense, both wild type and constitutively active Rho proteins transform NIH 3T3 cells (22, 23) and provide metastatic properties *in vivo* (24). Furthermore, these GDP/GTP binding molecular switches are overexpressed in a variety of human tumors such as colon, breast, lung, testicular germ cell, and head and neck squamous cell carcinoma, contributing to both tumor proliferation and metastasis (18, 25, 26).

The family of human Rho GTPases include RhoA, Rac-1, and Cdc42 as prototypes (27). Rho GTPases interact and activate a large number of effector molecules, triggering a complex network of intracellular signaling cascades. Well-known effectors to RhoA include the ROCK kinase, involved in both RhoA-induced cytoskeletal changes and in RhoA-induced transformation and metastasis (28–32). As well, the serine/threonine kinase PKN that is a PKC-related protein is mediating RhoA-induced actin reorganization, endocytosis, and malignant transformation through mitogen-activated protein kinase (MAPK) activation (31, 33). In addition to these well-known effectors to RhoA, other effector molecules have been described such as citron, citron kinase (a kinase containing a domain variant of citron), rhophilin, Rhotekin, m-Dia, IQGAP and phospholipase D, a lipid related enzyme of the Kennedy pathway, also involved in malignant trasformation and tumorigenesis (18).

Here, we provide definitive evidence of the oncogenic potential of choline kinase. Indeed, choline kinase behaves as an oncogene by itself and potently synergizes when combined with RhoA, an upstream regulator of the enzyme.

Materials and Methods

Cell cultures and transfections. All cell lines used in this study were maintained under standard conditions of temperature (37°C), humidity (95%), and carbon dioxide (5%). Human primary mammary epithelial cells, HMEC (Clonetics, Innogenetics, Madrid, Spain) were grown in MEBM supplemented with a bullet kit (Clonetics). Human embryonic kidney cells (Hek293T), Madin-Darby canine kidney Madin-Darby canine kidney cells (MDCK), and Rat-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY). NIH3T3 fibroblasts were cultured in DMEM supplemented with 10% newborn calf serum. Transfection of human HMEC, MDCK, and Rat-2 cells was carried out using the LipofectAMINE Plus Reagent from Life Technologies as described by the manufacturer. Transfection of human Hek293T was carried out by the calcium phosphate method as previously described (7). The amount of plasmidic DNA was kept constant at 2 to 4 μg with the corresponding empty vector.

Choline kinase assays and Western blotting. Choline kinase assays were done as previously described (2). Western blots of cell extracts using
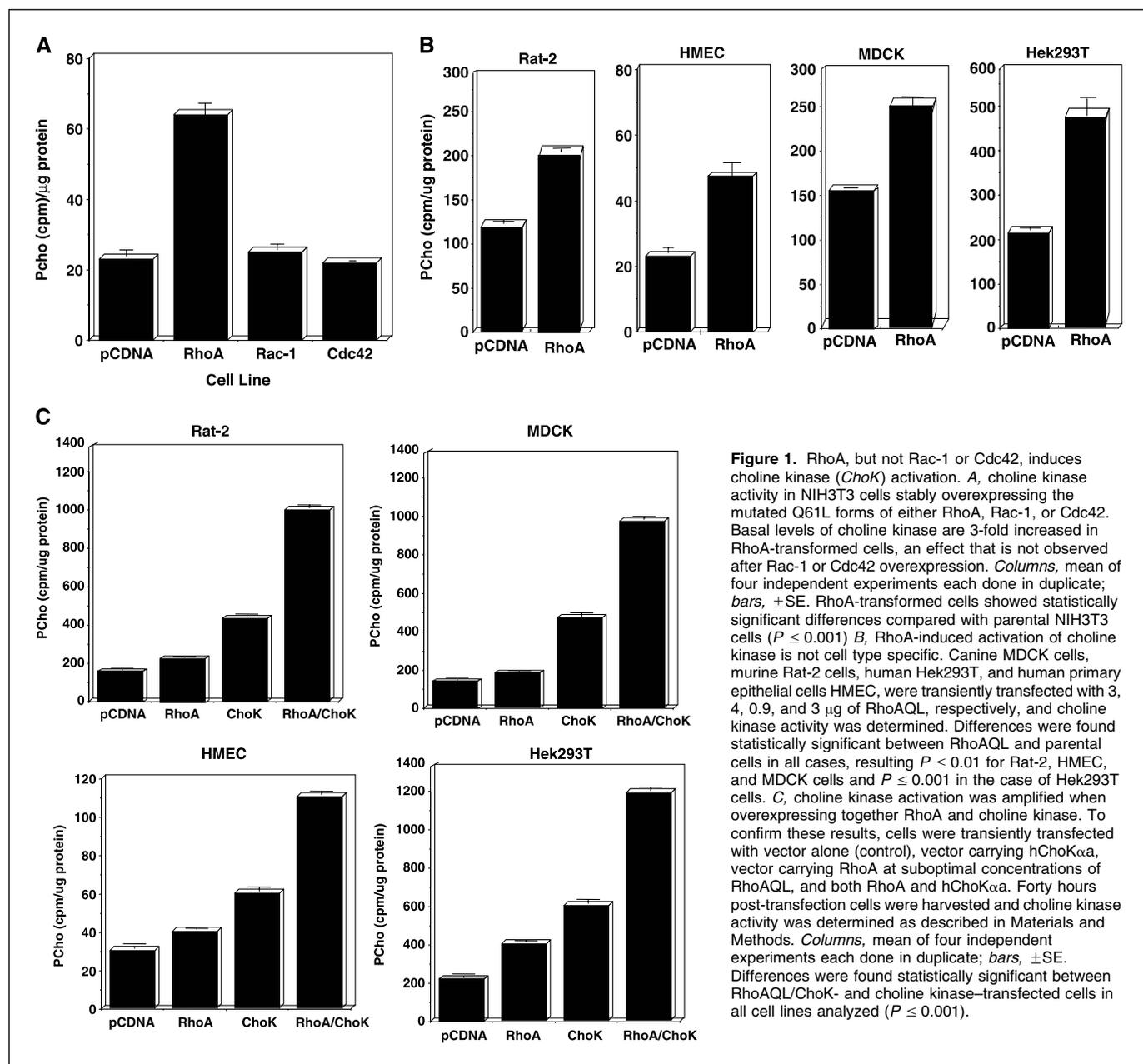
Figure 1. RhoA, but not Rac-1 or Cdc42, induces choline kinase (*ChoK*) activation. *A,* choline kinase activity in NIH3T3 cells stably overexpressing the mutated Q61L forms of either RhoA, Rac-1, or Cdc42. Basal levels of choline kinase are 3-fold increased in RhoA-transformed cells, an effect that is not observed after Rac-1 or Cdc42 overexpression. *Columns,* mean of four independent experiments each done in duplicate; *bars,* ±SE. RhoA-transformed cells showed statistically significant differences compared with parental NIH3T3 cells ($P \leq 0.001$) *B,* RhoA-induced activation of choline kinase is not cell type specific. Canine MDCK cells, murine Rat-2 cells, human Hek293T, and human primary epithelial cells HMEC, were transiently transfected with 3, 4, 0.9, and 3 μg of RhoAQL, respectively, and choline kinase activity was determined. Differences were found statistically significant between RhoAQL and parental cells in all cases, resulting $P \leq 0.01$ for Rat-2, HMEC, and MDCK cells and $P \leq 0.001$ in the case of Hek293T cells. *C,* choline kinase activation was amplified when overexpressing together RhoA and choline kinase. To confirm these results, cells were transiently transfected with vector alone (control), vector carrying hChoKαa, vector carrying RhoA at suboptimal concentrations of RhoAQL, and both RhoA and hChoKαa. Forty hours post-transfection cells were harvested and choline kinase activity was determined as described in Materials and Methods. *Columns,* mean of four independent experiments each done in duplicate; *bars,* ±SE. Differences were found statistically significant between RhoAQL/ChoK- and choline kinase–transfected cells in all cell lines analyzed ($P \leq 0.001$).

the indicated antibody were done as previously described (13). Antibodies against RhoA and anti-Flag and anti-Myc antibodies to detect RhoA effectors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody generated against the purified recombinant human ChoKαa protein expressed in *Escherichia coli* as a glutathione *S*-transferase (GST)–fused protein was used in this study. A full description of the generation and the properties of this monoclonal antibody is described elsewhere.[3]

Anchorage-independent growth in soft agar, *in vivo* tumorigenic assay, and MN58b treatment. Anchorage-independent growth assay was done at 5 weeks of incubation as previously described by plating 50,000 cells per 60-mm dish (8). For the *in vivo* tumorigenic assays, cells were resuspended in DMEM just before inoculation (10^6 cells per 0.1 mL) and injected s.c. in *nu/nu* immunosuppressed mice. Tumor growth was monitored weekly for 60 days. Mice were kept under standard laboratory conditions according to the guidelines of the Spanish Government. When tumors reached a mean volume of 0.1 cm^3, mice were randomized to control and treated groups. Treatments with MN58b were done as previously described (11), and tumor volume was calculated by the formula: $V = (D \times d^2) / 2$. MN58b was generated according to previously reported procedures (11).

Statistical analysis. Statistical analysis was done by the Tudey test, using the GraphPad InStat V2.04. All factors were treated as single categorical variables, and all reported Ps are two sided. Statistical significance was defined as $P < 0.05$.

Results

RhoA, but not Rac1 or Cdc42, induces choline kinase activation. Several members of the Rho and Ras families of GTPases are potent upstream activators of phospholipase D (15).

We have previously reported that oncogenic Ras proteins induce the activation of both phospholipase D and choline kinase by independent mechanisms (7). We explored the possible regulation of choline kinase by members of the Rho family of GTPases. Choline kinase activity in NIH3T3 cell lines stably overexpressing mutated Q61L versions of RhoA, Rac-1, or Cdc42, the three prototypes of this family was measured (17, 27). Basal levels of choline kinase were greatly increased in RhoA-transformed cells, an effect that was not observed in cells overexpressing Rac1 or Cdc42 (Fig. 1*A*). To investigate if RhoA-induced activation of choline kinase observed in NIH3T3 cells was cell type specific, we transiently transfected RhoA, the human ChoKα (here referred as choline kinase for simplicity) in four additional different cell lines, Rat-2 (rat epithelial cells), MDCK, Hek293T (human

embryonic kidney cells), and the primary, senescent HMEC. RhoA induced a clear and statistically significant activation of endogenous choline kinase in all the cell lines analyzed ($P \leq 0.01$; Fig. 1*B*). Finally, choline kinase activation was synergistically amplified when overexpressing together RhoA and choline kinase, indicating that RhoA activates choline kinase in a cell-independent manner (Fig. 1*C*). This activation was also found statistically significant ($P \leq 0.001$).

Activation of choline kinase by RhoA is not mediated by direct protein-protein interaction but is mediated by ROCK. We next investigated whether the RhoA-induced constitutive increase in choline kinase activity was due to a direct interaction of both proteins, as previously reported for the Rho-mediated activation of phospholipase D. Addition of RhoA recombinant
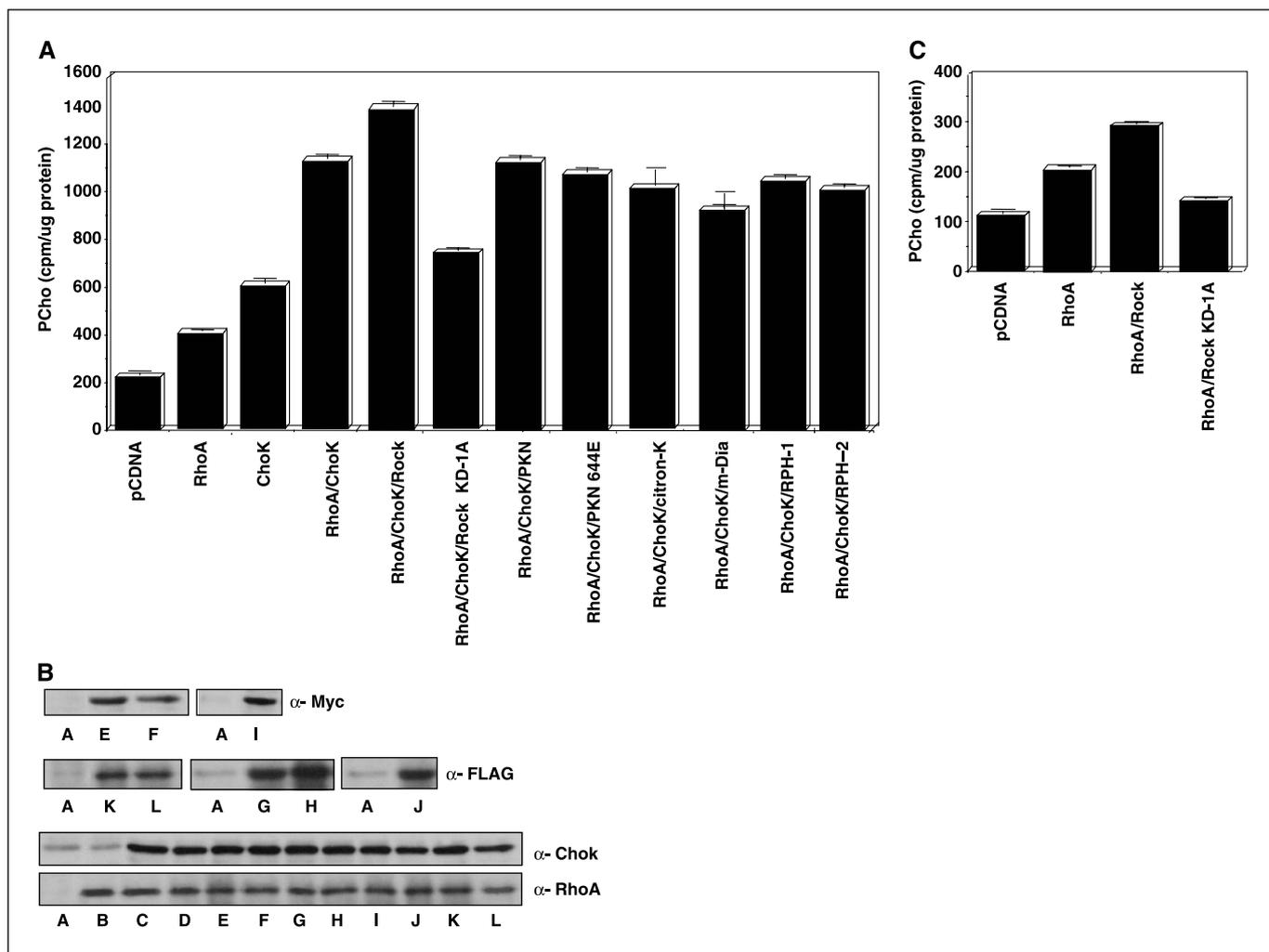

Figure 2. RhoA-induced choline kinase (*ChoK*) activation is mediated by ROCK. *A,* Hek293T cells were transiently transfected with the hChoKα, the Q61L mutant of RhoA, the indicated effectors to RhoA, or the dominant-negative mutants ROCK (ROCK KD-IA) and PKN (PKN K664E). Forty hours post-transfection, choline kinase activity was determined as previously described. Representative experiment of five independent experiments each done in duplicate. Differences were found statistically significant between RhoA/ChoK and both choline kinase or RhoA expression ($P \leq 0.001$). In addition, choline kinase activity was significantly activated when overexpressing RhoA/ChoK/ROCK ($P \leq 0.01$), and RhoA-induced choline kinase activation was statistically significantly blunted in the presence of the dominant-negative form of ROCK, ROCK KD-1A ($P \leq 0.01$). By contrast, expression of other effectors to RhoA had no significant effect ($P > 0.05$) in choline kinase activity. *B,* expression of human ChoKα, RhoAQL, and the different effectors to RhoA in Hek293T cells tested by Western blotting analysis. RhoA and ChoKα expression was detected using specific antibodies. Expression of Flag-tagged wtPKN, dominant-negative PKN K664E, m-Dia, and RPH-1 and 2, was detected by a specific Flag antibody, and Myc-tagged wtROCK, dominant-negative ROCK KD-IA and citron-K using a specific Myc antibody. *Lanes A-L,* correlative DNAs in (*A*). *C,* the involvement of Rock in RhoA-induced activation was confirmed using the endogenous choline kinase in these cells. Statistically significant differences were found between both RhoA/ROCK and RhoA expression ($P \leq 0.01$) and between RhoA/ROCK KD-1A and RhoA expression ($P \leq 0.001$). *Columns,* mean of five different experiments each performed in duplicate; *bars,* ±SE.
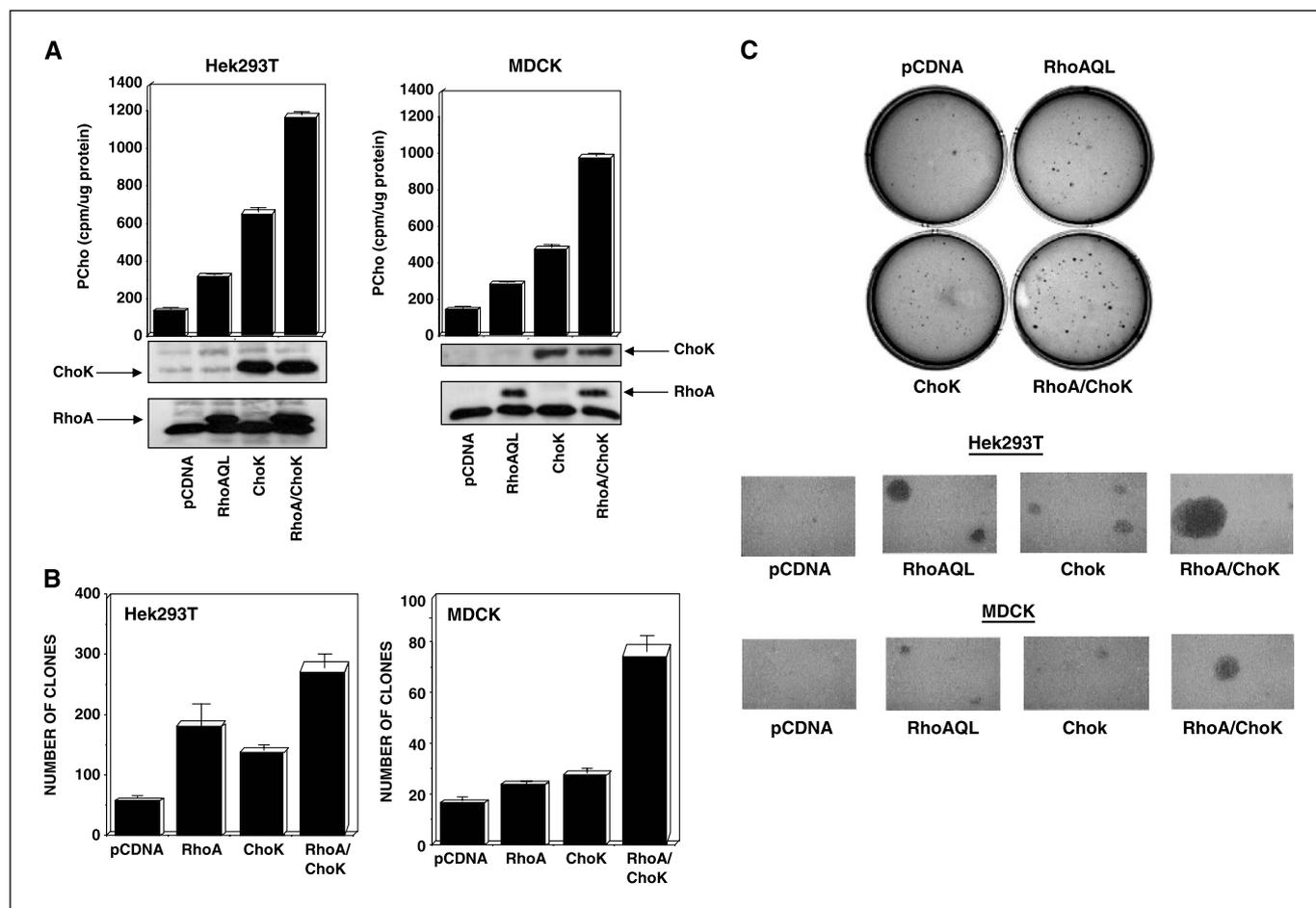
Figure 3. Choline kinase (*ChoK*) induces and potentiates RhoA oncogenic transformation of human Hek293T and canine MDCK cells. *A,* RhoA-induced choline kinase activation. Hek293T and MDCK cells were transiently transfected with either empty pCDNA vector as control, RhoAQL, human ChoKαa, or both RhoAQL and ChoKαa. Differences were found statistically significant between RhoAQL- and choline kinase–transfected cells ($P \leq 0.001$ for Hek293T and $P \leq 0.01$ for MDCK cells), and between RhoAQL/ChoK- and choline kinase–transfected cells ($P \leq 0.001$) in both cases. Expression levels were determined by Western blot analysis. *B,* RhoA and ChoKαa promote anchorage-independent growth in MDCK and human Hek293T cells. RhoA/ChoK-transfected cells display a statistically significant increase ($P \leq 0.001$) in the number of clones with respect to only RhoAQL or choline kinase–expressing cells or to parental cells. *Columns,* mean of three independent experiments each done in triplicate; *bars,* ±SE. *C,* coexpression of RhoA and ChoKαa results in a significant increase in both growth rate and size of the clones compared with those induced by RhoA or ChoKαa alone. Representative photographs of one experiment of three independent experiments done in triplicate and with similar results. *Top,* Hek293T cells.

protein to NIH3T3 cells extracts, resulted in significant increase in choline kinase activity of up to a 50% increase, an increase that was dose dependent, whereas addition of purified Rac1 or Cdc42 to NIH3T3 extracts had no effect on choline kinase activity (data not shown). As negative controls, similar amounts of bovine serum albumin or GST (purified as the Rho proteins) were also added with no significant effect on choline kinase. Precipitation of GST-RhoA previously incubated with human recombinant choline kinase was carried out. No significant differences were observed when comparing choline kinase activity of this precipitate with that of control, GST alone previously incubated with recombinant choline kinase (data not shown). Similarly, no interaction of choline kinase and RhoA was observed in a precipitate of GST-hChoK previously incubated with recombinant RhoA (data not shown). Further controls indicated that RhoA and choline kinase were efficiently pulled down under these conditions, and that a lack of a physical interaction was the explanation for these negative results (data not shown).

RhoA signaling relies on a large number of effector proteins that are activated when it is in the GTP-loaded form, triggering a large number of signaling cascades through which it exerts its biological effects. Among the most studied effectors to RhoA are the families of ROCK and PKN (17) that mediate cytoskeletal changes induced by RhoA and have also been implicated in RhoA-induced tumorigenesis and metastasis. In addition, there are other well-known effectors to RhoA such as citron kinase, m-Dia, or rhophilin-1 and rhophilin-2, although they have not been found involved in RhoA-induced transformation or tumorigenesis. We next studied the implication of these other effectors in RhoA-induced choline kinase activation. Wild-type ROCK (ROCKwt) and its dominant-negative mutant (ROCK KD-IA), the wild-type PKN (PKNwt) and its dominant-negative mutant (PKN K664E), as well as citron kinase, m-Dia, rhophilin-1, and rhophilin-2, were expressed in Hek293T cells, along with RhoA or choline kinase, and choline kinase activity was determined. Cells expressing oncogenic RhoA displayed an almost 2-fold increase in choline kinase activity with respect to control cells (Fig. 2*A*). Expression of ROCKwt with RhoA further increased choline kinase activation and this activation was statistically significant ($P \leq 0.01$). Accordingly, dominant-negative ROCK efficiently blocked RhoA-induced choline kinase activation

($P \leq 0.001$). On the other hand, expression of PKNwt, citron kinase, mDia, or rhophilin-1, and rhophilin-2 had no effect over choline kinase activity (Fig. 2*A*) besides their efficient expression (Fig. 2*B*). Cotransfection of ROCK proteins with RhoA showed similar results, although at a lower total activity levels, when using the endogenous choline kinase present in Hek293T cells (Fig. 2*C*). These results show that ROCK, but not PKN, citron kinase, m-Dia, rhophilin-1, nor rhophilin-2, affects RhoA-mediated choline kinase activation.

Choline kinase potentiates RhoA-induced oncogenic transformation. We next investigated whether activation of choline kinase had any effect on the biological function of RhoA. Transient transfection experiments of the human Hek293T and the canine epithelial MDCK cells were carried out. RhoA resulted in choline kinase activation, an effect that was amplified when overexpressing RhoA together with choline kinase, confirmed by protein expression characterization (Fig. 3*A*). Transfected cells were plated on soft agar plates and their ability to grow under anchorage-independent conditions determined. The number of clones grown in soft agar in each case was estimated after 5 weeks (Fig. 3*B*). Expression of constitutively active RhoA induced 2.9-fold increase in Hek293T and 1.3-fold increase in MDCK in the number of colonies. Surprisingly, overexpression of choline kinase alone was sufficient to induce 2.3-and 1.4-fold increase in the number of colonies, respectively, in these cell systems, Hek293T and MDCK. Coexpression of RhoAQL and choline kinase resulted in a further increase of >4-fold in the number of colonies in both cell lines and an increase in the mean size of the colonies (Fig. 3*C*). These results suggest that choline kinase is sufficient to induce oncogenic transformation displaying a transforming potential similar to that of RhoA and that choline kinase potentiates RhoA-induced anchorage-independent growth.

Choline kinase enhances RhoA-mediated tumorigenesis *in vivo*. We next analyzed both the tumorigenic potential of choline kinase–transfected Hek293T cells and the putative role of this enzyme in RhoA-mediated tumorigenesis *in vivo*. Immunosuppressed mice (*nu/nu*) were injected with 1×10^6 Hek293T cells

Table 1. ChoK efficiently potentiates RhoA-induced tumorigenicity

DNA	Tumor incidence (%)	Tumor volume (cm^3)
PCDNAIIIb	0/24 (0)	0
RhoAQL	8/24 (33)	0.6 ± 0.4
ChoK	8/30 (26)	0.7 ± 0.4
RhoAQL/ChoK	10/30 (33)	2.7 ± 0.3

NOTE: Mice were injected s.c. with 1×10^6 of either Hek293T-pCDNAIIIb, Hek293T-RhoAQL, Hek293T-ChoKαa, or Hek293T-RhoAQL/ChoKαa transfected cells. Tumor growth was monitored weekly up to 50 days after injection. Whereas none of the control animals induce any detectable tumor, Hek293T-RhoAQL-, Hek293T-ChoKαa-, and Hek293T-RhoAQL/ChoKαa-transfected cells induced tumor growth in 26% to 33% of the cases. Both, tumor volume and growth rate of RhoAQL/ChoKαa cells were significantly higher than those of RhoAQL or ChoKαa cells. Differences were found significant between both RhoA and ChoKαa-transfected cells with respect to control cells ($P \leq 0.01$) and between RhoA/ChoKαa expressing cells with respect to the expression of only RhoA or ChoKαa ($P \leq 0.001$).

transfected with either RhoAQL alone, choline kinase alone, or both. Tumor growth was monitored weekly up to 45 days after injection. As controls, mice were injected under same conditions with Hek293T cells transfected by the empty pcDNAIIIb vector. Hek293T-RhoAQL-, Hek293T-ChoK-, and Hek293T-RhoAQL/ChoK-transfected cells were all able to induce tumors (Table 1). However, both the tumor volume and growth rate of RhoAQL/ChoK cells were significantly increased respect to those of RhoAQL or choline kinase cells alone. Whereas RhoAQL and choline kinase tumors reached 0.7 and 0.6 cm^3, respectively, RhoAQL/choline kinase cells reached a mean tumor volume of 2.7 cm^3 (Fig. 4*A*), >4-fold bigger, indicating a relevant synergism between choline kinase and RhoA. Thus, overexpression of choline kinase is sufficient to induce tumors and strongly facilitates RhoA-induced tumorigenesis *in vivo*. Finally, Hek293T-ChoK- and Hek293T-RhoAQL/ChoK-induced tumors were surgically extracted and both choline kinase activity and expression levels were determined with respect to control Hek293T cell extracts. As shown in Fig. 4*B* and *C*, all tested tumors displayed increased choline kinase activity and expression similar to that observed in the transfected cells before inoculation (Fig. 3), proving that overexpression of choline kinase paralleled tumorigenesis *in vivo*.

MN58b inhibits choline kinase–induced tumorigenesis *in vivo*. A conclusive support that choline kinase overexpression was the cause for the generation of tumors can be provided if choline kinase–induced tumors were susceptible to *in vivo* growth inhibition by treatment with the choline kinase–specific inhibitor MN58b (29–31). Hek293T cells overexpressing human choline kinase were injected s.c. (1×10^6 cells) in nude mice, and MN58b was given i.p. in sterile 0.9% NaCl. Treatment was initiated when tumors reached a volume of 0.1 cm^3 and consisted of five daily consecutive doses of 5 mg/kg, separated by 9 days. Control mice received equivalent volumes of vehicle alone, following an identical schedule, and tumors were monitored twice a week. Inhibition of choline kinase resulted in a statistically significant strong inhibition of the tumor growth ($n = 5$; $P < 0.05$) with an estimated mean reduction in tumor volume of 80% (Fig. 4*D*). These results show that tumors were indeed generated by overexpression of choline kinase and that cell proliferation of tumor cells was dependent on choline kinase activity. Similar results were obtained when cell lines derived from tumors generated by Hek293 cells transfected with both RhoA and choline kinase were inoculated into mice and treated with MN58b. A statistically significant reduction of 45% in tumor growth was observed ($n = 5$; $P = 0.05$). This result was expected because RhoA can induce tumors through activation of tumorigenic signaling cascades independent of choline kinase (16–21).

Discussion

ChoKαa belongs to a family of at least five known human choline and choline/ethanolamine kinases (ChoKαa, ChoKαb, ChoKβ1, ChoKβ2, and HCEKV). Although very little is known on their significance and mechanisms of regulation, choline kinase activity has been linked to human carcinogenesis (34, 35). We have used ChoKαa in our study and show that it has oncogenic activity. Furthermore, we provide evidence that ChoKαa is susceptible of specific regulation by RhoA but not other members of the family such as Rac-1 or Cdc42.

Rho GTPases are master pieces in the regulation of a large variety of cellular functions (16, 17). They act as molecular

switches that control microtubule- and actin-based complexes, epithelial cell junctions, lipid metabolism, and signaling events such as MAPK and transcription factors regulation. Consequently, Rho GTPases are able to play important roles in tumor biology and other human pathologies (16–20). Although no point mutations of Rho GTPases in human tumors have been clearly related to tumor

Figure 4. Choline kinase (*ChoK*) enhances RhoA-mediated tumorigenesis *in vivo*. *A,* RhoAQL and human ChoKαa promote tumor growth *in vivo*. Hek293T cells transfected with empty pCDNAIIIb vector, RhoAQL, or Chokαa alone and cotransfected with both RhoAQL and ChoKαa. After transfection, 1×10^6 cells were inoculated s.c. in athymic mice. Tumor volume was monitored weekly. Promotion of *in vivo* tumorigenesis by choline kinase and RhoAQL in nude mice was found statistically significant ($P \leq 0.001$). Overexpression of hChoKαa strongly facilitates RhoA-induced tumorigenesis *in vivo* with statistically significant value ($P \leq 0.001$). *B,* surgically extracted tumors generated by choline kinase overexpression display an increase in choline kinase activity (*top*; $P \leq 0.001$) and expression (*bottom*). *C,* the specific choline kinase inhibitor MN58b efficiently blocks tumor growth induced by choline kinase overexpression. 1×10^6 Hek293T cells overexpressing human choline kinase were injected s.c. in *nu/nu* mice and when tumor reached a mean volume of 0.1 cm³, mice were treated i.p. with MN58b (5 mg/kg body weight). From day 10 to the rest of treatment, reduction of tumor volume in treated mice was found statistically significant ($n = 5$; $P \leq 0.01$). All experiments shown were done three independent times with similar results.

formation, overexpression of these GTPases or some upstream or downstream element of Rho signaling has been identified in a large variety of tumors, including pancreatic cancers, breast cancer, melanomas, colorectal carcinomas, testicular germ cancer, head and neck squamous cell carcinomas, leukaemias, osteosarcomas, gastric cancer, thyroid papillary carcinomas, hepatocellular carcinomas, ovarian cancers, neuroblastomas, prostate cancer, bladder cancer, Hodgkin's lymphoma, and renal carcinomas (17–20). A complex network of effectors to Rho proteins have been identified and their role in human cancers is starting to be understood. Among them, the families of ROCK/Rho kinase and PAK are linked to tumor development and metastasis (17–20). Others, such as IQGAP, mDia, PKN, ACK1/2, and phospholipase D are starting to be linked to transformation. However, the precise mechanism for the oncogenic activity of Rho GTPases is still not fully understood. Here, we provide strong evidence that choline kinase may be a new relevant molecule in RhoA-induced oncogenic transformation.

Activation of choline kinase by RhoA is not mediated by direct protein-protein interaction such as that described for phospholipase D (7), another enzyme involved in the regulation of phosphatidylcholine synthesis and degradation. Furthermore, we have previously reported that activation of phospholipase D does not influence choline kinase activity (7). Instead, this regulation is carried out through ROCK activity, one of the well-known effectors to RhoA. Other Rho effectors such as PKN, citron kinase, m-Dia, rhophilin-1, and rhophilin-2 have no effect on choline kinase activity under similar conditions. These results suggest that ROCK is a mayor contributor to RhoA-induced activation of ChoKαa in Hek293T cells. The precise mechanism for Rock-induced choline kinase activation is still not fully resolved, although preliminary evidence suggests that ROCK may not directly interact with choline kinase in a stable conformation (data not shown). Furthermore, the involvement of other Rho effectors in other cell systems cannot be excluded. Indeed, Rho proteins show cell specificity for signaling through MAPK (extracellular signal-regulated kinases, p38, and c-*jun*-NH2-kinase) and transcription factors (19), and this could also be true for choline kinase activation.

We have also investigated the effect of choline kinase activation on the biological function of RhoA and show that both proteins synergize for transformation of human Hek293T and canine MDCK cells. A significant increase in the number and size of anchorage-independent colonies induced by RhoA was observed when choline kinase was overexpressed in these systems, suggesting that choline kinase is an important molecule in
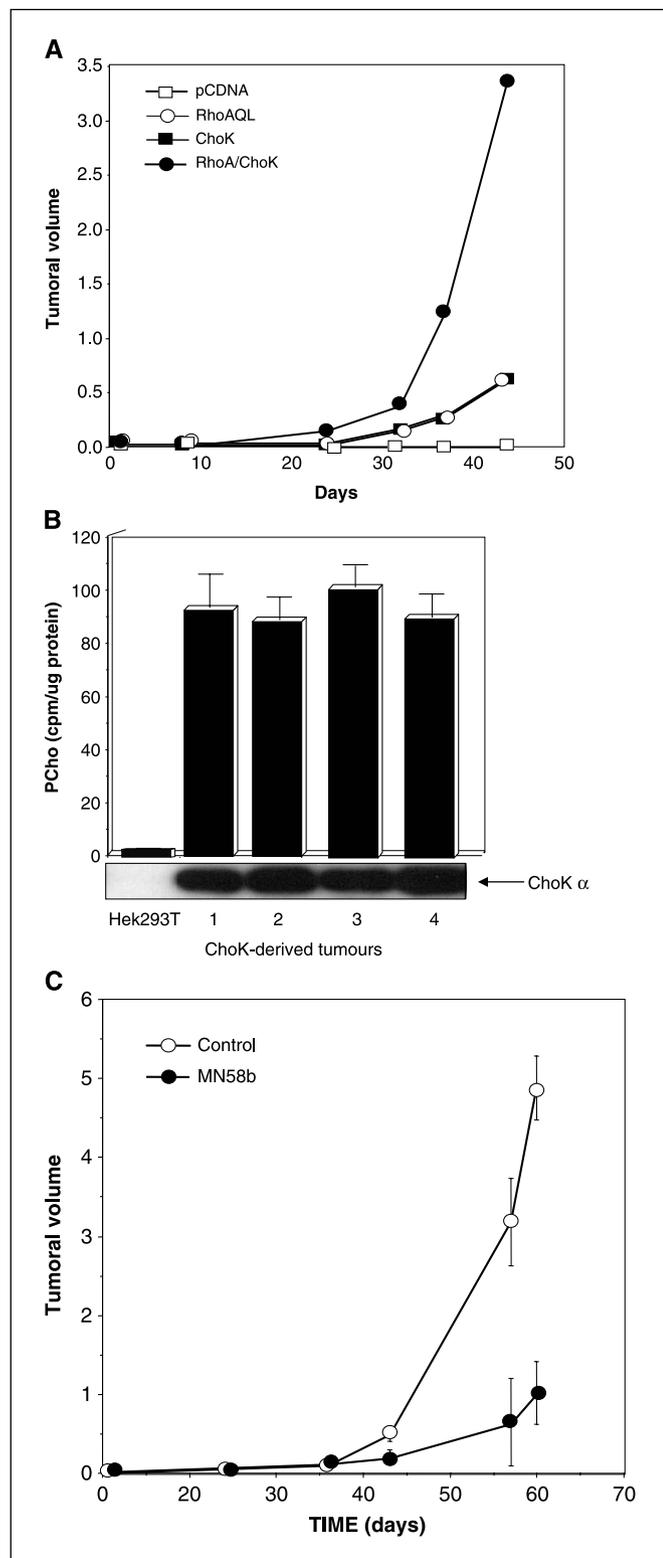
RhoA-mediated transformation. In addition, choline kinase potentiates RhoA-induced tumorigenesis *in vivo* when inoculated in the nude mice system. Coexpression of RhoA and choline kinase increased up to 4-fold both tumor volume and tumor growth rate, indicating that this enzyme is an important molecule that efficiently synergizes with the oncogenic activity of RhoA. Choline kinase was sufficient by itself to induce both anchorage independent growth and tumorigenicity with an oncogenic potential similar to that of RhoA alone. These results are in line with recent evidence that overexpression of choline kinase in human primary epithelial cells is sufficient to induce entry into the S phase (36). A further strong demonstration that choline kinase was the driving force in the observed transforming activity is that its *in vivo* oncogenic activity was drastically inhibited by MN58b, a specific choline kinase inhibitor (10–14). Because choline kinase has been recently shown to be increased in several important human tumors (1, 2) and is modulated by oncogenes (7, 8, 10), our results are also relevant for the validation of choline kinase inhibition as a useful anticancer therapy.

In conclusion, we provide here definitive evidence that human ChoKα has oncogenic activity by itself, and synergistically enhances the transforming properties of the RhoA GTPase. Furthermore, specific choline kinase inhibition efficiently interferes with its *in vivo* oncogenicity. These results provide very strong support for a novel antitumor strategy based on ChoKα inhibition.

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