Calcineurin Inhibitors Activate the Proto-Oncogene Ras and Promote Protumorigenic Signals in Renal Cancer Cells

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Abstract

The development of cancer is a major problem in immunosuppressed patients, particularly after solid organ transplantation. We have recently shown that calcineurin inhibitors (CNI) used to treat transplant patients may play a critical role in the rapid progression of renal cancer. To examine the intracellular signaling events for CNI-mediated direct tumorigenic pathway(s), we studied the effect of CNI on the activation of proto-oncogenic Ras in human normal renal epithelial cells (REC) and renal cancer cells (786-O and Caki-1). We found that CNI treatment significantly increased the level of activated GTP-bound form of Ras in these cells. In addition, CNI induced the association of Ras with one of its effector molecules, Raf, but not with Rho and phosphatidylinositol 3-kinase; CNI treatment also promoted the phosphorylation of the Raf kinase inhibitory protein and the downregulation of carabin, all of which may lead to the activation of the Ras-Raf pathway. Blockade of this pathway through either pharmacologic inhibitors or gene-specific small interfering RNA Raf pathway. We found that CNI treatment significantly increased the level of activated Ras in human renal cancer cells. Finally, it was observed that CNI treatment increased the growth of human renal tumors in vivo, and the Ras-Raf pathway is significantly activated in the tumor tissues of CNI-treated mice. Together, targeting the Ras-Raf pathway may prevent the development/progression of renal cancer in CNI-treated patients. [Cancer Res 2009;69(23):OF1–8]

Introduction

Immunosuppressive medications are essential for the prevention of allograft rejection and also for the treatment of different inflammatory diseases (1, 2). However, patients receiving immunosuppressive therapy have a greater risk of developing cancer compared with the general population (3–8). Recent studies have established that calcineurin inhibitors (CNI) may play a major role for the increased incidence of cancers in these patients (9–13). CNI, such as cyclosporine (CsA) and tacrolimus (FK506), may suppress the immune system so that the immune surveillance mechanism is impaired (6, 9, 14). Immunosuppression predisposes patients to a variety of viral infections that lead to malignant transformations of different tissues (7, 15). In addition, they may also have direct tumorigenic effects. CNI can promote tumor growth through transforming growth factor-β production (9, 11, 16). We and others have reported that CNI may induce tumor growth through overexpression of the angiogenic cytokine vascular endothelial growth factor (VEGF; refs. 10, 17). Recently, we have shown that CNI may promote the proliferation of human renal cancer cells through altered expression of the chemokine receptor CXCR3 (18). Although several effector molecules have been identified to be responsible for CNI-mediated tumor growth, the intracellular signaling mechanism(s) for these direct tumorigenic pathways has not been defined.

The major and classic function of CNI is to suppress T-cell activation responses through downregulation of the calcineurin-NFAT pathway (19). Although the calcineurin-NFAT pathway is well characterized in immune cells, components of this signaling pathway exist in many other cell types (20). The tumorigenic effects of CNI may be mediated either through suppression of the calcineurin-NFAT pathway in T cells or through some other direct mechanism(s) independent of immune cells. In support of direct mechanism(s), it has been shown that the treatment of tumor xenografts in T-cell–deficient and B-cell–deficient severe combined immunodeficient (SCID) mice with CNI enhanced tumor growth and progression (9). CNI may also activate/inhibit genes important for cell cycle regulation, apoptosis, and oncogene/tumor suppressor function (21). There are some reports that oncogenes may become activated in immunosuppressed transplant patients (21–23). However, very little is known about the signaling mechanism(s) of CNI-mediated direct tumorigenic pathways.

The ras family of proto-oncogenes encodes small proteins that transduce mitogenic signals from tyrosine kinase receptors (24, 25). Ras proteins act as molecular switches that cycle between active GTP-bound and inactive GDP-bound forms (26–28). The three isoforms of Ras—H-Ras, K-Ras, and N-Ras—are ubiquitously expressed in mammalian cells (29). The hyperactive Ras can promote the growth of cancer cells without being mutated, where it may be activated by persistent upstream signaling events (30–32). Activated Ras proteins transmit their signals to a cascade of protein kinases that have mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) as the substrate, such as MEK kinase, c-Raf-1, and B-Raf, culminating in the activation of MAPK (33). On activation, Ras may primarily function to promote the translocation of Raf-1 from the cytosol to the plasma membrane, where subsequent Ras-independent events trigger Raf-1 kinase activation (34). However, Ras may also mediate its action through Raf-independent pathways, including Rho and phosphatidylinositol 3-kinase (PI3K) pathways (35–38).

In the present study, we show a novel tumorigenic pathway in which CNI promotes the activation of Ras and its downstream effector molecules in human renal cancer cells. CNI-mediated Ras activation plays a critical role in renal cancer cell proliferation.
Materials and Methods

Reagents. CsA (Novartis) and FK506 (Astellas) were purchased from the Children’s Hospital Boston pharmacy. Rapamycin was gifted to the laboratory by Wyeth-Ayerst Research. Raf-1 kinase inhibitor I (RKi: 5-iodo-3-[[3,5-dibromo-4-hydroxy-phenyl]methylene]-2-indolino]e) and farnesyl transferase inhibitor (FTI) were purchased from Calbiochem. The gene-specific small interfering RNAs (siRNA) for H-Ras, K-Ras, N-Ras, and carablin along with their controls were purchased from Qiagen.

Antibodies. The antibodies for Ras, H-Ras, K-Ras, N-Ras, Raf, RKIP, and phospho-RKIP were purchased from Santa Cruz Biotechnology. The Rho antibody was purchased from Upstate. The carabin antibody was purchased from ProSci, Inc. The antibodies for PI3K, ERK, and phospho-ERK were purchased from Cell Signaling Technology, Inc. The β-actin antibody was obtained from Sigma.

Cell culture. The human renal cancer cell lines (786-0 and Caki-1) were obtained from the American Type Culture Collection. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories). Human renal proximal tubular epithelial cells (REC) were purchased from Clonetics and cultured in complete epithelial medium (REGM BulletKit).

Measurement of active/GTP-bound Ras. The active/GTP-bound form of Ras in the cell lysates was measured by using an EZ-Detect Ras activation kit (Pierce). This kit uses specific Ras-binding domain (RBD) of Raf-1 that can specifically bind active GTP-bound form of Ras. The cell lysates were incubated with glutathione S-transferase (GST)-Raf-1-RBD and a SwellGel immobilized glutathione disc. The eluted samples were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (NEN Life Sciences Product, Inc.), and probed with either anti-Ras or isoform-specific Ras antibody.

Immunoprecipitation assays. Immunoprecipitations were performed with 0.5 mg of total protein at antibody excess. Immunocomplexes were captured with protein A-Sepharose beads (Amersham Pharmacia Biotech), and bead-bound proteins were subjected to Western blot analysis using specific antibody.

Western blot analysis. Protein samples were run on SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with specific primary antibody and subsequently incubated with peroxidase-linked secondary antibody. The reactive band was detected by chemiluminescent substrate (Pierce).

Cell proliferation assay. Cells (5 × 10^3) were seeded and grown in 96-well plates. [3H]thymidine (0.5 μCi/well) was added for the final 15 h before cell harvesting. [3H]thymidine incorporation was measured using a microplate scintillation and luminescence counter (Perkin-Elmer/Wallace).

In vivo tumor development. Human renal cancer cells (786-0) were injected s.c. either in immunodeficient nude (nu/nu) mice or in SCID-Beige mice. Either CsA (10 mg/kg/d) or the vehicle was then administered i.p. to these mice. Tumor volume was measured using a digital caliper at regular intervals. The volume was estimated by following standard method (18) using the formula \( V = \pi/6 \times a^2 \times b \), where \( a \) is the short and \( b \) is the long tumor axis. Mice were killed at designated times after injection. All animal works were approved by the Animal Care and Use Committee at Children’s Hospital Boston.

Statistical analyses. Statistical evaluation for data analysis was determined either by t test for two groups of data or by one-way ANOVA for three or more groups. Differences with \( P < 0.05 \) were considered statistically significant.

Results

CNIs promote the activation of Ras in human renal epithelial cells. We first evaluated whether CNIs can induce Ras activation in human renal tubular epithelial cells (REC) in vitro. We treated REC with CsA in both time-dependent (1 μg/mL for 3 and 6 hours) and dose-dependent (1 and 5 μg/mL for 6 hours) manner. Vehicle-treated cells served as controls. To measure the activation status of Ras in these cell lysates, we performed affinity pull-down reactions with the RBD of Raf that can specifically bind active GTP-bound form of Ras as described in Materials and Methods. The eluted samples were subjected to Western blot analysis using Ras-specific antibody. CsA treatment markedly induced the activation of Ras in both time- and dose-dependent manner compared with controls (Fig. 1A, top; Supplementary Fig. S1A). However, there was no change in the expression of total Ras following CsA treatment (Fig. 1A, bottom).

Next, we examined whether CsA can activate Ras in human renal cancer cells as observed in REC. 786-0 and Caki-1 cells were treated with either increasing concentrations of CsA or vehicle. We found that, similar to REC, CsA treatment markedly increased the activation of Ras in both 786-0 and Caki-1 cells compared with vehicle-treated controls (Fig. 1B, top; Supplementary Fig. S1A). There was no change in the expression of total Ras (Fig. 1B, bottom).

We also evaluated the effect of FK506 (another CNI) on the activation of Ras. We treated REC and Caki-1 cells with either increasing concentrations of FK506 or vehicle. As observed with CsA treatment, FK506 also increased the activation of Ras in both the cell types (Fig. 1C; Supplementary Fig. S1A); there was no change in the level of total Ras following FK506 treatment. Together, these results suggest that CNI play a major role in the activation of Ras in human normal renal epithelial and renal cancer cells.

CNIs primarily activate H-Ras in vitro. Here, we set out to determine which particular isoform(s) of Ras is activated following CNI treatment. REC, 786-0, and Caki-1 cells were treated with either increasing concentrations of CsA or vehicle, and the activation status of Ras isoforms (H-Ras, K-Ras, and N-Ras) in these cells was measured by affinity pull-down reactions using RBD of Raf followed by Western blot analysis using isoform-specific Ras antibody. As shown in Fig. 2 (top) and Supplementary Fig. S1B, CsA markedly induced the activation of H-Ras compared with vehicle-treated controls; there was minimal change in the activation of K-Ras and N-Ras. There was no change in the amount of total Ras isoforms (H-Ras, K-Ras, and N-Ras) in these cells following CsA treatment (Fig. 2, bottom). These results suggest that CNI primarily activates H-Ras in human renal epithelial and renal cancer cells in vitro.

CNI-induced and Ras-mediated signals are channeled through the Raf pathway. We evaluated whether CNI could promote the association between Ras and one of its effector molecules (Raf, Rho, and PI3K). We treated REC with increasing concentrations of CsA. By immunoprecipitation, we observed that CsA treatment markedly increased the association between Ras and Raf compared with vehicle-treated control (Fig. 3A, left). However, there was no significant change in Ras-Rho or Ras-PI3K association following CsA treatment (Fig. 3A, middle left and middle right). We confirmed that CsA treatment also increased the Ras-Raf association in these cells in a time-dependent manner (Fig. 3A, right).

We observed that similar to REC, CsA treatment increased the association between Ras and Raf in 786-0 renal cancer cells (Fig. 3B, left). We also found that CsA treatment primarily increased the association between H-Ras and Raf in these cells (Fig. 3B, middle left); however, there was no significant change in K-Ras–Raf or N-Ras–Raf associations (Fig. 3B, middle right and right). A similar finding in terms of CsA-induced H-Ras–Raf association was observed in REC (data not shown).

It has been established that Raf kinase inhibitory protein (RKIP) acts as an endogenous inhibitor of the Raf-1/MEK pathway (39). Nonphosphorylated RKIP normally inhibits Raf and therefore
blocks Raf-mediated signaling events (40). To examine the effect of CNI on RKIP, we treated 786-0 and Caki-1 cells with CsA (1.0 and 5.0 μg/mL) or with the vehicle for 6 h. C, REC and Caki-1 cells were treated with FK506 (0.01 and 0.1 μg/mL) or with the vehicle for 6 h. Cells (A–C) were lysed, and the active GTP-bound form of Ras in the cell lysates (with equal amount of proteins) was measured by affinity pull-down reactions with GST-Raf-1-RBD followed by Western blot analysis using Ras-specific antibody (top). Bottom, the amount of total Ras in the cell lysates was measured by direct Western blot analysis using Ras-specific antibody. The relative intensity of each active Ras band to that of total Ras was measured by densitometry, and the values are listed under the blots. Representative of three independent experiments with similar findings.

Figure 1. CNI induces renal cancer cell proliferation in vitro. The 786-0 cells were treated either with CsA or with vehicle in the presence or absence of pharmacologic Ras inhibitor (FTI) and then subjected to a cell proliferation assay. CsA increased the proliferation of renal cancer cells compared with vehicle-treated control, and FTI significantly reduced CsA-induced cell proliferation (Fig. 4A). Similar to CsA, FK506 also increased the proliferation of renal cancer cells, and FTI treatment inhibited FK506-mediated cell proliferation (Supplementary Fig. S2A). To examine whether a mammalian target of rapamycin inhibitor can counteract the effect of CNI, we observed that rapamycin significantly inhibited CsA-mediated renal cancer cell proliferation (Supplementary Fig. S2B).

Next, we evaluated the role of specific Ras isoform(s) in CNI-induced renal cancer cell proliferation in vitro. We used gene-specific siRNAs for H-Ras, K-Ras, and N-Ras that specifically and...
To assess cell proliferation. As shown in Fig. 4, Ras pathway (Supplementary Fig. S3) is also primarily mediated through the H-Ras signaling. A similar result in REC and Caki-1 cells where CsA-induced cell proliferation was very little decrease in CsA-induced cell proliferation following transfection with either K-Ras or N-Ras siRNA. We observed a significant decrease in CsA-induced cell proliferation; however, there was very little decrease in CsA-induced cell proliferation following transfection with either K-Ras or N-Ras siRNA. We observed a similar result in REC and Caki-1 cells where CsA-induced cell proliferation is also primarily mediated through the H-Ras signaling pathway (Supplementary Fig. S3A and B).

To examine if Raf is critical in CNI-induced renal cancer cell proliferation, we treated 786-0 cells with either CsA or vehicle in the presence or absence of RKI. RKI significantly inhibited CsA-induced cell proliferation (Fig. 4D). Together, these results suggest that the Ras-Raf pathway plays a major role in CNI-induced renal cancer cell proliferation.

CNI downregulate the expression of carabin, a negative regulator of the ras pathway. It has been reported that a novel molecule called carabin can inhibit not only the calcineurin pathway but also the Ras signaling pathway (41). Thus, the level of expression of carabin may play a significant role in regulating the cellular status of Ras activation. To explore the possible mechanism(s) of CNI-induced Ras activation, we first investigated the effect of CNI on the expression of carabin. REC and 786-0 cells were treated with either CsA or vehicle, and the expression of carabin was evaluated by Western blot analysis. In both cell types, the expression level of carabin was decreased following CsA treatment compared with vehicle-treated control (Fig. 5A, left and middle). Similarly, we found that the expression of carabin was also downregulated in 786-0 cells following FK506 treatment (Fig. 5A, right).

Next, we examined the effect of carabin knockdown on the proliferation of renal cancer cells in vitro. The 786-0 cells were transfected with either the gene-specific siRNA of carabin or the control siRNA and then subjected to a cell proliferation assay. The knockdown of carabin increased the proliferation of renal cancer cells compared with controls (Fig. 5B). The knockdown of carabin was confirmed by Western blot analysis (Fig. 5B, right). We also observed that the knockdown of carabin primarily increased the level of active H-Ras in 786-0 and Caki-1 cells (Fig. 5C); there was very minimal change in the activation of K-Ras and N-Ras. Together, carabin may act as a negative regulator of cancer cell proliferation. We suggest that CsA-induced and Ras-mediated increase in renal cancer cell proliferation may involve downregulation of carabin.

The CNI CsA promotes activation of the Ras-Raf pathway in human renal tumor tissues in vivo. We have recently shown that in immunodeficient (nu/nu) mice, the growth of human renal tumors (786-0) was significantly accelerated following CsA treatment (10 mg/kg/d, a dose that can significantly prolong allograft survival in BALB/c mice) compared with vehicle-treated controls (10). We have also observed that CsA promoted an enhanced growth of

![Figure 3](image-url)
human renal tumors in SCID-Beige mice that do not have any residual natural killer cells (Supplementary Fig. S4). Here, we examined the status of the Ras-Raf pathway in the tumor tissues from CsA-treated mice. We observed that similar to our in vitro findings, CsA treatment in both SCID-Beige and nude mice significantly increased the activation of Ras in the tumors compared with vehicle-treated controls (Fig. 6A). However, in contrast to our in vitro findings, we observed that the activation status of all the Ras isoforms (H-Ras, K-Ras, and N-Ras) was increased in tumor tissues following CsA treatment (Fig. 6B); this is possibly due to in vivo microenvironment, where CsA can regulate multiple factors in tumor cells as well as in other cell types that may result in the activation of all the Ras isoforms.

Next, we wished to evaluate which Ras effector molecules are involved in CsA-induced Ras activation in these tumor tissues. We observed that CsA treatment increased the association of Ras and Raf compared with vehicle-treated control (Fig. 6C); however, there was no significant change in the association of either Ras-Rho or Ras-PI3K in the tumor tissues. We also measured the phosphorylation status of RKIP in these tissues. As shown in Fig. 6D, CsA treatment significantly increased the phosphorylation of RKIP compared with vehicle-treated controls.

As ERK is a potential downstream target of the Ras-Raf pathway (33), we examined the phosphorylation status of this kinase in these tumor tissues. CsA treatment significantly increased the level of phospho-ERK compared with control (Supplementary Fig. S5). There was no change in the level of total ERK. Together, these experiments suggest that CsA treatment may promote accelerated growth of human renal tumors in vivo likely through the induction of the Ras-Raf-ERK signaling cascade.

**Discussion**

CNIs may promote cancer development through tumorigenic pathways. In the present study, we show a novel mechanism by which CNI can promote the growth of human renal cancer through the activation of the proto-oncogene ras.

Kidney cancer has been reported to be one of the major cancers among transplant recipients (8). Nephrotoxicity is the most common and limiting side effect of CNI treatment in transplant patients and gives rise to both acute and chronic kidney damage (42). It has been shown that CsA can induce some proto-oncogenes (c-fos and c-jun), which may partially be responsible for long-term nephrotoxicity (23). However, there was no correlation between the proto-oncogenic activation, nephrotoxicity, and renal tumor development.

Ras proteins play a key role in tumor development (25, 28). Here, for the first time, we have shown that CNI can activate the

![Figure 4. CsA promotes renal cancer cell proliferation through the activation of Ras.](image-url)

A. 786-0 cells were treated with FTI (1 μmol/L) or with the vehicle for 12 h and then treated with CsA (1 and 5 μg/mL) or with vehicle (control) for 72 h. B. 786-0 cells were transfected with 25 nmol/L of the control siRNA, H-Ras siRNA, K-Ras siRNA, or N-Ras siRNA for 48 h. The expression of H-Ras, K-Ras, N-Ras, and β-actin in the cell lysates was measured by Western blot analysis using isoform-specific Ras antibodies or anti-β-actin. Representative of three independent experiments. C. 786-0 cells were transfected with 25 nmol/L of either control siRNA, H-Ras siRNA, K-Ras siRNA, or N-Ras siRNA for 48 h and then treated with CsA (1 μg/mL) or vehicle alone for 48 h. D. 786-0 cells were treated with RKI (1 μmol/L) for 12 h and then treated with either CsA (1 μg/mL) or vehicle for 48 h. In A, C, and D, the cells were subjected to cell proliferation assay by measuring [3H]thymidine incorporation within the cells. Data reflect three independent experiments. Columns, average of triplicate readings (cpm) of the samples; bars, SD. A, **, P < 0.01 versus vehicle-treated cells; *, P < 0.01 versus CsA-treated and FTI-untreated cells. C, **, P < 0.05 versus control siRNA-transfected cells; ***, P < 0.05 versus control siRNA-transfected and CsA-treated cells. NS, not statistically significant versus control siRNA-transfected and CsA-treated cells. D, **, P < 0.01 versus vehicle-treated cells; ***, P < 0.05 versus CsA-treated and RKI-untreated cells.
proto-oncogene ras in human renal epithelial and renal cancer cells without altering the expression of total Ras. It is possible that CNI may directly/indirectly modulate the expression/activity of either guanine nucleotide exchange factors or GTPase-activating proteins that regulate Ras activity (24, 25). Our in vitro studies suggest that CNIs primarily activate H-Ras and induce renal cancer cell proliferation. Although the Ras isoforms are ubiquitously expressed and highly conserved, they may exhibit different biological functions (29, 43). In support of our findings, Best and colleagues (44) showed that H-Ras–transformed renal epithelial cells have an altered growth pattern and morphology that correlates with the characteristics of renal cell carcinoma. Chin and colleagues (45) showed that H-Ras activation is sufficient in both the genesis and maintenance of solid tumors. However, in contrast to our in vitro findings, we observed that all the Ras isoforms (H-Ras, K-Ras, and N-Ras) are activated in tumor tissues from CNI-treated mice. We suggest that within in vivo microenvironment, CNI can regulate multiple factors that may act in autocrine/paracrine manner to promote activation of all the Ras isoforms.

There is a possible cross-talk between the calcineurin and the Ras pathway, and it has been shown that a novel molecule named carabin may act as an endogenous inhibitor for both the pathways (41). In this study, we have observed that CNI may downregulate the expression of carabin in renal epithelial and renal cancer cells and can promote cell proliferation. We suggest that CNI-mediated downregulation of carabin may act as one of the possible mechanisms for Ras activation in renal epithelial cells following CNI treatment. In the future, it will be interesting to examine if knock-down of carabin can promote tumor growth in vivo through activation of Ras.

The activation of the Ras-Raf-MEK-ERK pathway contributes to the development of different types of human tumors, including renal cancer (25, 46). We have observed that CNI-induced and Ras-mediated signals are channeled through the Raf pathway. A prerequisite for Raf-1 activation in many signaling pathways is an interaction with the ras proto-oncogene product (39). Recent studies suggest that the Raf-Raf pathway is assembled by some specific scaffolding proteins, such as the kinase suppressor of Ras (KSR) and connector enhancer of KSR (47). RKIP is a member of the phosphatidylethanolamine-binding proteins and represents a new class of modulators for the Ras-Raf-MEK signaling cascade (39). It can interrupt the Raf-mediated signaling by dissociating the interaction between Raf-1 and its substrate MEK (40). More recently, RKIP has emerged as an important suppressor of metastasis (48). In this study, we have found that CsA promotes an increased association between Ras and Raf in human renal cancer cells, and it also inactivates RKIP through increased phosphorylation of the protein. We suggest that CNI-mediated inactivation of RKIP may play a major role in channeling the Ras signals through the Raf pathway to promote augmented tumor growth.

We have recently shown that CsA can induce the overexpression of VEGF in human renal cancer cells and promote a rapid progression of posttransplantation cancer (10). It is now established that the activation of Ras is one of the critical factors for VEGF

Figure 5. CNIs downregulate the expression of carabin. A, REC and 786-0 cells were treated with CsA (1 and 5 μg/mL; left and middle), and 786-0 cells were treated with FK506 (0.1 μg/mL; right) for 6 h. Vehicle-treated cells served as controls. The expression of carabin (top) and β-actin (bottom) in the cell lysates was measured by Western blot analysis using anti-carabin and anti-β-actin, respectively. Representative of three independent experiments. B, 786-0 cells were transfected with either carabin siRNA (10 and 25 nmol/L) or control siRNA for 72 h, and the cells were subjected to cell proliferation assay by measuring [3H]thymidine incorporation within the cells. Data reflect three independent experiments. Columns, average of triplicate readings (cpm) of the samples; bars, SD. *, P < 0.05 versus control siRNA–transfected cells. Right, the knockdown of carabin by siRNA (25 nmol/L) was confirmed by Western blot analysis using anti-carabin. C, 786-0 and Caki-1 cells were transfected with either carabin siRNA (25 nmol/L) or control siRNA for 72 h. The active GTP-bound form of Ras isoforms and also the level of total Ras isoforms in the cell lysates were measured by immunoprecipitation and Western blot analysis as described in Fig. 2. Representative of three independent experiments.
Thus, our findings in this study suggest that CsA-induced Ras activation might be one of the key factors for VEGF overexpression in renal cancer cells.

In summary, our study identifies the activation of the proto-oncogenic ras as a central mediator of CNI-induced renal cancer progression. In addition, it shows that the Raf-MEK-ERK pathway acts as the key downstream effector for the CNI-induced and Ras-mediated signals. Thus, targeting this protumorigenic pathway may allow new opportunities for the development of novel anticancer drugs, particularly for the treatment of transplant patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

Figure 6. CsA activates the Ras-Raf signaling pathway in tumor tissues in vivo. Human renal cancer cells (1.0 × 10⁶; 786-0) were injected s.c. either in SCID-Beige mice (n = 5 in each group) or in nude (nu/nu) mice (n = 10 in each group), and they were treated either with CsA (10 mg/kg/d) or with vehicle as control. Tumors were harvested at day 28 (SCID-Beige mice) and day 30 (nu/nu mice) following tumor injection. A, top, the active GTP-bound form of Ras in the tissue lysates (with equal amounts of proteins) was measured by affinity pull-down reactions with GST-Raf-1-RBD followed by Western blot analysis using Ras-specific antibody; bottom, the amounts of total Ras were measured by direct Western blot analysis using Ras-specific antibody. B, the active GTP-bound form of Ras isoforms (top) and also the level of total Ras isoforms (bottom) in the tissue lysates were measured by immunoprecipitation and Western blot analysis as described in Fig. 2. C, the tissue lysates were immunoprecipitated with anti-Raf (top left), anti-PI3K (top right), or anti-Rho (bottom), and Western blots (Blot) were performed with anti-Ras (top left, top right, and bottom), anti-Raf (top left), anti-PI3K (top right), or anti-Rho (bottom). D, the amount of phospho-RKIP (top) and β-actin (bottom) in the tissue lysates was measured by Western blot analysis using anti-phospho-RKIP and anti-β-actin, respectively. A to D, representative of three different tissue samples of both CsA- and vehicle-treated groups.


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