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Ras-dependent Oncolysis with an Adenovirus VAI Mutant

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ABSTRACT

Adenovirus synthesize proteins that interact with oncogene and tumor suppressor gene products to set the cell for virus replication. Mutant viruses defective in these functions replicate selectively in cancer cells and represent new tools to treat cancer. We report a selectivity strategy based on deletions of adenovirus Virus-Associated (VA) RNAs. In normal cells, these RNAs are necessary for virus replication because they inactivate the RNA-dependent protein kinase protein kinase R, a kinase that otherwise would block protein translation in response to infection. However, downstream effectors of Ras can also inactivate protein kinase R, and therefore, the need for VA RNA genes should be bypassed in cells with an active Ras pathway. We demonstrate here that a VAI RNA mutant presents a Ras-dependent replication and can be used for oncolytic virotherapy of pancreatic tumors.

INTRODUCTION

Viral therapy of cancer is a long established concept that is engaging renewed attention. The mechanisms that confer a natural oncotropism to certain viruses are being elucidated, and other viruses are engineered to display such a selectivity. One reason behind this trend is the modest clinical benefit of cancer gene therapy, thwarted by the difficulty to reach all of the cells within a tumor. Oncolytic viruses retain their replication capabilities, amplifying the input dose and helping to spread the virus to adjacent cells.

A common central pathway that confers tumor selectivity to viruses is the IFN pathway (1). On binding to their receptors, α, β, and γ IFNs engage a signal transduction cascade that results in the translocation of Stat1 to the nucleus where it activates the transcription of IFN-stimulated genes. One of these genes encodes for PKR,[2] the double-stranded RNA-activated serine/threonine protein kinase (2), which is central to the antiviral role of IFN. Viral dsRNAs can bind to two PKR molecules, leading to intermolecular transphosphorylation and activation of its kinase activity (3). Activated PKR phosphorylates the α subunit of eIF-2α, leading to the inhibition of protein synthesis and viral replication. As a mechanism of defense against this host antiviral response, most viruses have evolved diverse PKR inhibitory functions to counteract viral replication restrictions and become refractory to IFN-mediated blockage (1). Among these strategies is the production of RNAs, such as adenovirus VA RNAs, that bind to PKR acting as dsRNA antagonists.

Defects in the IFN pathway are common in tumor cells, which are accompanied by growth and survival benefits (4). Viruses that are readily blocked by IFN find themselves protected in such cells. This is the case of the avian paramyxovirus Newcastle disease virus (5) and vesicular stomatitis virus (6). Nonetheless, in tumor cells that have a certain level of PKR before IFN priming or a partially defective IFN pathway, viral yields are reduced through PKR activation. It is known, however, that downstream effectors of Ras can revert PKR activation (7). Viruses that cannot revert PRK activation by themselves become in this way dependent on an active Ras pathway. Such is the case of reovirus, which lacks effective PKR counteracting mechanisms (8). A virus can also be rendered Ras dependent if its PKR-inactivating functions are deleted. This has been recently achieved mutating the influenza A virus NS1 protein, which sequesters dsRNAs (9). A similar effect has been achieved mutating the ICP34.5 protein of Herpes Simplex Virus, a protein that activates the PKR phosphatase PP1 and redirects its activity to dephosphorylate eIF-2α (10).

In the present study, we apply this strategy to achieve Ras-dependent replication with adenovirus. Adenovirus is a major player in current virotherapy efforts (11). Tumor cells and cells infected with adenoviruses share many traits with respect to signal transduction pathways, cell cycle regulation, and apoptosis inhibition. These similarities have facilitated the design of tumor selective adenoviruses. Adenoviruses that cannot block p53 or Rb are leading examples (12–14). Here, we take advantage of the common function of Ras and adenovirus VAI RNA to inactivate PKR. Adenovirus infection leads to production of dsRNA as a result of bidirectional transcription of the genome, thus activating PKR. However, adenovirus prevents this activation using small RNAs that bind to PKR but do not result in activation (15). These VA RNAs are produced in the late phase of the viral cycle from independent RNA polymerase III promoters. Adenovirus type 5 contains two virus-associated RNA genes, VAI and VAII. VAI accumulates to higher levels and has more PKR blocking activity than VAII. Based on the observation that viruses that are unable to counteract PKR activation replicate to higher levels when PKR is inactivated by Ras, we hypothesized that a VAI mutant adenovirus should show such an oncotropic trait. We demonstrate here that the activation of the Ras pathway allows the normal replication of an otherwise defective VAI mutant adenovirus and that these mutants can be used to treat tumors with an active Ras pathway.

MATERIALS AND METHODS

Cells and Virus. HEK293, and human pancreatic tumor cell lines BxPC-3 (wild-type Ras) and Panc-1 (mutant Ras), were obtained from the ATCC (Manassas, VA) and grown in DMEM containing 5% FBS. Cells were transfected using the calcium phosphate precipitation with 24 μg of vector or pCMV-RasV12/pCMV-RasN17 (Clontech, Palo Alto, CA). NP-18 (wild-type ras) and NP-9, NP-31, NP-29 (mutated K-ras) human pancreatic tumor cell lines were established in our laboratory from perpetuated xenografts orthotopically implanted in nude mice (16). Human fibroblasts were kindly provided by Antonio Tugores (Almirall-Prodesfarma, Barcelona, Spain). Primary cultures of human hepatocytes were obtained using a two-step perfusion technique and seeded on collagen-coated plates (17). Wild-type Ad5 (H5*wt*300) was obtained from ATCC, and mutant dl331 was a kind gift from Dr. T. Shenk. It has been described previously (18). It contains the dl330 deletion (29-bp deletion at 29.5 map units within VAI RNA-coding region), which eliminates the block B from the intragenic promoter sequence of VAI precluding VAI RNA synthesis, and it also lacks the 79–84 map unit *Xba*I D fragment (324 deletion, dl324).

Immunoblot Analysis. Control and infected cells were lysed at indicated times with lysis buffer [10 mM Tris–HCl (pH 7.4), 400 mM NaCl, 5 mM NaF, 10% glycerol, 5 mM EDTA, 0,5% Nonidet, 1 mM sodium orthovanadate, 4 mM DTT, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10

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[2] The abbreviations used are: PKR, protein kinase R; dsRNA, double-stranded RNA; eIF, eukariotic initiation factor; ATCC, American Type Culture Collection; VA, virus-associated; FTI, Farnesyltransferase inhibitor; Rb, retinoblastoma; ERK, extracellular signal-regulated kinase.

μg/ml aprotinin]. For PKR phosphorylation status analysis, aliquots were immunoblotted with a mouse anti-total PKR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or immunoprecipitated with the same antibody followed by immunodetection with an anti-phospho-PRK (Biosource, Camarillo, CA). Analysis of ERK activation was performed by probing blots with mouse anti-total ERK1/2 (Zymed, San Francisco, CA) or anti-phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA) antibodies.

Viral Yield Assay and *in Vitro* CPE. For viral replication assays, 293 cells were growth to 80% confluence and subsequently infected at an m.o.i. of 3 with either dl331 or Adwt. At indicated times after infection, supernatants were collected and subjected to titer determination on HEK 293 cells. Pancreatic tumor cells lines were infected at the same m.o.i., and 3 days postinfection, both cells and supernatants were collected and subjected to three cycles of freezing and thawing. Cell lysates were titered on HEK 293 cells.

For CPE assays, cells were seeded in 0.5% FBS and infected 24 h later at doses ranging from 50,000 to 0.001 vp/cell. At day 5 postinfection, plates were stained for total protein content (BCA assay; Pierce, Rockford, IL), and the absorbance was quantified. Viral particles per cell that produced 50% growth inhibition (IC_{50}s) were estimated from dose-response curves by standard nonlinear regression (GraFit; Erithacus Software Ltd., Surrey, United Kingdom) using an adapted Hill equation.

Suppression of Tumor Growth *in Vivo*. s.c. NP-9, NP-18, and BxPC3 tumors were established by injection of 12.5×10^6 cells into the flanks of 6–8-week-old male nude mice. Animals were kept and manipulated in accordance with recommendations from the Federation of European Laboratory Animal Science Associations for the proper use of laboratory animals. Once tumors had reached 100–200 mm^3, mice were randomized ($n = 8$–10), and tumors were directly injected with 10^9 vp of Adwt, dl331, or vehicle (PBS). Tumor size was determined twice weekly and compared for statistical significance using the Student's unpaired *t* test.

Adenovirus Hexon Immunodetection. Immunofluorescence was performed using OCT-embedded tumor sections obtained at day 21 after virus administration. NP-9 tumor sections were treated with mouse anti-hexon (2Hx-2 hybridoma; ATCC) and Alexa Fluor 488-labeled goat antimouse (Molecular Probes, Eugene, OR) antibodies and counterstained with 4′,6-diamidino-2-phenylindole. The slides were analyzed under a fluorescent microscope (Olympus BX51).

RESULTS

Replication of VAI Mutant Adenovirus dl331 Is Sensitive to PKR Activation. H5dl331 (dl331) mutant adenovirus contains a 29-bp deletion within the intragenic control region of the VAI RNA gene (map unit 30 of Ad5) and fails to synthesize this PKR-inactivating RNA (18). In the absence of VAI, activated PKR phosphorylates initiation factor eIF2 and blocks protein translation. It is expected that, in contrast to wild-type Ad, dl331 will not prevent PKR activation, and virus production will be impaired. We used 293 cells to check this hypothesis and validate dl331. 293 cells were infected with 3 m.o.i. of Adwt (Ad5wt300) or dl331, and cell extracts were obtained 24 h after infection. Total PKR was immunoprecipitated, and PKR activation was detected by Western blot with an anti-PhosphoPKR antibody. Infection with Adwt or dl331 did not change total PKR levels, demonstrating that PKR is not regulated at the level of protein synthesis. In contrast, PKR phosphorylation increased after dl331 infection, an effect not observed with Adwt (Fig. 1*A*). When virus production was measured, we found that dl331 yield was reduced ≤20-fold compared with Adwt (Fig. 1*B*). These virus production differences correlated with CPEs. Compared with Adwt, the amount of dl331 needed to obtain a 50% reduction in cell viability (IC_{50}) was 20-fold higher (Fig. 1*C*). These results are consistent with a dl331 replication block as a consequence of PKR activation.

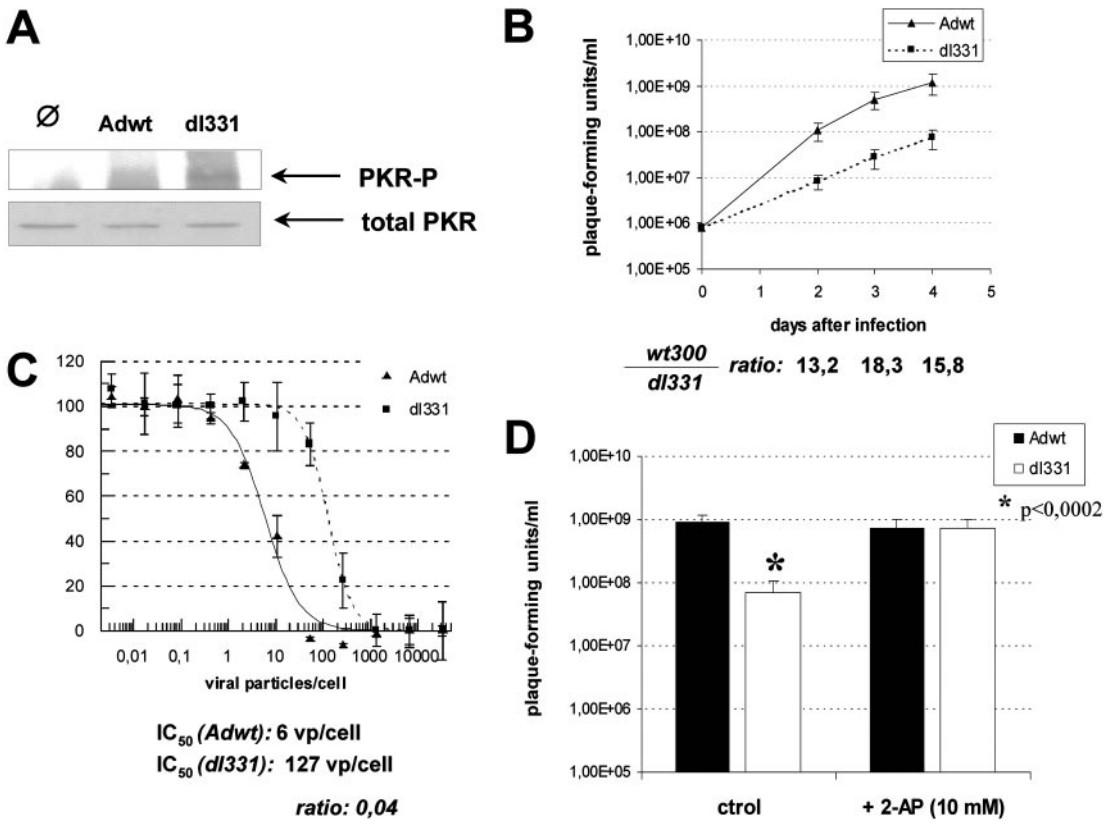

Fig. 1. Dependence of VAI-mutant on PKR activation. *A*, phosphorylation status of PKR after exposure to Adwt or dl331. 293 cell lysates were prepared 20 h after infection with 3 m.o.i. of each virus. Aliquots were immunoprecipitated with an anti-total PKR Ab and subjected to immunoblot analysis for phospho-PKR (pT^{451}). *B*, growth kinetics of mutant and wt adenoviruses. 293 cells were infected at 3 m.o.i., and viral yield in the supernatants at the indicated times after infection was measured by plaque assay on 293 cells. *C*, comparative CPE in 293 cells at day 5. Dose-dependent reduction in cell viability after infection with Adwt or dl331 mutant. Viral particles/cell required to cause a 50% cell death (IC_{50}) were deducted from adjusted curves using Grafit software. *D*, effect of PKR inactivation by 2-AP on growth of Adwt or dl331 on 293. Cells were infected as in *B* and exposed to 10 mM 2-AP 2 h after infection. Viral yield in culture supernatants was determined 2 days after infection.

To demonstrate the role of PKR phosphorylation in dl331 inhibition, we blocked PKR phosphorylation with 2-AP, a serine-kinase inhibitor that specifically inhibits PKR autophosphorylation (19). In 2-AP-treated cells, dl331 and Adwt yields were equivalent (Fig. 1*D*). Analysis of cytopathic curves in the presence of 2-AP showed similar IC_{50}s for both viruses (0.3 and 0.5 vp/cell for dl331 and Adwt, respectively). The CPE of both viruses resulted equivalent also when assayed in cells that do not express PKR, such as glioma U118 cells (20). In this cell line, IC_{50}s were 229 and 296 vp/cell for dl331 and Adwt, respectively. Taken together, these results demonstrate the requirement for PKR inactivation to rescue the dl331 VAI mutation.

An Active Ras Pathway Complements the VAI Mutation. Since activated Ras has been demonstrated to induce the expression of an inhibitor of PKR activation (7), we studied the dependence of the VAI mutant dl331 replication on activated Ras. We transfected subconfluent cultures of 293 cells with plasmids expressing a constitutive active (H-ras V12) or a dominant negative (H-ras N17) form of H-ras. Western blot analysis of the α-phosphorylated form of Erk, a downstream effector of Ras, revealed that these transfections modulated Ras activity in 293 cells (Fig. 2*A*). Activation of the Ras pathway with H-ras V12 rescued dl331 replication to the level of Adwt. Although the ratio of dl331 to Adwt virus yields was close to 0.1 in control mock-transfected 293 cells, in H-ras V12 cells, this ratio was 2.5 (Fig. 2*A*). In contrast, on Ras inhibition by H-ras N17 transfection, dl331 replication was further repressed, and virus yield of dl331 relative to Adwt was 0.0625.

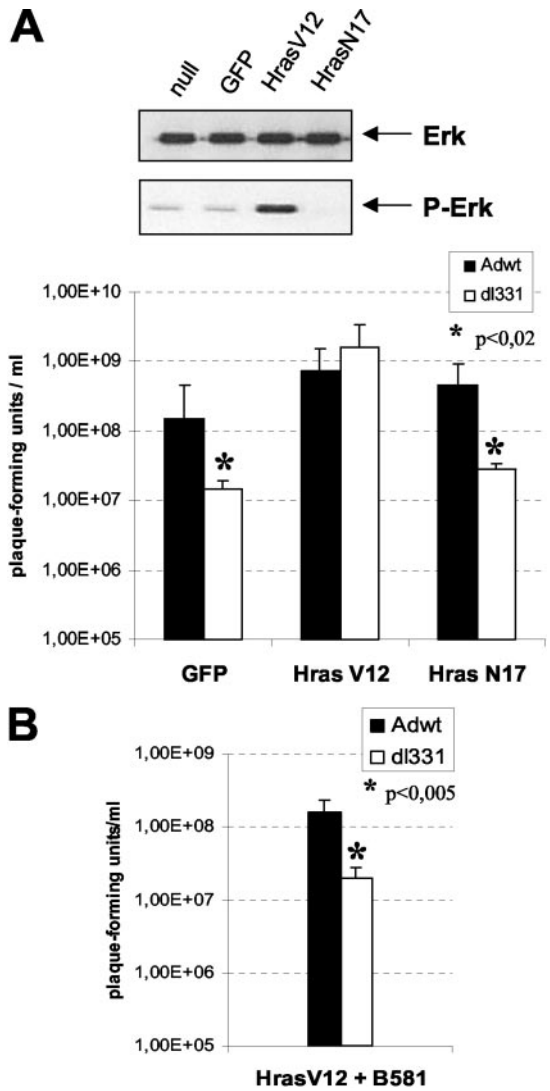

Fig. 2. Effect of activated Ras pathway on VAI-mutant replication. *A*, transfection of 293 cells with mutant H-ras forms. Cells were transfected with 4 μg of each plasmid using a standard calcium precipitate technique. Total Erk and phospho-Erk protein levels were determined 96 h after transfection and 24 h after serum deprivation. Nondeprived cultures were infected 72 h after transfection with Adwt or dl331 mutant, and viral yield was determined from supernatants 96 h postinfection by plaque assay on 293. *B*, inhibition of Ras^{Val12}-activated pathway by B581 reverses dl331 growth. Activated H-Ras^{Val12}-transfected 293 cells infected with Adwt or dl331 were exposed 2 h after infection to the FTI B581 (final concentration 50 μM). Culture supernatants were collected 2 days after infection, and viral yield was determined.

Table 1 *Cytopathic effect of dl331 and Adwt in different mutant pancreatic tumor cell lines*

Cell line	K-*ras* status	IC_{50} (vp/cell) Adwt	IC_{50} (vp/cell) *dl331*	ratio dl331/Adwt
NP-9	GGT[a] → GAT (1:0)[b]	165	290	1.77
NP-31	GGT → GAT (1:1)	60	30	0.5
NP-29	GGT → TGT (1:1)	895	1140	1.26
Panc-1	GGT → GAT (1:1)	352	212	0.57

[a] GGT, glycine; GAT, aspartic acid; TGT, cystein.
[b] 1:0 indicates mutation in homozygosis, whereas 1:1 indicates that cells contain a wild-type allele.

FTIs inhibit the function of oncogenic Ras. In particular, the CAAX peptidomimetic compound B581 (FTI-1) has been demonstrated to be active especially in H-ras-transformed cell lines, because this Ras form is the most dependent on farnesylation (21). In 293 cells transfected with H-Ras V12, where dl331 shows no deficiency, incubation with B581 after infection reduced 10-fold the dl331 to Adwt relative virus yields (Fig. 2*B*). Therefore, blocking Ras in cells with an active Ras pathway eliminates the permissiveness for dl331 replication.

VAI Mutant Production in Human Pancreatic Tumor Cells and Human Normal Cells. Our results show that activation of Ras signaling pathway is able to rescue the VAI mutation. Thus, this mutation should confer adenovirus replication selectivity in Ras-activated tumor cells. To test this hypothesis, we used pancreatic cancer cell lines with different status of Ras: (*a*) NP-9 cells with both alleles of K-ras gene mutated; (*b*) NP29, Panc-1, and NP31 with K-ras mutation in one allele; and (*c*) NP-18 and BxPC-3 that are wild-type ras (Table 1). As shown in Fig. 3*A*, NP-18 and BxPC-3 cells retain the ability to activate the Ras pathway when stimulated with serum. In contrast, NP-9, NP29, and NP31 cells have an activated Ras pathway that is not modulated after serum stimulation.

Pancreatic cell lines present a low level of expression of CAR, and consequently, they are difficult to infect (Ref. 22; *e.g.*, 50% transduction was achieved at 100 m.o.i. in NP9 cells compared with 1 m.o.i. for 293 cells). To normalize for infectivity differences, dl331 and Adwt were compared within each cell line. In NP-18 and BxPC-3 (wild-type Ras), the amount of dl331 produced was 18.8- and 14.1-fold lower than that of Adwt (ratio dl331 to Adwt 0.053 and 0.07), whereas in NP-29, NP-9, and NP-31 (mutated K-ras), the proportion of dl331 to Adwt was, respectively, 1, 0.55, and 0.33 (Fig. 3*A*). When PKR phosphorylation status was analyzed in NP-31 cell line, no increment in phospho-PKR was detected after dl331 infection, nor after infection with Adwt (Fig. 3*C*), indicating that in this tumor cell line, the VA1 mutation is also complemented, and PKR is unable to block adenovirus replication.

We compared the relative cytotoxicity of both viruses in four human pancreatic tumor cell lines containing K-ras mutations (NP-9, NP-31, NP-29, and Panc-1). Equivalent IC_{50}s were obtained for all cell lines tested, with dl331 to Adwt ratios close to 1 (Table 1). To further demonstrate the selectivity of dl331, we measured virus production in normal human fibroblasts and hepatocytes (Table 2). In fibroblasts, dl331 produced 15-fold less virus than Adwt. In hepato-

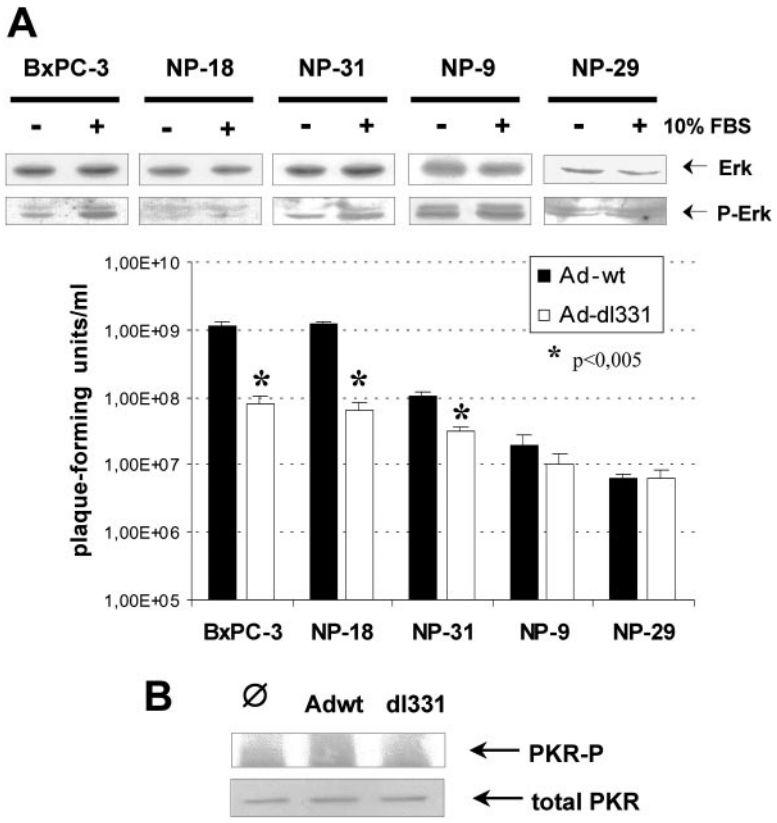

Fig. 3. Ras-activated human tumor cells are permissive to VAI-mutant. *A*, replication of dl331 mutant in human pancreatic tumor cell lines correlates with its Ras-pathway status. Status of activation of Ras pathway in different human pancreatic cells lines was characterized based on their ability to phosphorylate Erk1/2 when exposed to serum for 30 min after serum deprivation. Tumor cell lines were infected with Adwt or dl331 mutant at 1500 vp/cell. Three days after infection, both cells and supernatant were collected, and viral yield was determined by plaque assay on 293 cells. In *B*, Dl331 doesn't activate PKR in a Ras-mutated tumor cell. Phosphorylation status of PKR after exposure to Adwt or dl331 was determined in cell lysates of NP-31 human pancreatic tumor cells prepared 20 h after infection as in 1*A*.

cytes, which are more permissive to adenoviruses than fibroblasts, a 75-fold attenuation was observed.

Efficacy of *VAI* Mutant against Pancreatic Tumors *in Vivo*. We subsequently assessed the antitumoral potency of Ad VAI dl331 mutant in a model of s.c. Ras-activated tumors. We chose to implant NP9 cells as a tumor model because they form tumors that are very refractory to chemotherapy treatment, mimicking the traits of pancreatic cancer (23). These cells also express very low levels of adenovirus receptor CAR as found in most human pancreatic tumors (24, 25). NP-9 tumor xenografts in nude mice were injected with a single dose of 1.10^9 viral particles of dl331 or Adwt or with PBS. Tumors decreased in tumor size from day 12 after injection in both dl331 and Adwt groups. At day 21, dl331-mediated oncolytic effect was as marked as with Adwt; tumors were, respectively, 38.4 and 39.7% smaller than the control vehicle-treated tumors ($P < 0.02$ for both dl331 and Adwt with respect to PBS; Fig. 4*A*). Of note, dl331 has the same antitumoral activity as Adwt, used as a control of Ras-independent oncolysis. This indicates that dl331 mutation is complemented in Ras-activated tumors *in vivo*.

Intratumoral viral replication was demonstrated by immunofluorescence against adenovirus hexon. The presence of positive hexon staining in dl331-treated tumors at day 21 after treatment indicates virus replication within tumor cells (Fig. 4*B*).

The effect of dl331 in pancreatic tumors with wild-type ras (BxPC3 and NP18) was also studied (Fig. 5). In both tumor models, dl331 was less oncolytic than Adwt, confirming the defective replication in cells with wild-type ras as observed *in vitro* (Fig. 3). However, although dl331 showed no oncolytic effects in BxPC3-derived tumors, some oncolytic effects were observed in NP18-derived tumors. As virus production is similar *in vitro* (Fig. 4), different growth properties of these tumors could account for the *in vivo* heterogeneity.

Table 2 *Viral yield of dl331 and Adwt in normal human cells*

	Human hepatocytes		Human fibroblasts	
Vector	Mean[a] (pfu/ml)	SD	Mean (pfu/ml)	SD
Ad-wt	$1{,}9.10^7$	$6{,}0.10^6$	$1{,}8.10^7$	$3{,}3.10^6$
dl331	$2{,}5.10^{5b}$	$5{,}7.10^4$	$1{,}1.10^{6b}$	$2{,}3.10^5$
ratio dl331/Ad-wt	0,013		0,06	

[a] Mean yield values for the indicated virus determined from cell lysates obtained at day 4 postinfection by plaque assay.
[b] $P < 0.005$ *versus* Ad-wt yield by Student's *t* test.

DISCUSSION

We propose the use of an adenovirus 5 mutant that contains a 29-bp deletion in the coding region of VAI gene (dl331 mutant) as a conditionally replicative oncolytic adenovirus for Ras-activated tumors. The selectivity of this virus stems from: (*a*) the inability of dl331 to block the activation of the double-stranded RNA-activated protein kinase (PKR) and, therefore, to prevent cellular anti-viral response; and (*b*) the demonstration that oncogenic Ras induces an inhibitor of PKR (7). Based on this, it is hypothesized that the need for PKR inactivation can be bypassed in cells with a constitutively active Ras pathway and that dl331 can selectively propagate in these cells.

As hypothesized, VAI mutated virus propagates poorly in cells with a normal Ras pathway, such as 293 cells. The degree of impairment (20-fold) is in agreement with previous results (26, 27), and it has been attributed to an inefficient initiation of protein translation in the late phase of infection. Of note, the growth phenotype of dl331 correlates with an increase in cellular PKR phosphorylation, which was otherwise blocked by Adwt infection. We further demonstrate the dependence of dl331 mutant on PKR activation, adding a PKR inhibitor, 2-AP. This drug is a specific inhibitor of PKR autophosphoryl-

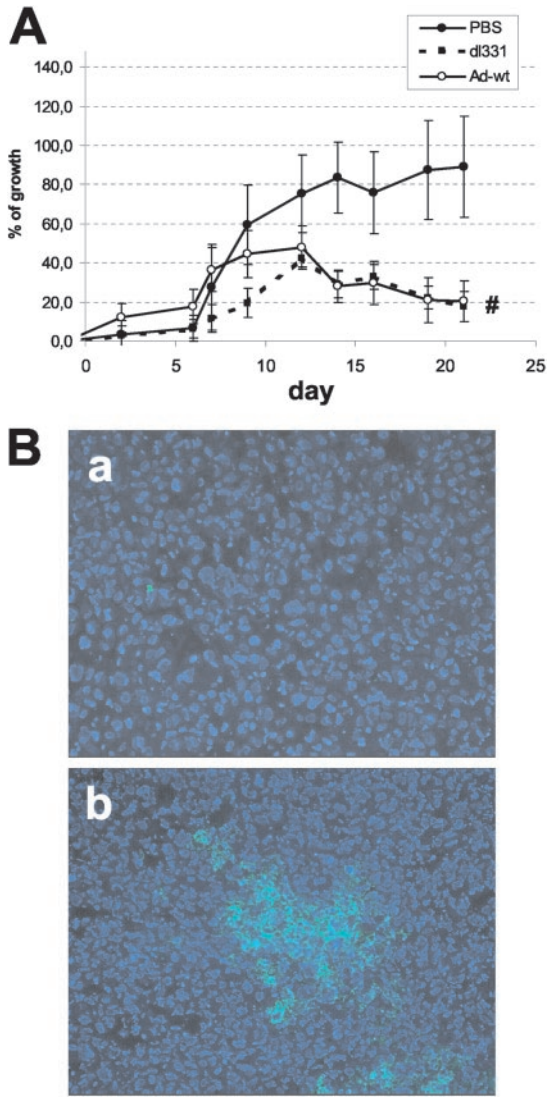

Fig. 4. VAI-mutant is effective in a xenograft tumor model of *ras*-mutated pancreatic cancer. In *A*, NP-9 xenografts in nude mice were treated with a single i.t. injection of 1.10^9 viral particles of Adwt (○) or dl331 mutant (■) or with PBS alone (●), $n = 8–10$ mice/group. Percentage of growth ± SE is plotted. Both viruses show a similar oncolytic activity than results in smaller tumors compared with PBS-treated group (#, $P < 0,02$). *B*, adenovirus intratumoral replication assessed by hexon detection in tumor xenografts. Frozen sections of tumors injected with PBS (*a*) or dl331-mutant (*b*) were treated with an antihexon antibody and counterstained with 4′,6-diamidino-2-phenylindole (×100). Sections taken from tumors treated with dl331 were positive for adenovirus presence (*green dots* in *b*).

ation and has been used previously to demonstrate Ras selectivity for different viruses, such as reovirus (8–10). Treatment with 2-AP restores dl331 viral yields and virus-induced cytotoxicity to the levels of Adwt, suggesting that the VAI gene mutation is responsible for the dl331 phenotype. Furthermore, a direct relation of ectopic activation and dl331 mutant titers was observed. It is worth mentioning that the mutant is produced to levels equal or higher than Adwt when an oncogenic form of H-Ras (Hras-V12) is expressed, suggesting that this selectivity strategy does not compromise oncolytic potency. These data strongly support the role of Ras pathway in negatively regulating endogenous PKR activity and validate Ras-activated cells as permissive cells for dl331 mutant. In contrast, dl331 is attenuated ≤75-fold in normal human cells, such as hepatocytes.

The same correlation between viral yield and Ras status was found in different human pancreatic tumor cells fostering the possibility to use VA mutants for the treatment of tumors with an active Ras pathway. The potency of dl331 oncolysis is also encouraging in view of the limited efficacy of nonreplicative adenoviruses as gene delivery vectors when applied to the same pancreatic tumor models (22, 23, 28). Dl331 shows also ras dependency *in vivo*. This mutant demonstrated to have antitumoral efficacy *in vivo* when administered intratumorally in xenografts of Ras-activated pancreatic cancer cells with a potency similar to Adwt. Moreover, we were able to detect dl331 intratumoral propagation, which is in contrast to other approaches using conditionally replicative adenoviruses that are known to be less potent than Adwt in pancreatic tumors (29).

It has been described that pancreatic tumors are refractory to Ad infection because of a lack of the primary Ad receptor, CAR (24). However, integrins, used as a secondary receptor for internalization, are expressed, especially those of $\alpha_v\beta_5$ type. The cell line-dependent differences in infectivity have not been directly addressed in this study. Instead, an Adwt control has been included as a reference of virus production and cytotoxicity. In these conditions, our results show that dl331 has reduced virus production and cytotoxicity in pancreatic tumor cells compared with 293. Several approaches can be envisioned to bypass CAR deficiency and increase the propagation and oncolytic efficacy of dl331 in ras-activated pancreatic tumor cells, such as the insertion of an RGD integrin-binding motif into the fiber. Previously, we have demonstrated that this genetic modification al-

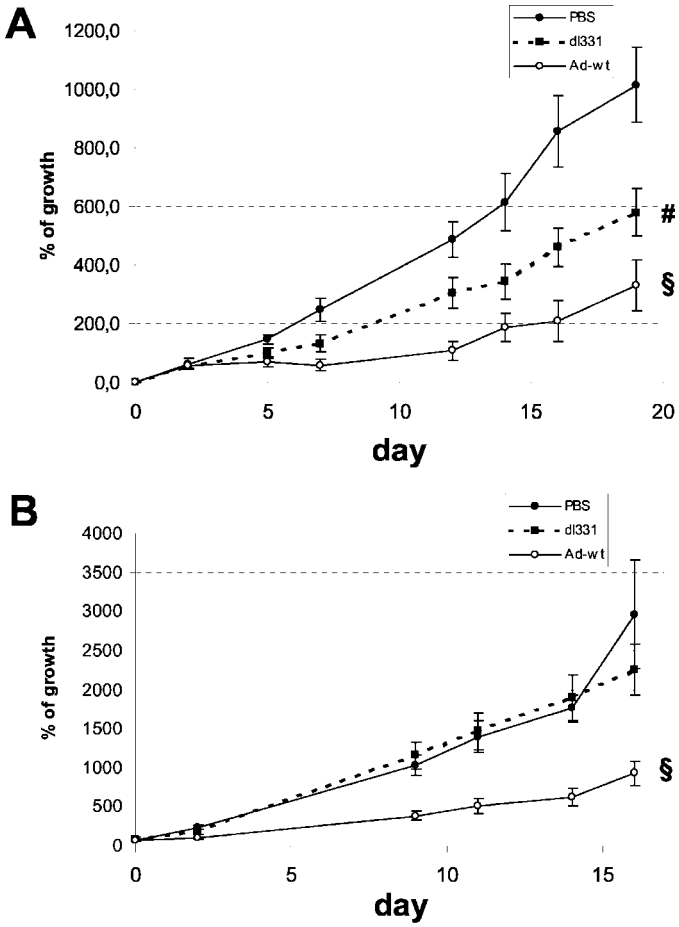

Fig. 5. VAI-mutant effectiveness is impaired in xenograft tumor models of wild-type ras pancreatic cancer. NP-18 (*A*) and BxPC-3 (*B*) xenografts in nude mice were treated with a single i.t. injection of 1.10^9 viral particles of Adwt (○) or dl331 mutant (■) or with PBS alone (●), $n = 8–10$ mice/group. Percentage of growth ± SE is plotted. In these wt ras tumor models, dl331 demonstrated to have an oncolytic activity significantly different from Adwt, that in both cases results in smaller tumors compared with PBS-treated group (§, $P < 0,05$ compared with PBS and dl331-injected tumors; #, $P < 0.05$ compared with PBS-injected tumors).

lows for CAR-independent Ad entry and increases the potency of oncolytic vectors (30). In fact, the oncolytic vector AdΔ24RGD, with a mutation that impairs E1a binding to Rb and with RGD in the HI loop of the fiber, is currently one of the most potent conditionally replicating adenoviruses (30–32).

In addition to VAI, adenovirus expresses VAII to inhibit IFN antiviral response. The role of VAII in PKR inactivation becomes evident in the absence of VAI, when transcription of VAII gene increases by 12-fold (26). In fact, the double deletion of VAI and VAII further reduces virus replication to 1 of 60 with respect to Adwt (26). However, the significant Ras dependence demonstrated with the single VAI mutant indicates that VAII activity does not block PKR to a level sufficient to replace the blocking function of Ras. Conversely, it remains to be studied whether the PKR inactivation mediated by the Ras pathway will be efficient enough to complement the double VAI and VAII defects. We are currently evaluating if double VAI-VAII mutants are not defective in cells with an active Ras pathway.

Previously, we have designed mutations to obtain tumor selective replication based on alterations of the Rb pathway (13). Because this pathway is affected in 100% of tumors, those CRAds may have a broad application. In contrast, Ras is found mutated in 30% of all tumors, pancreatic cancer being the tumor type with the highest incidence of Ras mutations (90%), followed by colon (40%), thyroid (50%), and lung adenocarcinomas (30%). Pancreatic cancer is then an attractive candidate to apply this strategy, specially given the poor response observed with the current therapies (33). However, the selectivity of this strategy is not restricted to tumors with *ras* mutations. Alteration of upstream elements in the Ras pathway, such as by overexpression of epidermal growth factor and platelet-derived growth factor receptors, can also activate this pathway even in the absence of *ras* mutations. Altogether, it is accepted that 80% of all tumors have this pathway activated (34). In addition, defects in the IFN pathway can also lead to loss of activation of the PKR function, allowing replication of dl331. Deletions in several IFN regulatory factors as well as alterations in ISGF3 components have been associated with the development of tumors such as melanoma, leukemia, and lymphomas (35).

The increasing knowledge of the adenoviral genome and its interaction between viral and cellular components has promoted the broad use of adenoviruses to generate oncolytic vectors; some of them are currently in clinical trials. The strategy presented here opens a new way of achieving tumor-specific replication based on the differential requirement of PKR control for adenoviruses mutated in VA RNA genes. Prospects to further improve the selectivity for an active Ras pathway could include double VAI-VAII mutations or the addition of Ras-specific RNA stabilization signals[3] and Ras-activated promoters to control the expression of key viral genes. The VA RNA mutation approach can also be combined with other described strategies to achieve selective replication based on promoter regulation of viral genes or deletions of Rb and p53-binding sites. In addition, strategies aimed to increase potency, such as expression of cytotoxic genes, can also be considered.

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