Antiviral action of the tumor suppressor ARF

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Oncogenic viruses frequently target the pathways controlled by tumor suppressor genes, suggesting an extra function for these proteins as antiviral factors. The control exerted by the tumor suppressor *Arf* on cellular proliferation is crucial to restrict tumor development; however, a potential contribution of *Arf* to prevent viral infectivity has remained unexplored. In the present study, we investigated the consequences of loss or increased expression of *Arf* on viral infection. Our results reveal that ARF expression is induced by interferon and after viral infection. Furthermore, we show that ARF protects against viral infection in a gene dosage-dependent manner, and that this antiviral action is mediated in part by PKR through a mechanism that involves ARF-induced release of PKR from nucleophosmin complexes. Finally, *Arf*-null mice were hypersensitive to viral infection compared to wild-type mice. Together, our results reveal a novel and unexpected role for the tumor suppressor ARF in viral infection surveillance.

The EMBO Journal (2006) 25, 4284–4292. doi:10.1038/sj.emboj.7601302; Published online 7 September 2006
Subject Categories: cell cycle; microbiology & pathogens
Keywords: antiviral activity; IFN; ARF; NPM; PKR

Introduction

Tumor suppressor genes, the sensors of multiple forms of cellular stress, are regulated to induce cell-cycle arrest, senescence or apoptosis. Viral infection induces interferon (IFN) synthesis, which stimulates the expression of hundreds of genes that can be denominated as viral-stress-inducible genes. Some viruses, while trying to evade this cellular response encode for proteins that inhibit tumor suppressor pathways and in this manner contribute to tumor formation, whereas other viruses succumb to cellular defense mechanisms. It has been speculated that these tumor suppressors

may form part of inducible antiviral response mechanisms (Reich *et al*, 1988; Hawkins and Vaux, 1994; Moore *et al*, 1996; Neil *et al*, 1997; Weinberg, 1997). The activation of some of these tumor suppressors by IFN treatment (Mecchia *et al*, 2000; Takaoka *et al*, 2003), a factor with potent antioncogenic and antiviral activities, strengthens this speculation. In particular, the activation of the tumor suppressor genes *Pml* and *p53* by IFN has been demonstrated (Lavau *et al*, 1995; Grotzinger *et al*, 1996; Borden, 2002; Takaoka *et al*, 2003) and a role for these tumor suppressors in the antiviral defense of the cell has been established (Chelbi-Alix *et al*, 1998; Takaoka *et al*, 2003; Munoz-Fontela *et al*, 2005). An induction of *Arf* by both viral infection and IFN treatment has been reported (Ries *et al*, 2000; Sandoval *et al*, 2004). However, a putative role for *Arf* in the prevention of virus infection has not been proven yet.

The tumor suppressor *Arf* (encoding p14ARF in humans and p19ARF in mice) is among the most important oncogenic stress sensors in mammalian cells, being often mutated in cancer (Ruas and Peters, 1998; Sharpless and DePinho, 1999). ARF localizes mainly in nucleoli where it interacts with MDM2 (Pomerantz *et al*, 1998; Zhang *et al*, 1998), inhibiting its E3 ligase activity (Honda and Yasuda, 1999), and as a consequence stabilizes and activates p53. In addition, ARF interacts with nucleophosmin (NPM) (Bertwistle *et al*, 2004; Brady *et al*, 2004), a protein frequently over-expressed in a variety of human malignancies (Itahana *et al*, 2003).

In this report, we have analyzed the role of *Arf* as a sensor of viral stress. We demonstrate the induction of the tumor suppressor *Arf* in primary mouse embryo fibroblasts (MEFs) after vesicular stomatitis virus (VSV) infection. In addition, we show that *Arf* is a critical component of the antiviral response, protecting the cell and the animals against virus infection. The protective effect of *Arf* is exerted, at least in part, through the activation of the double-stranded RNA-dependent protein kinase, PKR, and is mediated by interaction with NPM.

Results

Arf protects against viral infection in a gene dosage-dependent manner

To address directly a putative contribution of *Arf* to protection against viral infection, we analyzed the cytopathic effect (CPE) caused by VSV infection of MEFs derived from animals with different *Arf* gene dosages. We took advantage of a novel mouse model with a transgenic gene dose of *Ink4a/Arf* (Matheu *et al*, 2004). ‘Super *Ink4a/Arf*’ mice, carrying the endogenous allele plus the transgenic allele, manifest a higher resistance to cancer compared to normal, nontransgenic mice. Moreover, cells derived from ‘super *Ink4a/Arf*’ mice present an increased resistance to *in vitro* immortalization and oncogenic transformation, demonstrating that a modest increase in the ARF tumor suppressor results in a beneficial, antitumoral activity (Matheu *et al*, 2004). MEFs from *Ink4a/*

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Received: 10 June 2006; accepted: 27 July 2006; published online: 7 September 2006

Arf-null, wild-type or 'super *Ink4a/Arf*' mice were infected with increasing amounts of VSV and cell viability was quantified, as a measure of virally triggered CPE. As shown in Figure 1A, reduced VSV-induced cytolysis was detected with increasing gene dosages of *Arf*. The cells derived from the *Ink4a/Arf*-null mice were approximately 10 times more sensitive to be destroyed by the virus than the wild-type cells, whereas cells derived from the transgenic 'super *Ink4a/Arf*' animals showed around 100 times increased viability. This result correlated well with the amounts of viral progeny as measured by titration of the supernatants from VSV-infected cultures (MOI of 10) after all cells had died as a result of the infection (Figure 1B). Viral production in wild-type cells was around one log lower than in *Ink4a/Arf*-deficient cells and more than one log higher than in 'super *Ink4a/Arf*' cells. Importantly, *Arf*-null cells (lacking *Arf* and retaining *Ink4a*) showed a similar resistance to viral infection than the doubly deficient *Ink4a/Arf* cells in both experiments, thus pointing to *Arf* as responsible for the observed antiviral effect. Further supporting this notion, MEFs derived from the *Ink4a*-null mouse (lacking *Ink4a* but retaining *Arf*) showed a similar infectivity and viral production than wild-type cells (Supplementary Figure S1A).

To extend the above results to other cell types, we decided to make use of a human osteosarcoma-derived cell line with IPTG-inducible *Arf* expression (NARF2) that has been previously described (Stott *et al*, 1998). Addition of IPTG to the cell medium resulted in expression of ARF (see for example Figure 3C) with concomitant protection against VSV infection as measured by a cell viability assay (Figure 1C, upper panel). This effect is not attributable to the IPTG treatment, as IPTG has no effect *per se* on VSV infection (Supplementary Figure S1B).

Recently, an antiviral action of p53 mediating the IFN-induced response has been described (Takaoka *et al*, 2003; Munoz-Fontela *et al*, 2005). This p53-mediated protection against viral infection is achieved through increased induction of apoptosis and a resulting decreased viral yield. In our case, we observed *Arf*-mediated increased protection against viral infection through reduced CPE (as deduced from the increased cell viability) and viral progeny reduction, thus

suggesting that the antiviral activities of ARF and p53 are mechanistically different. To examine the potential contribution of p53 in the observed antiviral action, NARF-E6 cells, expressing papillomavirus oncoprotein E6 to block p53 activity (Stott *et al*, 1998), were used in an analogous manner. Importantly, IPTG-induced expression of ARF (Figure 1D) resulted in antiviral protection independently of p53 status (Figure 1C, lower panel). In agreement, p53 protein levels were induced to a similar extent by VSV infection in both wild-type and *Arf*-null cells, as shown by Western blot

Figure 1 Inhibition of VSV infection by *Arf*. (A) Resistance to VSV-induced cytolysis by *Arf* MEFs derived from doubly deficient *Ink4a/Arf*-null (square), *Arf*-null (circle), wild-type (triangle) or transgenic 'super *Ink4a/Arf*' (rhomb) mice were infected in triplicate with VSV at the indicated MOI, and, 24 h after infection, cell viability was quantified as described in Materials and methods. Indicated values are means of triplicate wells. The standard deviation of the measurements was within 15% in each assay. Results shown were obtained using MEFs derived from two different wild-type or transgenic embryos. The same results were obtained in at least three different experiments. (B) Reduction in the viral yield by *Arf*. Virus yield assay of the supernatant of the indicated MEFs after viability of all the cells subsequent to VSV infection (10 MOI) was lost. Comparison between means was performed using the Student's *t*-test showing significant differences between groups ($P < 0.05$). (C) *Arf* inhibits VSV infection by a p53-independent pathway. NARF2 (upper panel) or NARF-E6 (lower panel) cells incubated in the presence or absence of IPTG were infected with VSV at the indicated MOI and, 24 h after infection, cell viability was determined. (D) NARF-E6 cells were treated with IPTG and the expression of p14ARF is shown. (E) MEFs isolated from *Arf*-null or wild-type mice were infected with VSV at an MOI of 5 PFU/cell and, 6 h after infection, the p53 protein levels were determined.

(Figure 1E). Together, these results point to *Arf* as responsible for the observed increased resistance to VSV infection observed in the cell viability assays and suggest that this occurs through a p53-independent mechanism.

To substantiate the above results, we decided to analyze the antiviral activity of *Arf* using other viruses different from VSV. In particular, we used Sindbis virus, which similar to VSV, is sensitive to IFN β ; and poliovirus, which is insensitive to IFN. We detected *Arf*-mediated protection against Sindbis virus but not against poliovirus (Supplementary Figure S2A and B). Thus, this protection seems to be specific of IFN-sensitive viruses such as VSV or Sindbis virus.

This ARF-induced antiviral protection was not the result of a different rate of proliferation or withdrawal from the cell cycle, as FACS analysis of the cells showed similar cell cycle profiles (Supplementary Figure S3). Moreover, the proliferative capacity of these MEFs has been tested before, with no alteration in their growth rates (Matheu *et al*, 2004). In summary, we have observed a clear protective activity of ARF against viral infection.

***Arf* is induced by viral infection**

Based on the protective role of *Arf* after viral infection, we reasoned that ARF levels could be induced by virus infection. Immunoblot analysis of cell extracts from MEFs infected for 6 h with VSV exhibited increased levels of the protein compared to mock-infected cells (Figure 2A, left panel). A similar increase in ARF expression was also observed after IFN β treatment (Figure 2A, right panel), in agreement with recent observations by others (Sandoval *et al*, 2004). In accordance with the immunoblot data, we verified by quantitative real-time PCR (Q-RT-PCR) an upregulation of *Arf* mRNA levels in wild-type cells as early as 1 h after infection with 5 PFU/cell VSV (Figure 2B). In contrast, *p16Ink4a* levels remained unchanged after viral infection, as measured by immunoblot and Q-RT-PCR (data not shown). To further corroborate these findings, we used a luciferase reporter assay in which the sequence from the promoter of human *Arf* gene (3.4 kb upstream of the ATG start codon) was placed before a luciferase reporter gene. Cells transfected with this construct displayed around two-fold upregulated luciferase expression at 8 h after viral infection (Figure 2C), whereas no significant differences were observed after transfection of a shorter version containing only 700 bp upstream of the ATG codon (data not shown). Inspection of human and mouse *Arf* promoters revealed the presence of consensus sequences for the IFN-stimulated response element (ISRE) and the IFN-regulatory factor 3 (IRF-3) binding domain. IRF-3 is known to bind to a consensus binding motif found in many virus-inducible genes and to participate in the transcriptional induction of IFN α and IFN β genes, as well as of a number of IFN-stimulated genes. A transcriptional activation by IFN β or vaccinia virus (VV) infection through the p19*Arf* gene-derived IRF-3 was verified by using a transient reporter gene assay (Supplementary Figure S4A). In summary, our results demonstrate that *Arf* transcription is induced after virus infection or IFN treatment and that this induction results in elevated levels of the protein.

PKR is involved in the antiviral action of *Arf*

The major cellular mechanism of defense against viral infection is the IFN-mediated antiviral host response, and in

Figure 2 VSV infection induces *Arf*. (A) MEFs were isolated from *Arf*-null or wild-type mice and the p19ARF protein (and actin as a control) levels were determined in noninfected cells or at 6 h after VSV infection at an MOI of 5 PFU/cell (left panel), and after IFN β treatment (right panel). (B) Wild-type MEFs were infected with VSV at an MOI of 5 PFU/cell and, 1 h after infection, total RNA was isolated. After reverse transcription, the samples were amplified by PCR using specific primers for *Arf* and actin, and analyzed. (C) Transactivation of the *Arf* promoter by VSV infection in a reporter assay. HeLa cells transfected with human *Arf* promoter (3.4 kb upstream of the ATG start codon) fused to the luciferase reporter gene were infected with VSV (5 PFU/cell) and, at the indicated times after infection, luciferase expression was determined. The averages and standard deviations of luciferase values from triplicates of a representative experiment are shown. Comparison between means was performed using the Student's *t*-test showing statistically significant differences at time 8 h (*) ($P = 0.0437$).

particular a functional PKR enzyme is required for the attenuation of VSV (Stojdl *et al*, 2000). Metabolic labeling of cells deficient for *Arf* revealed VSV protein synthesis and shut-off of cellular protein synthesis after infection at low MOI (Figure 3A). In contrast, viral proteins or cellular shut-off were not observed after infection at the same MOI as that of wild-type cells or after IFN β treatment of *Arf*-null cells. Thus, the *Arf* protective effect was reminiscent of the actions exerted by an activated PKR. We then tested the possibility that *Arf* protection could be mediated by different levels of IFN production. However, the determination of IFN levels in the supernatants of *Arf*-null and wild-type MEFs revealed no significant differences (Supplementary Figure S4B).

The involvement of PKR in *Arf*-mediated resistance to VSV infection was addressed by overexpressing ARF in immortal MEFs derived from wild-type or *pkr*-deficient mice, and that had lost p53 during immortalization. Whereas ARF overexpression in wild-type cells conferred protection against VSV infection, as expected, *pkr*-null MEFs did not exhibit ARF-mediated increased protection (Figure 3B). To further confirm the contribution of PKR to the *Arf*-mediated protection, we examined the phosphorylation levels of the well-

established downstream PKR target, eIF2 α (Gil *et al*, 1999). After VSV infection, the levels of eIF2 α -P were slightly but consistently higher in wild-type than in *Arf*-null cells (Figure 3C). Similarly, IPTG-treated NARF2 cells (that is, expressing ARF) presented elevated levels of eIF2 α -P after VSV infection compared to nontreated cells (Figure 3C). A similar situation was observed when we examined the phosphorylation levels of a different PKR target, Ikb α (Gil *et al*, 1999), at different times after VSV infection of the wild-type or *Arf*-null MEFs (Figure 3D). In summary, our observations provide evidence for an important contribution of PKR to the *Arf*-mediated protection against VSV infection.

To determine if the effect of *Arf* on the PKR pathway also operates when *Arf* is induced in response to a nonviral stimulus, such as oncogenic Ras (Palmero *et al*, 1998), MEFs derived from the *Arf*-deficient or wild-type mice were transduced with retroviral vectors encoding *H-rasV12* or empty vector and eIF2 α -P levels were determined. Expression of *H-rasV12* induced an increase in the eIF2 α -P levels, only when ARF was present, thus correlating the induction of *Arf* with eIF2 α -P levels (Supplementary Figure S5). Furthermore, activation of *Arf* by *H-rasV12* *in vivo* during DMBA/TPA-induced papilloma formation in mice (Kelly-Spratt *et al*, 2004; Collado *et al*, 2005), a well-established carcinogenic protocol driven by *H-ras* oncogenic mutation (Quintanilla *et al*, 1986), resulted in increased levels of eIF2 α -P (Supplementary Figure S5). Thus, *Arf* has a critical role in the activation of the PKR pathway upon viral and oncogenic stress.

NPM links *Arf* to PKR

ARF and PKR proteins have a common partner, NPM. In particular, ARF induces the relocalization of NPM to the nucleolus (Itahana *et al*, 2003; Brady *et al*, 2004), whereas NPM inhibits the activation of PKR (Pang *et al*, 2003). To assess the contribution of NPM to viral infectivity, we tested whether the protective effect of *Arf* could be recapitulated by knocking down NPM with a specific siRNA. For this, we used p14ARF-deficient human breast cancer MCF-7 cells after retroviral transduction of p19ARF or an anti-NPM siRNA. MCF-7 cells transduced with retroviruses encoding NPM-targeting siRNAs duplexes were protected against VSV infection to a similar extent to that observed after overexpressing ARF (Figure 4A). In agreement with the above, virus yield was reduced in the cell culture supernatants after RNA interference of NPM or after ARF overexpression (Figure 4B). A role for NPM in the resistance to VSV infection

Figure 3 *Arf* enhances the activation of the PKR pathway after VSV infection. (A) Virus-induced protein synthesis shut-off and viral protein synthesis are affected by *Arf* gene dosage. Untreated or IFN β -treated (500 U/ml) MEFs were infected (+) or left uninfected (-) with VSV at an MOI of 0.1 PFU/ml and, after 8 h, levels of protein synthesis were determined by [³⁵S]methionine labeling for 30 min and SDS-PAGE analysis. Synthesis of viral proteins is indicated in the autoradiogram by the arrows on the left. (B) Increased resistance to VSV infection after ARF expression requires PKR. Wild-type (upper panel) or *pkc*-deficient MEFs (lower panel) were transduced with retroviruses encoding p14ARF or empty vector and, after selection, cells were infected with VSV at different MOIs. Cell viability was determined 24 h after infection, as described in Materials and methods. (C) Activation of the PKR target eIF2 α (eIF2 α -P levels) is increased in the presence of *Arf*. *Arf*-null or wild-type primary MEFs and NARF2 cells incubated in the presence or absence of IPTG were infected with VSV at an MOI of 5 PFU/cell. At 6 h after infection, cells were lysed and subjected to immunoblot analysis with antibodies recognizing ARF, eIF2 α , eIF2 α -P or actin (as a loading control). The increase in the intensity of the eIF2 α -P bands observed after VSV infection with respect to uninfected cells was calculated by densitometry and expressed in arbitrary units. (D) Activation of the PKR target Ikb α (Ikb α -P levels) is increased in the presence of ARF. Kinetics of Ikb α phosphorylation on serine 32 upon VSV infection in *Arf*-null or wild-type primary MEFs was carried out. Cells were infected with VSV at an MOI of 5 PFU/cell and, at the indicated times after infection, cells were collected, extracts prepared and immunoblot analyses using antibodies that specifically recognize the phosphorylated form of Ikb α on serine 32 or actin were performed.

Figure 4 Increased resistance to VSV infection after ARF expression is mediated by NPM. (A) Inhibition of NPM expression or p19ARF overexpression induces resistance to virus infection in MCF-7 cells. MCF-7 cells transduced with retroviral vectors encoding for p19ARF, siRNA-NPM (iNPM), siRNA-GFP (iGFP) or empty vector (vector) were infected with VSV at the indicated MOI. After 24 h of infection, cell viability was determined. p19ARF, NPM or actin protein levels in the transduced cells are shown. (B) Reduction in the viral yield by ARF overexpression or NPM inhibition. Virus yield assay of the supernatant of the MCF-7 cells transduced with the indicated retroviral vectors after viability of all the cells was lost after VSV infection (5 MOI). (C) *In vivo* interaction between NPM and PKR is disrupted by ARF. Protein extracts obtained after immunoprecipitation against PKR (upper panel) or NPM (bottom panel) from VSV-infected NARF-E6 cells incubated in the presence or absence of IPTG were immunoblotted with antibodies against NPM, PKR or ARF to reveal co-precipitation. (D) Colocalization of PKR with NPM decreases after expression of ARF. NARF-E6 cells, incubated in the presence or absence of IPTG, were infected with VSV at an MOI of 5 PFU/cell and, 16 h after infection, cells were fixed and immunostained for PKR (green) or NPM (red).

was also suggested by the observation that downregulation of NPM levels by serum deprivation in NIH3T3 cells, an effect previously described by others (Chou and Yung, 1995), increased resistance to viral infection (data not shown). In addition, knockdown of NPM or ARF overexpression in MCF7 cells resulted in increased protection against Sindbis virus infection, whereas no effect was observed when poliovirus was used (Supplementary Figure S2C and D), supporting again both the involvement of NPM in the ARF-mediated protection and that this effect can be extended to other viruses.

To confirm our hypothesis of ARF disrupting NPM inhibitory interaction with PKR, we performed an immunoprecipitation assay with antibodies against PKR followed by immunoblot detection of NPM (Figure 4C, upper panel). As expected, an interaction between PKR and NPM was de-

tected. In addition, we observed that ARF expression after IPTG treatment of NARF-E6 cells considerably reduced the amount of NPM detected in the precipitates, thereby demonstrating that ARF disrupts the NPM/PKR complexes. This interaction between NPM and PKR in the absence of ARF was also confirmed after immunoprecipitation using anti-NPM antibody and immunoblot detection of PKR (Figure 4C, lower panel). In addition, incubation with anti-ARF antibody demonstrated the already reported interaction between NPM and ARF (Figure 4C, lower panel). However, an interaction between ARF and PKR was not observed after immunoprecipitation with antibodies against ARF and immunoblot detection using anti-PKR antibody (Supplementary Figure S6A).

Next, we hypothesized that ARF induction by VSV infection might prevent NPM to colocalize with, and thereby inhibit, PKR. To corroborate this, we analyzed by immunostaining

and confocal microscopy the localization of NPM and PKR in the NARF-E6 cells after VSV infection, in the presence or absence of IPTG (that is, after ARF-induced expression or not). Viral infection resulted in NPM re-localization to the nucleoplasm and cytoplasm where it was shown to clearly colocalize with PKR in the absence of *Arf* (Figure 4D). Induced expression of *Arf* by addition of IPTG to infected NARF-E6 cells caused a drastic shift of NPM to the nucleus where it accumulated mainly in nucleoli (Figure 4D), thereby releasing PKR from the inhibitory interaction with NPM. Thus, induction of *Arf* increases the amount of free PKR susceptible to be activated. Similarly, immunostaining analysis of PKR and NPM in VSV-infected MEFs revealed a higher degree of colocalization of these two proteins in *Arf*-deficient cells than in wild-type cells, reinforcing the view of ARF opposing NPM inhibition of PKR (Supplementary Figure S6B). Taken together, these results place NPM as a mediator between ARF and PKR in the antiviral action exerted by the tumor suppressor ARF.

ARF protects *in vivo* against viral infection

Some viruses, such as VV, resist the actions of IFN mainly by encoding inhibitors of PKR. This resistance is severely impaired when VV is deleted of the E3L gene (VV- Δ E3L) (Beattie *et al*, 1995), showing diminished replicative capacity. To assess the physiological relevance of the antiviral action of ARF and, at the same time, to prove the involvement of PKR, we decided to use infection with VV- Δ E3L. First, and after verifying an increase in ARF levels after infection with VV- Δ E3L (Supplementary Figure S7A), we corroborated *in vitro* that wild-type cells were more resistant to infection by VV- Δ E3L than *Arf*-null cells (Figure 5A), whereas infection with wild-type VV was not altered by *Arf* (data not shown). In addition, metabolic labeling of *Arf*-deficient cells clearly showed the production of VV proteins and shut-off of cellular protein synthesis, whereas wild-type cells did not show signs of viral protein synthesis or shut-off at the MOI tested (Figure 5B). Thus, *in vitro* data suggested again an involvement of PKR in the *Arf*-mediated antiviral effect and validated the use of VV- Δ E3L as a system to study *Arf* protection *in vivo*. *Arf*-null or wild-type mice were intranasally inoculated with VV- Δ E3L and monitored daily for weight loss, a parameter indicative of progression of the viral infection (Alcami and Smith, 1992). A clear and persistent weight loss was observed during the experiment for the *Arf*-deficient animals, whereas only a moderate and transient weight loss was measured in the wild-type mice group. Mann-Whitney test of the percentage of relative weight loss at 4 days after infection revealed statistically significant differences between both groups ($P=0.0219$) (Figure 5C). In addition, VSV inoculation of wild-type or 'super *Ink4a/Arf*' mice showed an increased protection against viral infection in the transgenic animals (Supplementary Figure S7B). 'Super *Ink4a/Arf*' mice exhibited a decreased death rate compared to wild-type animals. Furthermore, some of the 'super *Ink4a/Arf*' mice survived viral infection, whereas all wild-type animals succumbed to VSV infection (Supplementary Figure S7B). Overall, these results reveal that increased levels of ARF enhance the resistance of the cells or mice to be infected by IFN-sensitive viruses.

Figure 5 Arf mediates increased resistance to VV- Δ E3L infection. (A) Resistance to VV- Δ E3L-induced cytolysis after ARF expression. MEFs isolated from *Arf*-null or wild-type mice were infected in triplicate with VSV at the indicated MOI and, 24 h after infection, cell viability was examined by crystal violet staining as described in Materials and methods. A representative picture of a stained plate is shown. (B) Virus-induced protein synthesis shut-off and viral protein synthesis are affected by *Arf* gene dosage. MEFs derived from *Arf*-null or wild-type mice were infected or not with VV- Δ E3L at an MOI of 5 PFU/ml and, after 8 h, levels of protein synthesis were determined by [³⁵S]methionine labeling for 30 min and SDS-PAGE analysis. (C) Increased susceptibility of *Arf*-null mice to VV- Δ E3L infection. Analysis of the percentage of relative weight loss at 4 days after intranasal infection with VV- Δ E3L (5×10^7 PFU) of *Arf*-null or wild-type mice. Comparison between groups of mice was performed using the Mann-Whitney *U*-test and is represented by a box-and-whisker plot. The differences obtained were statistically significant ($P=0.0219$).

Discussion

A potential antiviral activity has been suggested for various genes with a demonstrated crucial role controlling cancer development (Chelbi-Alix *et al*, 1998; Takaoka *et al*, 2003; Munoz-Fontela *et al*, 2005). Induction of tumor suppressors by IFN (Mecchia *et al*, 2000; Takaoka *et al*, 2003) and inhibition by oncogenic viruses (Helt and Galloway, 2003; Collot-Teixeira *et al*, 2004; O'Shea and Fried, 2005) reinforce this idea. One of the most frequently mutated genes in cancer is the product of the *Arf* gene (Ruas and Peters, 1998; Sharpless and DePinho, 1999). Initially, ARF activity was linked to p53 stabilization after oncogenic stress (Sherr and Weber, 2000), but more recently p53-independent functions have been described (Weber *et al*, 2000), placing this tumor suppressor as a more general sensor of different types of stress.

Viral infection is the cause of cellular stress and the IFN system is the first barrier against viruses (Katze *et al*, 2002). Several reports have described the activation of ARF after the

expression of viral proteins (Yang *et al*, 2001; Pollice *et al*, 2004) or type I IFN treatment (Sandoval *et al*, 2004). Our study addressed the possibility that ARF acts as a viral stress sensor restricting virus infection. In this sense, we observed an induction of *Arf* expression after virus infection at the mRNA as well as at the protein level, suggestive of a physiological role for ARF during virus infection. Indeed, the analysis of the ARF promoter revealed the presence of IFN response elements such as IRF-3 and ISRE. Although the contribution of each of these elements needs further detailed analysis, our results clearly demonstrate the functionality of the *Arf*-derived IRF-3 binding site.

In this report, we clearly demonstrate an inverse correlation between susceptibility to viral infection of human and mouse cells and *Arf* gene dosage. This is reflected in both, a decreased cytopathic effect and a reduced production of viral particles with increasing amounts of Arf. This action is not merely the result of the known effects of Arf on proliferation, as analysis of the infected cells showed no alteration in the kinetics of the cell cycle.

The protective effect of ARF was initially shown for VSV infection but seems to be a general feature for IFN-sensitive viruses, as demonstrated after infection with Sindbis virus or with a VV deleted for the PKR inhibitory gene E3L. The involvement of PKR in the ARF-induced protection was suggested from the increased levels of the PKR downstream targets eIF2 α -P and I κ B α -P, after viral infection of wild-type MEFs compared to *Arf*-deficient cells, or in human cells after induced expression of ARF. VSV infection of *pkr*-null cells clearly placed this kinase downstream of the Arf-induced protection, as overexpression of ARF decreased viral replication only in cells with an intact PKR gene.

The involvement of PKR in this novel antiviral defense mechanism was not a surprise considering the well-known role of this kinase in innate immunity against viral infection (Samuel, 2001). In contrast, the molecular pathway employed by Arf to impinge on PKR was puzzling. As a first obvious candidate to mediate Arf actions, we tested the involvement of p53. However, the use of NARF-E6 cells demonstrates that the viral resistance induced by ARF is p53-independent. Again, this observation argues against the possibility that the antiviral action of ARF might be related to its cell cycle effects, as coexpression of papillomavirus E6 protein prevents ARF-mediated arrest (Stott *et al*, 1998). Nonetheless, this result does not imply a complete uncoupled antiviral action of ARF and p53. The previously reported antiviral action of p53 is a consequence of the increased apoptotic response upon viral infection that compromises cell viability and results in a decreased viral titer. We propose that independently of this, *Arf* is able to restrict viral infection by additional mechanisms that result in increased cellular integrity and reduced viral production (for a comparison of p53- and ARF-mediated antiviral effects, see Supplementary Figure S8).

In addition, a role for ATR mediating the effects of *Arf* has recently been described (Rocha *et al*, 2005). Treatment of NARF-E6 cells induced to express ARF by IPTG addition, with the ATR inhibitor caffeine resulted in no alteration of the degree of protection, suggesting that this kinase is not involved in the antiviral action mediated by *Arf* (Supplementary Figure S9).

We noticed from the literature that ARF and PKR have a common partner, NPM. It has been described that ARF sequesters NPM in the nucleolus (Brady *et al*, 2004), preventing its transit and intended function(s) elsewhere in the cell. Indeed, NPM has been shown to interact with and inhibit PKR (Pang *et al*, 2003), making it an attractive candidate to mediate the observed antiviral protection elicited by ARF. Our results support this hypothesis, as inhibition of NPM expression by specific siRNA-mediated gene knockdown augmented the cell resistance to be infected by VSV or Sindbis virus. Further experimental evidence was obtained when we observed by co-immunoprecipitation studies a diminished NPM interaction with PKR after ARF expression. Thus, ARF action on NPM would allow free PKR to exert its well-known antiviral activity. This view is supported by confocal analysis of NPM re-localizing away from PKR after induced expression of ARF. Our results provide a rationale for the existence of viral proteins such as adenovirus protein V that induces redistribution of NPM from the nucleolus to the cytoplasm or why hepatitis D virus delta antigen upregulates NPM expression (Huang *et al*, 2001; Matthews, 2001).

The physiological relevance of our findings was confirmed by the observation of an increased susceptibility to infection of *Arf*-null compared to wild-type mice when inoculated with VV- Δ E3L. The use of this mutant VV strain supported once again the involvement of PKR in the Arf-induced protection *in vivo*, as E3L is the main PKR inhibitor encoded by VV, and its deletion renders VV susceptible to the actions of an activated PKR (Brandt and Jacobs, 2001). In addition, an increased antiviral protection was observed when 'super *Ink4a/Arf*' mice were inoculated with VSV compared to wild-type animals, demonstrating *in vivo* the ability of extra *Arf* gene dosage to have a beneficial effect restricting virus infection.

In conclusion, we demonstrate here that ARF can be induced by viral infection and that the expression of ARF reduces viral infectivity. This antiviral effect depends, at least in part, on PKR activation mediated by its release from inhibitory complexes with NPM. These results provide a new link between tumor suppression and antiviral host defense, an important step to understand the tumorigenic activity of viruses and a crucial learning for the forthcoming use of viruses as therapeutic agents.

Materials and methods

Mice, cell cultures, virus and reagents

Arf-null mice (Kamijo *et al*, 1997), doubly deficient *Ink4a/Arf* animals (Serrano *et al*, 1996) and transgenic 'super *Ink4a/Arf*' mice (Matheu *et al*, 2004) of C57BL6/J pure background have been previously described. MEFs were isolated and cultured as described previously (Palmero and Serrano, 2001). Unless otherwise stated, all MEFs were used at the first 1–3 passages. NARF2 and NARF-E6 cells were kindly provided by Dr Gordon Peters (London Research Institute, Cancer Research UK) and cells derived from *pkr*^{-/-} mice were provided by Dr Charles Weissmann (University of Zurich, Switzerland). Human MCF-7, mouse L and green African monkey BSC-40 cells were cultured following a standard procedure. Infections were carried out using VSV of Indiana strain and virus yields were measured by plaque assays in BSC-40 cells. Retroviral plasmids for p14ARF and p19ARF (pLPC-p14ARF and pLPC-p19ARF) were generated by introducing restriction fragments from pBluescript vectors, carrying the corresponding cDNAs, into pLPC vector. NPM knockdown was accomplished by using retroviral vector pSUPER.retro-NPM (Brady *et al*, 2004) kindly provided by

Dr Jason D Weber (Washington University School of Medicine, Missouri). As a control, we used pSUPER.retro-GFP, a kind gift of Dr Madalena Tarsounas (University of Oxford, UK), targeting an irrelevant protein (GFP) (Tarsounas *et al*, 2004). Retrovirus production and transduction of the different target cells were carried out according to methods described previously (Gil *et al*, 2004).

Cell cytolysis induced by VSV

Cells were grown in 96-well plates to 100% confluence and then were infected with different VSV MOI. At 24 h after infection, the medium was removed and cytolysis was determined by crystal violet staining as described previously (García *et al*, 2002). The percentage of viable cells was calculated assuming the survival rate of uninfected cells to be 100%.

Transcriptional activity assays

HeLa cells were transfected with the human p14ARF (FL-ARF-luc) reporter (3.4 kb upstream of the p14ARF ATG start codon), or a truncated version with only 700 bp upstream of the ATG codon (FR-ARF-luc), cloned into the pGL3-basic luciferase reporter vector (Promega) (both reporter constructs were kind gifts from Dr Gordon Peters (London Research Institute, Cancer Research UK)). At 24 h after transfection, cells were infected with VSV at an MOI of 5 PFU/cell and, at different times after infection, cells were recovered and luciferase activities were analyzed by the dual luciferase assay (Roche Diagnostics). Experiments were carried out in triplicate and repeated at least twice.

Immunoblotting and immunoprecipitation

Confluent monolayers of cells were infected at an MOI of 5 PFU/cell or treated with IFN β (1000 U/ml) and proteins were extracted at 6 h after infection or 16 h after IFN treatment unless otherwise stated. For this, cells were lysed in 10 mM Tris-HCl (pH 8.0) containing 140 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors. Extracts were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the corresponding antibodies. The following antibodies were used: anti-p19ARF (Ab80, Abcam Ltd, Cambridge, UK), anti-NPM (Santa Cruz Biotechnology Inc., California, USA), anti-p53 (CM5, Novocastra), anti-eIF2 α (Santa Cruz Biotechnology), anti-eIF2 α -P (Biosource International), anti-phospho I κ B α serine 32/36 (Cell Signaling Technology), anti-actin (MP Biomedicals, Aurora, OH) and anti-p14ARF (a generous gift of Dr David Parry from DNAX, or Ab-2 LabVision, Neomarkers). For immunoprecipitation, an anti-human PKR antibody was used as described (Gil *et al*, 1999).

RNA analysis

MEFs were seeded onto 100-mm-diameter dishes and infected with VSV at an MOI of 5 PFU/cell. Then, cells were recovered and total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcription (RT-PCR) was performed using the reverse transcription system kit (Promega). Q-RT-PCR was performed using an ABI7700 instrument and SYBR Green system (Applied Biosystems). The following oligonucleotide primers were used: specific to mouse ARF, 5'-GCCGCACCGGAATCCT-3' (sense)

and 5'-TTGAGCAGAAGAGCTGCTACGT-3' (antisense); specific to β -actin, 5'-GGCACCACCTTCTACAATG-3' (sense) and 5'-GTGGTGGTGAAGCTGTAGCC-3' (antisense).

Analysis of viral protein synthesis

Confluent monolayers of cells were infected with VV- Δ E3L at an MOI of 5 PFU/cell or with VSV at an MOI of 0.1 PFU/cell. In the case of VSV, half of the cells were previously treated with IFN β (500 U/ml) for 16 h. Viral protein synthesis was measured by pulse labeling the cells from 8 to 8.5 h post-infection with methionine-free MEM supplemented with 50 μ Ci/ml of [35 S]methionine-cysteine labeling mix (PerkinElmer Life Sciences). After radiolabeling, the monolayers were lysed in Nonidet P-40 lysis buffer and analyzed by SDS-PAGE followed by autoradiography.

Immunofluorescence and confocal microscopy

NARF-E6 cells were seeded onto glass coverslips, incubated or not with IPTG (1 mM) and infected with VSV at an MOI of 5 PFU/cell. At 16 h after infection, cells were fixed and stained as described previously (Gil *et al*, 2004). Antibodies against PKR (Gil *et al*, 1999) or NPM were used, followed by Cy5- or fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories Inc., Baltimore Pike, West Grove and Sigma, respectively). Analysis of the samples was carried out with a Bio-Rad Radiance 2100 confocal laser microscope and images were stored and processed with Laser Pix software package (Bio-Rad Laboratories).

In vivo viral infections

Groups of seven age-matched male C57BL/6 Arf-null and wild-type mice were intranasally infected with 5×10^7 PFU of VV- Δ E3L in 50 μ l of PBS. The mice were examined and weighed daily to assess disease progression.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Gordon Peters, Jesús Gil, Charles Weissmann, Jason Weber, Madalena Tarsounas, Anton Berns and David Parry for providing reagents. We also thank the excellent technical assistance of Maribel Muñoz, Elisa Santos, Rosa Pérez and María Victoria Jiménez. ME is funded by the Spanish Ministry of Education and Science (BIO2002-03246) and by the European Union (QLK2-2002-01687 and QLK2-CT-2002-00954). Work at the laboratory of MS has been funded by the Spanish Ministry of Education and Science (SAF2002-03402) and by the European Union (INTACT and PROTEOMAGE). CR is funded by the Spanish Ministry of Education and Science (BIO2005-00599) and the Fundación Medica Mutua Madrileña.

The authors have no conflicting financial interests.

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