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Relevance of Akt phosphorylation in cell transformation induced by Jaagsiekte sheep retrovirus

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Abstract

Expression of the JSRV envelope (Env) is sufficient to transform immortalized rodent fibroblasts. A putative docking site for the PI3-K kinase (Y^{590}-X-X-M^{593}) in the cytoplasmic tail of the transmembrane domain of the JSRV Env is a major determinant of viral-induced cell transformation. Akt is constitutively phosphorylated in rodent fibroblasts transformed by the JSRV Env. However, recent data suggest that Y590 and M593 are not necessary for JSRV Env-induced transformation of the immortalized chicken fibroblasts cell line DF-1. In this study we found that JSRV-induced transformation of DF-1 cells is Akt-independent. In addition, a replication-competent avian vector expressing the JSRV Env (RCASBP(A)+JE) was also able to induce transformation of primary chicken embryo fibroblasts (CEF). Vectors expressing JSRV Env Y590 mutants were still able to induce CEF cells transformation but not as efficiently as the vectors expressing the wild-type Env. In CEF cells, as in DF-1 cells, only the expression of the wild-type Env induced constitutive phosphorylation of Akt. Thus, in chicken cells, the degree of transformation induced by the JSRV Env is maximum in the presence of Y590 and Akt phosphorylation. We addressed the significance of Akt phosphorylation in rat 208F cells transformed by the JSRV Env and showed that Akt is indeed activated and shows kinase activity. Inhibitors of the PI-3K/Akt pathway reproducibly decreased the transformation efficiency of the JSRV Env. In vivo, we found phosphorylated Akt only in nasal tumors induced by the enzootic nasal tumor virus (ENTV), a JSRV-related β-retrovirus. No evidence of Akt phosphorylation was found in lung tumor sections of sheep affected by pulmonary adenocarcinoma. As a whole, these results suggest that the activation of the PI-3K/Akt pathway contributes to the process of JSRV-induced cell transformation but most likely is not the primary determinant both in vitro and in vivo.

Keywords: Jaagsiekte sheep retrovirus; JSRV; sheep; lung cancer; adenocarcinoma; RCASBP; PI3-K; Akt; Envelope

Introduction

Jaagsiekte sheep retrovirus (JSRV) is the etiologic agent of a contagious lung cancer of sheep known as ovine pulmonary adenocarcinoma (OPA; also known as sheep pulmonary adenomatosis, Jaagsiekte, or ovine pulmonary carcinoma) (DeMartini and York, 1997; Palmarini and Fan, 2001; Palmarini et al., 1997). OPA is one of the major infectious diseases of sheep in many countries of the world and represents a unique animal model to study lung carcinogenesis. OPA occurs spontaneously in nature and has striking similarities to some forms of human lung adenocarcinomas (Palmarini and Fan, 2001; Perk and Hod, 1982). Lung cancer is the main cause of mortality among cancer patients worldwide (Jemal et al., 2002; Parkin, 1997; Wingo et al., 1998) but very few animal models are available to study its causes and the mechanisms of cell transformation.

The causative agent of OPA, JSRV, is a retrovirus with unique characteristics related to its tropism and to the mechanisms that it follows to induce cell transformation. JSRV is the only known retrovirus that is specifically expressed in

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the differentiated epithelial cells of the lungs (Palmarini et al., 1995; Platt et al., 2002). The JSRV long terminal repeats are preferentially expressed in cell lines derived from type II pneumocytes and Clara cells and interact with lung-specific transcription factors (McGee-Estrada et al., 2002; Palmarini et al., 2000a).

The expression of the JSRV envelope (Env) is sufficient to induce transformation of immortalized rodent fibroblasts (Maeda et al., 2001). This is a unique example of a functional retroviral envelope protein functioning essentially as an oncoprotein. The mechanisms of JSRV Env-induced cell transformation are not completely understood. In immortalized rodent fibroblasts, the cytoplasmic tail of the JSRV transmembrane domain (TM) is a major determinant of JSRV-induced cell transformation (Palmarini et al., 2001). In particular, a putative docking site (YXXM) for the phosphatidylinositol-3-kinase (PI3-K) is critical for transformation of mouse NIH3T3 and rat 208F cells. Mutations of tyrosine 590 (Y590) and methionine 593 (M593) render the JSRV Env unable to transform NIH-3T3 but leave unaltered its capacity to mediate viral entry. The activation of the PI3-K/Akt pathway leads to anti-apoptotic and proliferative stimuli and this pathway can be activated in a variety of cancers (Datta et al., 1999; Vivanco and Sawyers, 2002). We have found Akt phosphorylation in immortalized rodent fibroblasts transformed by JSRV and in the JS8 cell line, a cell line originated from an OPA tumor (Alberti et al., 2002; Palmarini et al., 2001). Phosphorylation of Akt in JSRV-transformed cells is dependent on Y590 and is abolished by PI3-K-inhibitors (Alberti et al., 2002). These data suggest that the PI3-K/Akt pathway might play a role in JSRV-induced cell transformation.

Interestingly, studies on a virus closely related to JSRV (enzootic nasal tumor virus, ENTV) (Cousens et al., 1999) have supported the relevance of the findings described above for JSRV. ENTV induces transformation of immortalized rodent fibroblasts such as 208F and NIH-3T3 (Alberti et al., 2002; Dirks et al., 2002). A tyrosine at position 590 of the ENTV Env is also critical for viral-induced cell transformation and phosphorylated Akt is detectable in 208F cells transformed by this virus (Alberti et al., 2002).

The JSRV Env can also transform the immortalized chicken fibroblast cell line DF-1 (Himly et al., 1998), but in this cell line Y590 is not necessary for transformation (Allen et al., 2002). This observation raises questions on the overall relevance of Y590 and the associated Akt phosphorylation in JSRV-induced transformation in vitro. In this study, we tried to dissect the role of Y590 and Akt phosphorylation in JSRV-induced cell transformation in immortalized cell lines, in primary cells, and in vivo. We show that DF-1 cells can be transformed by JSRV Env mutants that fail to induce Akt phosphorylation. We describe that the JSRV Env is able to induce transformation of primary chicken embryo fibroblasts (CEFs). Constructs expressing the JSRV Env Y590F mutants are not as efficient as the wild-type JSRV Env in transforming CEFs. We also demonstrate that in chicken cells (both primary or immortalized) the cytoplasmic tail of the JSRV transmembrane domain is a major determinant of viral transformation. We addressed the significance of Akt phosphorylation in JSRV-transformed rat 208F cells and showed that Akt is indeed activated and shows kinase activity. Furthermore, the addition of a PI3-K inhibitor (LY294002) attenuates the transforming process initiated by the JSRV Env in vitro. However, in vivo, Akt phosphorylation could be detected only in ENT tumors and not in lung tumors from sheep affected by OPA. Thus, we suggest that the activation of the PI3-K/Akt pathway likely contributes to JSRV-induced cell transformation but does not appear to be the primary determinant of this process.

Results

Morphological changes induced by the JSRV Env in the chicken DF-1 cell line are independent from tyrosine 590 and Akt phosphorylation

We have previously shown that Y590 in the JSRV Env is critical for transformation of immortalized rodent fibroblasts (Alberti et al., 2002; Palmarini et al., 2001). Y590 is part of a SH-2 domain that is a putative docking site for PI3-K. Phosphorylated Akt (the major downstream effector of PI3-K) is detected in rodent fibroblasts transformed by JSRV. However, it has recently been found that JSRV Env mutants in Y590 are still able to transform DF-1 (Allen et al., 2002), a cell line derived from immortalized chicken fibroblasts. This observation might lead to the conclusion that Akt phosphorylation is not necessary for transformation of chicken cells. To assess this point, we inserted various forms of the JSRV env in the replication-competent avian retroviral vector RCASBP(A). We routinely obtained with the empty RCASBP(A) vector titers of approximately 10^6 tissue culture infectious units (TCIU). The insertion of the JSRV Env (or of the various Env mutants) resulted in vectors with titers on the order of 10^2-10^3 TCIU. The decreased efficiency of replication of the constructed vectors derives most likely by the expression of two envelope proteins in the same cells (the Env of ALV and the Env of JSRV). By viral entry assays using MLV-based vectors expressing a marker gene and pseudotyped with the JSRV Env, we established that chicken cells do not express a functional receptor for JSRV (not shown).

Transformation assays were performed by transfecting DF-1 cells with the empty RCASBP(A) vector or with the vectors expressing the various forms of the JSRV Env. The transfected cells were passaged every 2-3 days and after four passages the cells that were originally transfected with the vectors expressing the JSRV Env (RCASBP(A)+JSE), or the mutants RCASBP(A)+JEY590D and RCASBP(A)+JEY590F showed extensive morphological changes with respect to the mock-transfected DF-1 or cells transfected

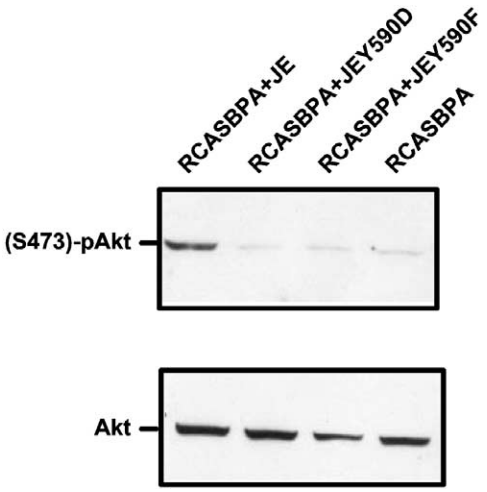

Fig. 1. Detection of phosphorylated Akt in DF-1 cells. Cell lysates of serum-starved DF-1 cells infected with the various RCASBP(A) vectors explained in the text were analyzed by SDS–PAGE Western blotting using a rabbit polyclonal antibody specific for Akt phosphorylated in S473 (Cell Signaling). Phosphorylated Akt is visible only in DF-1 cells transfected with RCASBP(A)+JE. The same lysates were analyzed by using an antiserum to Akt as a loading control.

with the empty RCASBP(A) vector, confirming recent results (Allen et al., 2002). Morphological changes consisted of the presence of refractile round cells across the culture that tended to grow at high density and detached easily from the solid support. When DF-1 transfected with RCASBP(A)+JE, RCASBP(A)+JEY590D, and RCASBP(A)+JEY590F were not split, they overgrew into clumps of cells rising above the monolayer (not shown). RCASBP(A)+JENruIen failed to transform DF-1 cells, suggesting that the cytoplasmic tail of the JSRV TM is a major determinant of cell transformation even in DF-1 cells. RCASBP(A)+JENruIen expresses a chimeric envelope formed by the SU, the ectodomain, and the membrane spanning domain of the TM of JSRV while the cytoplasmic tail is derived from the TM of enJS56A1 (a JSRV-related endogenous locus) (Palmarini et al., 2000b).

We tested for the presence of phosphorylated Akt in cell lysates obtained from serum-starved DF-1 cells infected with the various RCASBP(A) vectors described above. By Western blotting we detected phosphorylated Akt in DF-1 infected by RCASBP(A)+JE. These results indicated that Akt phosphorylation also occurs in DF-1 cells transformed by the JSRV Env and is not a phenomenon confined to JSRV-transformed rodent fibroblasts. As expected, only background levels of phosphorylated Akt were detected in DF-1 infected by RCASBP(A)+JENruIen or the empty vector RCASBP(A) (Fig. 1). Also DF-1 transformed by RCASBP(A)+JEY590D and RCASBP(A)+JEY590F did not show Akt phosphorylation beyond the background level seen in DF-1 infected with RCASBP(A). Consequently, at least in this cell line, the process of Akt phosphorylation can be uncoupled from the process of JSRV-induced cell transformation.

The JSRV Env transforms primary chicken embryo fibroblasts

DF-1 is an immortalized cell line (Himly et al., 1998) and presumably has already undergone genetic changes that allow continuous cell division. In our hands, the DF-1 cells do not show a strong contact inhibition and have the tendency to grow in soft agar. Thus, DF-1 might already possess some characteristics of transformed cells that compensate for the lack of Akt activation by the JSRV Y590 mutants. To address whether rodent and chicken cells respond differently to JSRV-induced transformation, we repeated the experiments described above in primary CEF. Three to four passages after transfections, morphological changes started to appear. CEFs transfected with RCASBP(A)+JE assumed an elongated morphology, were refractile, and tended to reach confluency earlier than mock-transfected CEF or CEFs transfected with RCASBP(A) or RCASBP(A)+JENruIen (Fig. 2). CEFs transfected with RCASBP(A)+JEY590F also had morphological changes, although they did not appear as dramatic as CEF transfected with RCASBP(A)+JE. RCASBP(A)+JEY590D showed only slight morphological changes difficult to distinguish from the mock-transfected CEFs. No changes with respect to the mock-transfected CEFs were observed with CEFs transfected with the empty RCASBP(A) vector or RCASBP(A)+JENruIen.

Y590 and Akt phosphorylation are associated with a fully transformed phenotype in CEFs

We assessed the capacity of the CEFs transfected with the RCASBP(A) vectors indicated above to form colonies in soft agar. Anchorage independence is one of the main criteria to define cell transformation in vitro. CEFs infected with RCASBP(A)+JE formed colonies in soft agar (Fig. 3). CEFs infected with the RCASBP(A)+JSEY590F and RCASBP(A)+JEY590D had 70 and 29%, respectively, efficiency of colony formation with respect to CEFs infected with RCASBP(A)+JE (e.g., representing 100%). The background given by the mock-transfected CEFs and CEFs infected with the empty vector RCASBP(A) was 3.2 and 6.2%, respectively (Fig. 3). CEFs infected with RCASBP(A)+JENruIen gave values slightly above background (13.6%). RCASBP(A)+JE not only induced a higher number of colonies, but also induced colonies with a greater diameter (size) with respect to RCASBP(A)+JSEY590F and RCASBP(A)+JEY590D. The average diameter of the colonies induced by RCASBP(A)+JE was 251 ($\pm$52) μm, while RCASBP(A)+JSEY590F and RCASBP(A)+JEY590D induced colonies with average diameters of 157 ($\pm$25) and 97 ($\pm$20) μm, respectively. The average diameter of the colonies induced by RCASBP(A)+JENruIen was 105 ($\pm$25) μm. Thus, in primary fibroblasts, Y590 influences the transforming potential of the JSRV Env (Fig. 3).

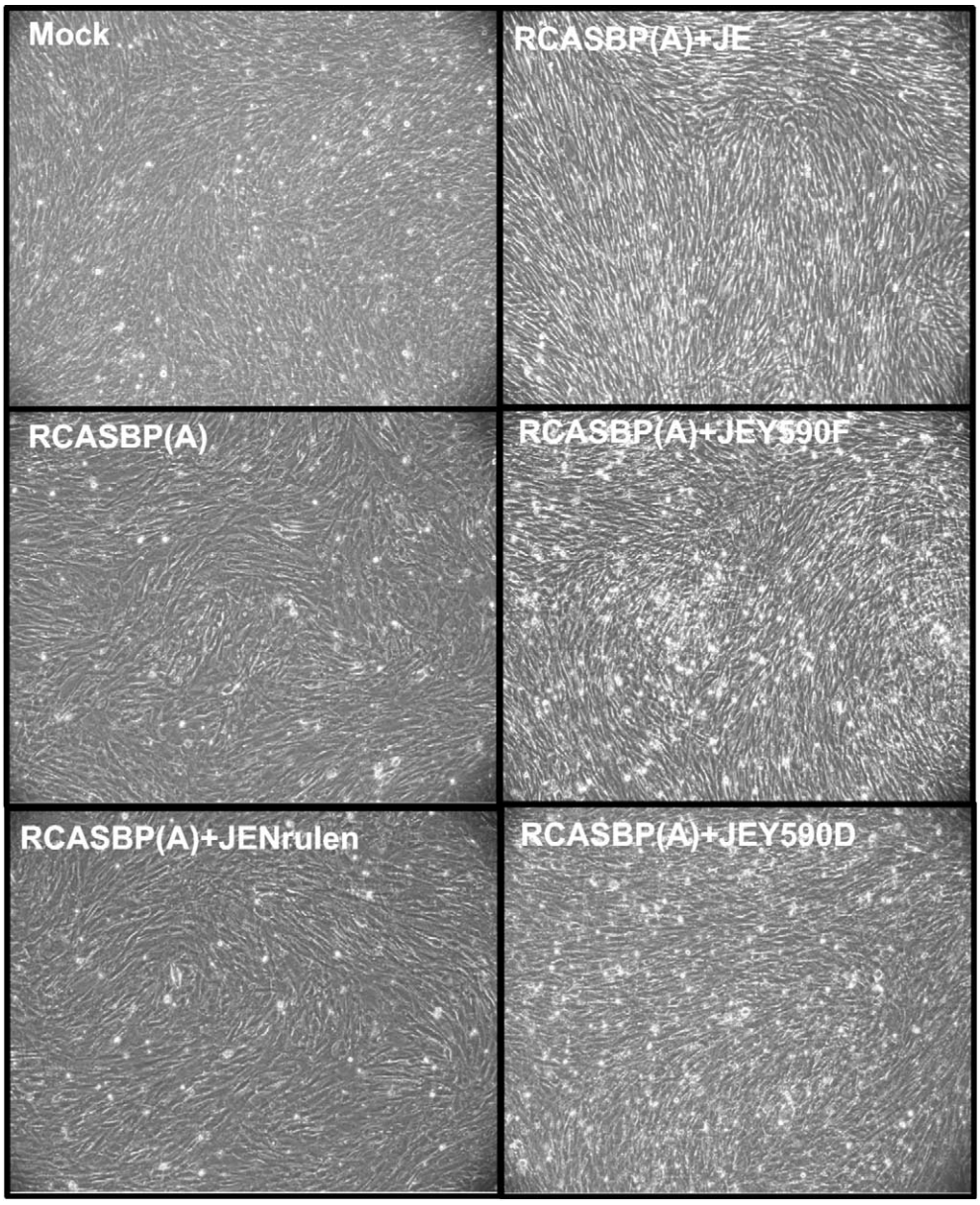

Fig. 2. Transformation of CEF cells by the JSRV Env. CEF cells were mock-transfected or transfected with the replication-competent retroviral vector RCASBP(A) expressing the various mutants/chimeras of the JSRV Env described in the text and passaged every 2-3 days. After five passages, CEF transfected with RCASBP(A)+JE shows a remarkable phenotypic change characterized by the presence in the culture of elongated, densely packed translucent cells. CEF transfected with RCASBP(A)+JEY590F shows morphological changes similar to but not as dramatic as those described above for CEF transfected with RCASBP(A)+JE. CEF cells transfected with RCASBP(A)+JEY590D show slight change in morphology with respect to the mock-transfected cells or cells transfected with the empty vector RCASBP(A) or RCASBP(A)+JENruIen.

By Western blotting on lysates prepared from serum-starved cells, we detected phosphorylated Akt only in CEFs transfected with the wild-type RCASBP(A)+JE (Fig. 4). These results confirm the previous observation that Y590 is directly linked to Akt phosphorylation in JSRV-transformed cells in vitro (Alberti et al., 2002; Palmarini et al., 2001). However, JSRV-induced transformation can occur without Akt phosphorylation, although it appears that the transformation process in primary cultures is not complete in the absence of Y590 and Akt phosphorylation.

Akt possess kinase activity in 208F cells transformed by JSRV

The results shown above prompted us to address the significance of Akt phosphorylation in 208F cells transformed by JSRV. In our previous studies we have shown that the expression of the wild-type JSRV Env in rodent fibroblasts is associated with Akt phosphorylation and that Akt phosphorylation can be inhibited by using the PI3-K inhibitor LY294002 (Alberti et al., 2002; Palmarini et al.,

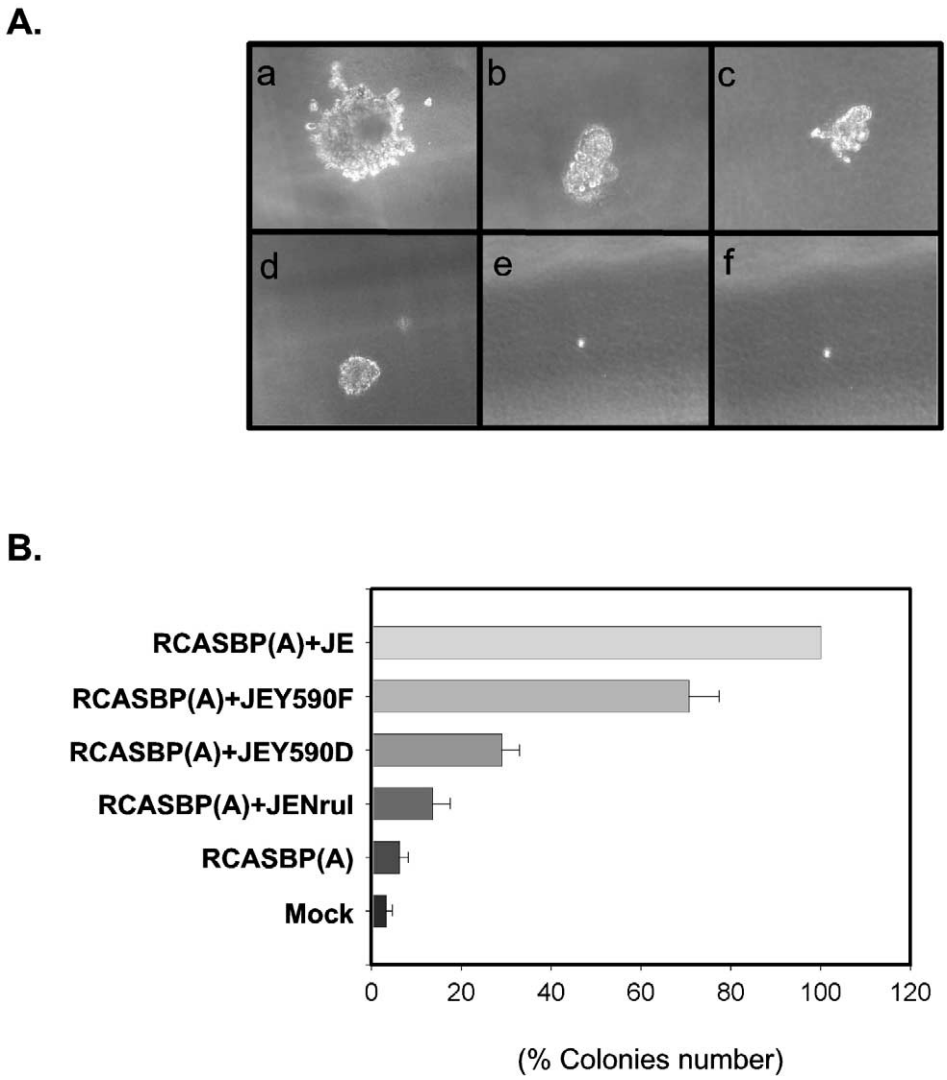

Fig. 3. Anchorage-independence assay. Anchorage-independence of CEFs cells shown in Fig. 3 was assessed by growth in soft agar as described under Materials and methods. (A) A representative example of the morphology and size of the colonies (magnification, ×100) of CEF cells infected with RCASBP(A)+JE (a), RCASBP(A)+JSY590F (b), RCASBP(A)+JSY590D (c), RCASBP(A)+JSNruIen (d), RCASBP(A) (e), and mock-infected (f). (B) A quantitative analysis of the various CEF cells by taking as 100% the number of colonies (counted 2 weeks after seeding) obtained with CEF infected with RCASBP(A)+JE. Values shown are the average of duplicate samples performed in two independent experiments. Bars indicate standard error.

2001). However we did not prove that Akt is active or that it has a role in JSRV-induced transformation. Akt encodes a serine/threonine kinase that acts on a variety of downstream effectors (Bellacosa et al., 1998), including GSK3β (Diehl et al., 1998). By kinase assays we show that Akt in 208F cells transformed by JSRV is active. Akt immunoprecipitated from a lysate of 208F cells transformed by JSRV was able to phosphorylate one of its downstream targets (GSK-3β) in vitro. Only a background of Akt kinase activity was detected in the parental 208F cells (Fig. 5).

Inhibition of the PI3-K/Akt pathway reduces the transformation potential of the JSRV Env in 208F cells

To further assess the role of the PI3-K/Akt pathway in JSRV-induced cell transformation, we transfected rat 208F cells with pCMV3JS21ΔGP and tested whether the addition of different concentrations of the PI3-K inhibitor LY294002 (1 to 10 μM) reduced the number of foci induced by the JSRV Env. LY294002 was added 1 day after transfection and re-added every other day with fresh media until the end of the experiment at Day 15.

LY294002 inhibited cell transformation induced by the JSRV-ENV in a dose-dependent manner. The results are summarized in Table 1, where they are shown by normalizing to 100 the number of foci induced by the JSRV Env without the addition of inhibitor. We found that the addition of LY294002 reduced the relative number of Env-induced foci to 64.5 (with 1 μM inhibitor) or virtually zero (with 10 μM LY294002). In parallel experiments, the number of foci induced by the oncogene *fos* was not decreased by the addition of 1 μM LY294002. Higher concentrations of

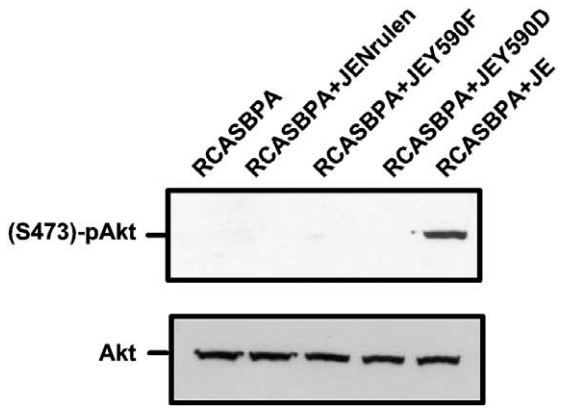

Fig. 4. Detection of phosphorylated Akt in CEF cells. Cell lysates of serum-starved CEF cells infected with the various RCASBP(A) vectors explained in the text were analyzed by SDS–PAGE Western blotting as described under Materials and methods. Akt phosphorylated in S473 is visible only in DF-1 cells transfected with RCASBP(A)+JE. The same lysates were analyzed by using an antiserum to Akt as a loading control.

inhibitor (5-10 μM) decreased the relative number of foci of approximately one-third. No toxic effect due to the addition of LY294002 was observed when concentrations of 1 or 5 μM of LY294002 were used. However, the addition of 10 μM of LY294002 induced some cell death as we reproducibly counted a smaller number of cells at the end of the experiment in the dishes where the drug was added in comparison to the dishes where no inhibitor was used (not shown). No toxic effects were seen when the initial addition of 10 μM of LY294002 was done 5 days after transfection (instead of 1 day as the experiments above). In the latter series of experiments, addition of LY294002 caused a 70% reduction in the number of foci induced by the JSRV Env, while the number of foci induced by *fos* was reduced of only approximately 30%.

These results suggest that the inhibition of the PI3-K/Akt pathway negatively interferes with the process of transformation of immortalized rodent fibroblasts by the JSRV Env. We cannot rule out that the addition of LY294002 negatively affects cell proliferation in general and this renders 208F cells less susceptible to cell transformation. Indeed, at higher concentrations of LY294002 we also noticed a reproducible inhibition of the transformation efficiency of the oncogene *fos* that is not supposed to function through the PI3-K/Akt pathway.

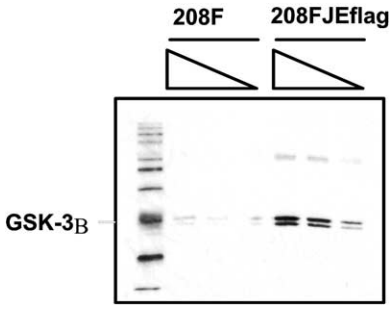

Fig. 5. Akt Kinase assay. Akt activation was assessed in cell lysates collected from 208F cells and from the JSRV-transformed 208F-JScflag cell line by using an Akt Kinase kit (Cell Signaling) as described under Materials and methods. The figure shows the level of phosphorylation of GSK-3β with increasing amounts of 208F JEflag immunoprecipitate.

Table 1
Inhibition of JSRV Env and *fos*-induced transformation of 208F cells by LY294002

LY294002 concentration	Induction of transformed foci			
	JSRV ENV	*n*[a]	*fos*	*n*
0	100[b]	6	100	6
1 μM	64.5 ($\pm$6.1)[c]	3	98 ($\pm$7.6)	3
5 μM	13.1 ($\pm$4.4)	3	41.1 ($\pm$2)	3
10 μM[d]	0.7 ($\pm$0.4)	6	33.4 ($\pm$4.3)	6
10 μM[e]	29.5 ($\pm$11.6)	4	72.1 ($\pm$13.8)	4

[a] n = number of experiments performed.

[b] Indicated values have been obtained by normalizing to 100 the number of foci induced in 208F cells transfected by either the JSRV expressing plasmid (pCMV3JS21ΔGP) or the *fos* expressing plasmid (pFBJ/R) without the addition of LY294002.

[c] Values represent the the average ($\pm$standard error) of the relative number of foci of three to six independent experiments as indicated under column *n*.

[d] These series showed an increased number of cell death in the dishes that had been treated with LY294002.

[e] In this series the inhibitor was added 5 days posttransfection and every other day thereafter until the end of the experiment.

Akt phosphorylation in naturally occurring OPA and ENT tumors

The mechanisms of JSRV-induced oncogenesis in vivo are likely more complex than the mechanisms of viral-induced cell transformation in vitro. To determine whether Akt is phosphorylated in tumors induced by sheep β-retroviruses, we tested OPA tumors from sheep (n = 10) and ENT tumors from both sheep (n = 5) and goats (n = 5) by immunohistochemistry. All the OPA samples resulted negative (Fig. 6). However, we detected a cytoplasmic staining in four of five ENT of sheep and two of five ENT of goats. The staining was intracytoplasmic and localized in one or, more often, several tumor cells in the same acini. Not all the tumor cells in the same section resulted positive. To verify the specificity of the staining, we preincubated the antiphosphorylated-Akt serum with serial dilutions of an Akt S473 peptide. The resulting preadsorbed antiserum completely lost the ability to stain the ENT tumor cells, confirming the specificity of the reaction (data not shown).

The data obtained indicate that Akt phosphorylation occurs, at least at a late stage of the disease, in ENT and not in OPA tumors. We cannot rule out that physiological differences between type II pneumocytes/Clara cells and the cells originating the nasal tumor could explain a difference in the immunohistochemical reactivity for phosphorylated Akt. However, tumors sections from both ENT and OPA

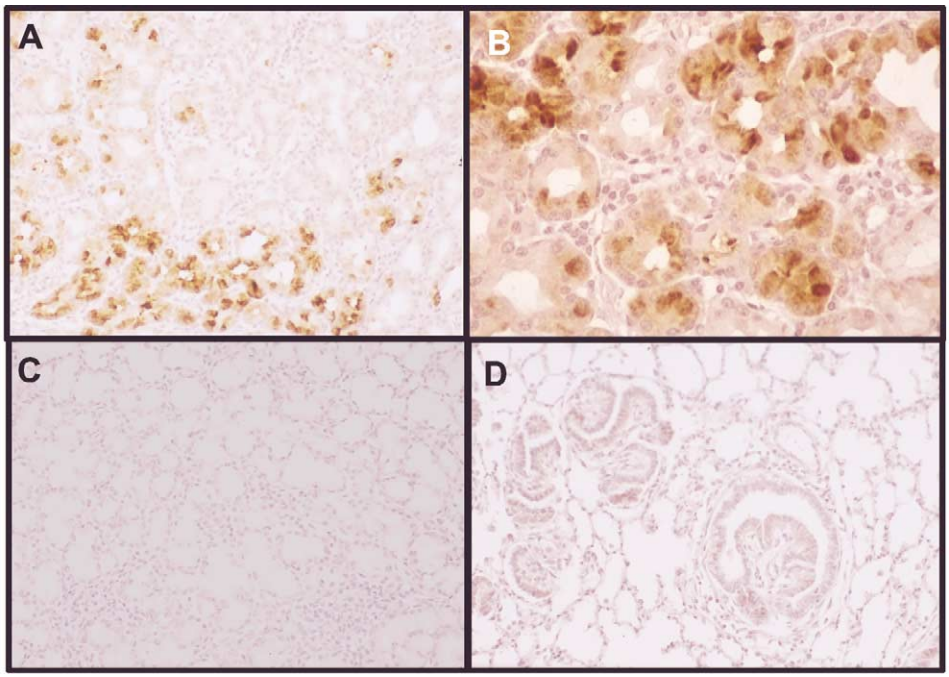

Fig. 6. Immunohistochemistry for the detection of phosphorylated Akt in vivo. (A) Low magnification (×100) micrograph of an immunohistochemistry for the detection of phosphorylated Akt, as described under Materials and methods, of Formalin-fixed tissue sections of a nasal adenocarcinoma from a goat affected by ENT. Positive cells show an intracytoplasmic brown color. Note that not all the tumor cells are positive for phosphorylated Akt. (B) Higher magnification (×400) of section shown in A. (C) No staining is observed when the primary antiserum was replaced with Tris buffer (magnification, ×200). (D) Formalin-fixed section of a lung adenocarcinoma from a sheep affected by OPA. No staining for phosphorylated Akt is observed (magnification, ×100). Carazzi's hematoxylin was used as a counterstain in all the panels shown above.

reacted equally well with an antiserum toward the JSRV CA (not shown).

Discussion

JSRV is a unique lung tropic retrovirus and studies on the mechanisms of viral-induced cell transformation in vitro and in vivo can function as a model to unravel the mechanisms of pulmonary carcinogenesis (Palmarini and Fan, 2001). In this article we have addressed the relevance of Akt phosphorylation in JSRV-induced cell transformation in vitro and in vivo. In immortalized rodent fibroblasts (mouse NIH3T3 or rat 208F), Y590 in the cytoplasmic tail of the JSRV Env is critical for viral-induced cell transformation (Palmarini et al., 2001). However, in the immortalized chicken cell-line DF-1, JSRV Env Y590 mutants were able to induce transformation as efficiently as the wild-type Env (Allen et al., 2002). In this study we have initially tested for Akt phosphorylation in DF-1 cells transformed by RCASBP-A vectors expressing the JSRV Env (RCASBP(A)+JSE) or the Env mutated in Y590 with either phenylalanine (RCASBP(A)+JEY590F) or aspartic acid (RCASBP(A)+JEY590D). We found phosphorylated Akt only in DF-1 transformed by RCASBP(A)+JE. These results confirmed that also in chicken cells expression of the JSRV Env and the presence of Y590 are associated with Akt phosphorylation. It appears that in chicken cells, in contrast to rodent cells, the process of JSRV-induced transformation is independent from the activation of the PI-3K/Akt pathway. However, a construct (RCASBP(A)+JENruIen) expressing the JSRV Env with the cytoplasmic tail of a JSRV-related endogenous virus (enJS56A1) (Palmarini et al., 2000b, 2001) did not induce transformation of DF-1, suggesting that also in these cells the cytoplasmic tail of the JSRV TM is a major determinant of cell transformation.

DF-1 is an immortalized cell line. In our hands DF-1 cells do not show a high degree of contact inhibition and tend to grow in soft agar, suggesting that they might already possess some characteristics of transformed cells that could compensate, for example, for the lack of Akt activation initiated directly or indirectly by the wild-type JSRV Env. Consequently, we analyzed primary CEF as they have not undergone the genetic changes characteristics of immortalized cells. RCASBP(A)+JE was able to induce marked morphological changes also in CEF. Primary cells are more difficult to transform with respect to immortalized cells. RCASBP(A)+JEY590F induced morphological changes but they tended to be not as dramatic as those induced by RCASBP(A)+JE. RCASBP(A)+JEY590D induced slight changes that were difficult to differentiate from the mock-transfected CEF or from CEF transfected with the empty RCASBP(A) vector. CEF infected with RCASBP(A)+JENruIen showed some moderate growth

in soft agar above background, which may indicate that part of the transforming potential of the JSRV Env is retained in this construct.

As in DF-1, only CEFs transfected with RCASBP(A)+JE induced Akt phosphorylation. In addition, CEF transfected with the RCASBP+JE vector gave a higher number of colonies in soft agar than those induced by RCASBP(A)+JEY590F and RCASBP(A)+JEY590D. The size of the colonies was also larger in CEF transfected with RCASBP(A)+JE. Thus, Y590 of the JSRV Env and activation of the PI3-K/Akt pathway are necessary to achieve the maximum degree of transformation induced by the JSRV Env.

These data prompted us to address the significance of the PI3-K/Akt pathway in rodent cells transformed by the JSRV Env. First, we assessed whether in JSRV-transformed cells Akt phosphorylation was indeed a parameter of Akt activation. Akt immunoprecipitated from JSRV-transformed 208F cells showed kinase activity on a recombinant GSK-3β protein (a downstream target of Akt) (Harwood, 2001). Notably, we have detected phosphorylated GSK-3β as well in the JS-8 cell line (Jassim, 1988), a cell line derived from an OPA-affected sheep, and in rodent fibroblasts transformed by the JSRV Env (A. Alberti and M. Palmarini, unpublished data).

These experiments demonstrate that the JSRV Env is indeed able to induce Akt activation. In a previous study we have shown that Akt phosphorylation in rodent fibroblasts transformed by JSRV is dependent on PI3-K kinase activity. Here we have also shown that the addition of a specific PI3-K inhibitor (LY294002) reproducibly reduces the number of JSRV-induced foci of transformed cells with respect to control plates where no inhibitor was added. Transformation by the oncoprotein Fos also was affected by high concentrations of LY294002. Consequently, we cannot rule out that the inhibition of the PI-3K/Akt pathway has a general negative effect on cell transformation that is not necessarily linked to specific inhibition of cell signaling activated by the JSRV Env.

Different cofactors can contribute to JSRV-induced transformation in different cells/cell lines originated from different animal species. However, there is a high likelihood that the primary events determining JSRV Env-induced cell transformation are the same in chicken and rodent cells. The results shown in this study suggest that activation of the PI3-K/Akt pathway is an important cofactor that contributes to JSRV-induced transformation but is not the critical event necessary for this process to occur.

In vivo, the mechanisms of oncogenesis in sheep affected by OPA are most likely more complex than the mechanisms of cell transformation in vitro. The possibility that classical insertional activation mechanisms contribute to JSRV-induced tumorigenesis has not been ruled out, although the short incubation period seen in some instances in experimental OPA cases does not favor this hypothesis. In nature, however, OPA has a long incubation period and thus it is likely that several steps are necessary to achieve viral-induced cell transformation. The JSRV Env might be relevant only for one of these several steps. We have analyzed by immunohistochemistry naturally and experimentally induced OPA lung tumors and we could not detect phosphorylated Akt in the tumor cells. An obvious explanation could be the lack of sensitivity of the immunohistochemical reaction. However, using the same method and antiserum, we have detected phosphorylated Akt in nasal adenocarcinoma induced by the ENTV (Cousens et al., 1999) in both sheep and goats. ENTV is a β-retrovirus closely related to JSRV whose envelope is able to induce cell transformation and Akt activation in the same way as JSRV (Alberti et al., 2002; Dirks et al., 2002). Experiments are in progress to understand in vivo the relevance of Y590 and to understand whether the JSRV Env is alone sufficient to induce cell transformation.

Materials and methods

Cells

All the cells used in this study were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37°C, 5% CO_2, and 95% humidity. 208F cells are an immortalized rat fibroblast cell line (Quade, 1979) (kindly provided by Dusty Miller). 208F JEflag is a cell line derived from a focus of 208F cells transformed by pCMV3JS21ΔGPcflag (see below). DF-1 cells are an immortalized chicken embryo fibroblasts (ATCC No. CRL-12203) cell line. Primary CEF were obtained by standard procedures (Vogt, 1969) from 9- to 11-day-old SPF chicken embryos obtained by SPAFAS (North Franklin, CT). The embryos were all derived from line 22, which has a C/O phenotype and consequently are permissive to ALV-A entry and replication. 293-GP and 293-GP-luc are 293-based cell lines expressing M-MuLV Gag and Pol (Clontech). 293-GP-AP were obtained by selection of 293-GP transfected with the retroviral vector pLAPSN (Miller et al., 1994) (kindly provided by Dusty Miller) expressing the human placental alkaline phosphatase. NIH-3T3-Hyal2 stably express human Hyal-2 and are susceptible to JSRV infection (Palmarini et al., 2001).

Plasmids

The pCMV3JS21ΔGP plasmid expresses the $JSRV_{21}$ (Palmarini et al., 1999) Env protein and has been described previously (Maeda et al., 2001; Palmarini et al., 2001). pCMV3JS21ΔGPcflag expresses the JSRV Env fused at the carboxyl-terminal of the protein with a FLAG epitope. Plasmids pCMV3JS21ΔGPY590D and pCMV3JS21ΔGPY-590F express the JSRV Env with Y590 mutated, respectively, into aspartic acid or phenylalanine (Palmarini et al., 2001). pSUxTMxNruIen has the SU and most of the TM

from $JSRV_{21}$ Env and the carboxyl-terminal portion of the VR3 region (including the cytoplasmic tail of TM) from the endogenous JSRV-related virus enJS56A1 as previously described (Palmarini et al., 2001). The RCASBP(A) vector is a replication-competent retroviral vector derived from Rous sarcoma virus (Federspiel and Hughes, 1997) and was kindly provided by Stephen Hughes. The JSRV Env or the mutant envelopes carrying Y590D, Y590F, and the chimeric envelope SuxTMxNruIen were inserted by standard procedures (Ausbel et al., 2000) into the *Cla*I site of RCASBP(A) to obtain RCASBP(A)+JE, RCASBP(A)+JE590D, RCASBP(A)+JE590F, and RCASBP(A)+JENruIen. pFBJ/R expresses the fos oncogene and was kindly provided by Dusty Miller (Miller et al., 1985).

Virus titration

RCASBP(A) and the derived vectors were transfected into DF-1 cells. After passaging the transfected cells for one to two passages, the supernatants were collected and stored at −70°C. Serial dilutions of the virus stocks were used to infect CEF or DF-1 cells and virus antigens were revealed by using an antigen-capture enzyme-linked immunosorbent assay (Synbiotic Corp., San Diego, CA) as instructed by the manufacturer. The final virus titer was expressed as TCIU (tissue culture infectious units) and calculated according to established procedures (Reed and Muench, 1938).

Entry assays

Susceptibility of CEF cells to JSRV entry was assessed by the ability of the JSRV Env to pseudotype MLV-based vectors (Rai et al., 2000). 293-GP-AP were transfected with the expression plasmids for the JSRV Env. Supernatants were collected and stored at −70°C. Subsequently, NIH-3T3-Hyal2 or CEF cells were exposed to 10-fold serial dilutions of the vector supernatants in the presence of Polybrene. Two days postinfection cells were fixed and stained for alkaline phosphatase positive foci. The viral titer is expressed as alkaline phosphatase focus forming units (AP^+ FFU). Experiments were performed twice.

Transformation assays

Transformation assays in DF-1 and CEF cells were performed by transfecting 1×10^6 cells with 28 μg plasmid DNA in a 10-cm-diameter dish. Cells were subsequently split when they reached confluency and passaged every 2-3 days for a variable number of passages (n = 4-7). To assess the effect of the PI3-K inhibitor LY294002 in JSRV-induced cell transformation, we performed transformation assays in 208F cells essentially as previously described (Miller et al., 1984). Cells (5×10^5 per 6-cm-diameter dish) were transfected with 10 μg of plasmid pCMV3JS21ΔGP or pFBJ/R (expressing the oncoprotein fos) using the CalPhos transfection kit (Clontech) as recommended by the manufacturer. One day after transfection, the cells were trypsinized and split into four 35-mm-diameter dishes. At this time, fresh media with 1, 5, or 10 μM of LY294002 (Sigma) was added to two dishes of transfected cells every 2 days for the duration of the assay (2 weeks). In the remaining two dishes media without the addition of LY294002 were also changed every 2 days. In an additional set of experiments, 10 μM LY294002 was added at Day 5 posttransfection (instead of Day 1 as above). Each set of experiments was performed at least three times. Foci of transformed cells were counted 15 days posttransfection. Mock-transfected cells were used to control possible toxic effects exerted by LY294002 and the total cell number was also determined at the end of each experiment.

Colony assays

Growth assays in soft agar were performed to assess anchorage independence using 6-cm-diameter petri dishes essentially as described previously (Macpherson and Montagnier, 1964). Each dish was coated with 2 ml of a base layer formed by 0.5% agar, 1× D-MEM, 20% FBS, and 10% tryptose phosphate buffer (TPB). Cells (2×10^4) were then suspended in 2 ml of incubation media containing D-MEM, 20% FBS, 10% TPB, and 0.3% agar and plated over the base layer. Cells were incubated for 2 weeks at 37°C, 5% CO_2, 95% humidity, and 2 ml of incubation media was added to the culture every 3-4 days. Colonies with a diameter above 50 μm were counted at the end of the experiment. Each experiment was done in duplicate and performed independently two times. The average size of the colonies was assessed in one of the experiments by measuring the diameter of 10% the total number of the colonies for each cell type by using the Image-Pro-Express software (Media Cybernetics).

Western blotting

For the detection of Akt, cells were grown at 37°C and 5% CO_2 in DMEM and 10% FBS as indicated above until they reached approximately 80% confluence. Cells were then washed twice with PBS and grown for another 24 h in medium lacking FBS. Cells were then lysed in buffer containing 0.5% NP-40, 50 mM HEPES buffer (pH 7.8), 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium ortovanadate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and one protease inhibitor cocktail tablet "Complete" (Roche) per 50 ml of lysis buffer. Following standard procedures, 5-10 μg of cell lysates were subjected to SDS–polyacrylamide gel electrophoresis/Western blotting (Ausbel et al., 2000). Filters were incubated with polyclonal rabbit antisera to either Akt (Cell Signaling) or Akt phosphorylated in Serine 473 (Cell Signaling) and detection was achieved using a donkey anti-rabbit Ig labeled with horseradish peroxidase (Amersham) followed by an enhanced

chemiluminescence detection system (Supersignal; Pierce) as recommended by the manufacturers.

Akt-kinase assay

Akt kinase activity was tested by using a nonradioactive Akt kinase assay kit (Cell Signaling). Briefly, decreasing amounts of cell lysates from 208F cells and 208F-JScflag were immunoprecipitated using an Akt antibody crosslinked to hydrazide agarose beads. The resulting immunoprecipitation was incubated in the presence of ATP with a recombinant GSK-3 fusion protein. Phosphorylation of GSK-3 is measured by Western blotting using a Phospho-GSK-3α/β (Ser 21/9) antibody and detected by a secondary antibody labeled with horseradish peroxidase, followed by an enhanced chemiluminescence detection system (Supersignal; Pierce).

Immunohistochemistry

Lung tumor samples from sheep affected by OPA ($n = 15$), nasal tumor samples from sheep affected by ENT ($n = 5$), or goats affected by ENT ($n = 5$), and normal olfactory mucosa were collected at the postmortem examination, fixed in Formalin, and embedded in paraffin by standard histological techniques. Sections measuring 4-6 μm were used to detect phosphorylated Akt (S-473) using a rabbit polyclonal antibody developed specifically for immunohistochemical applications as recommended by the manufacturer (Cell Signaling). Immunohistochemistry was performed by using a commercially available avidin–biotin peroxidase complex kit (Vectastain, ABC; Vector Laboratories) and diaminobenzidine as substrate. Sections were counterstained with Carazzi's hematoxylin. Controls included sections that were stained in parallel with the rabbit anti-Akt(S-473) serum preabsorbed with serial dilutions of an Akt S473 peptide (provided by Cell Signaling) and sections stained with a rabbit anti-JSRV major capsid protein as previously published (Palmarini et al., 1995).

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