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Transformation of Madin-Darby Canine Kidney Epithelial Cells by Sheep Retrovirus Envelope Proteins

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Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) induce epithelial tumors in the airways of sheep and goats. In both of these simple retroviruses, the envelope (Env) protein is the active oncogene. Furthermore, JSRV Env can transform cultured cells by two distinct mechanisms. In rat and mouse fibroblasts, the cytoplasmic tail of JSRV Env is essential for transformation, which involves activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the virus receptor hyaluronidase 2 (Hyal2) is not involved. In contrast, in the BEAS-2B human bronchial epithelial cell line, transformation is mediated by JSRV Env binding to Hyal2 followed by Hyal2 degradation and activation of the receptor tyrosine kinase RON, the activity of which is normally suppressed by Hyal2. Here we show that JSRV and ENTV Env proteins can also transform Madin-Darby canine kidney (MDCK) epithelial cells, but by a mechanism similar to that observed in fibroblast cell lines. In particular, the cytoplasmic tail of Env is required for transformation, the PI3K/Akt pathway is activated, expression of RON (which is not normally expressed in MDCK cells) does not affect transformation, and canine Hyal2 appears uninvolved. These results show that the JSRV and ENTV Env proteins can transform epithelial cells besides BEAS-2B cells and argue against a model for Env transformation involving different pathways that are uniquely active in fibroblasts or epithelial cells.

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) are two closely related simple retroviruses that cause ovine pulmonary adenocarcinoma (OPA) and enzootic nasal tumor (ENT), respectively, in sheep and goats (8). OPA and ENT are prevalent worldwide except Australia and New Zealand, and they impose significant economic impact, especially in South Africa where OPA accounts for as many as 70% of all sheep tumors (19, 25). Morphologically OPA closely resembles human bronchioloalveolar carcinoma (4, 21), a subclass of pulmonary adenocarcinoma (28). Whether JSRV or a related human retrovirus is involved in some forms of human lung cancer remains to be determined.

Concentrated JSRV virus has been shown to induce OPA in newborn sheep in as little as 10 days (26), showing that JSRV is an acutely transforming retrovirus. Several groups have demonstrated the Env protein of JSRV can transform fibroblast cell lines derived from mice (16), rats (23), and chickens (2, 33), as well as an immortalized bronchial epithelial cell line from a human (5). No other viral genes besides Env had transforming activity in cultured fibroblasts (16), indicating that Env plays an essential role in OPA. Similarly, the Env protein of ENTV can transform rodent fibroblasts (1, 7) and is thus likely to be the key oncogene responsible for ENT.

Interestingly, the JSRV Env protein transforms cultured cells by two distinct mechanisms. In fibroblasts, the cytoplasmic tail of Env protein likely interacts with a yet unidentified cellular factor(s) to trigger the phosphatidylinositol 3-kinase (PI3K)/Akt oncogenic signaling pathway (14, 20), and the virus receptor hyaluronidase 2 (Hyal2) is not involved in cell transformation (3, 12). In contrast, in BEAS-2B human epithelial cells, the Env protein indirectly activates the receptor tyrosine kinase RON, leading to transformation (5). Hyal2 is normally found associated with RON in these cells, and Env binding to Hyal2 causes Hyal2 degradation, release of RON from the complex, and constitutive activation of RON, leading to transformation. While it remains to be determined if the cytoplasmic tail of JSRV Env protein is required for BEAS-2B cell transformation, signaling through the RON pathway is critical since expression of a kinase-dead mutant of RON blocked transformation. Together these results suggested that Env transformation occurs by a fundamentally different mechanism in epithelial cells from that observed in fibroblasts and indicated the need for further study of Env transformation in epithelial cells, the natural target for oncogenesis in animals.

It should be pointed out that the BEAS-2B cell line was established by infection with an adenovirus 12/simian virus 40 hybrid virus and exhibits some features of transformation in prolonged culture, even though it does not generate tumors in nude mice (24). In addition, BEAS-2B cells strictly require a specific serum-free medium (LHC-8) to maintain a relatively normal morphology. These properties, plus low transfection efficiency, make BEAS-2B cells a difficult cell line for transformation assays. We therefore explored the use of other epithelial cell lines to further study transformation by the JSRV and ENTV Env proteins.

It has previously been demonstrated that epithelial cells of the Madin-Darby canine kidney (MDCK) line can be transformed by oncoproteins such as v-src (10), v-ras (30), and Epstein-Barr virus latent membrane protein 1 (11). Here we demonstrate that JSRV and ENTV Env proteins can also transform MDCK cells and that the mechanism of transforma-

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tion is similar to that observed in fibroblasts rather than that observed in BEAS-2B epithelial cells.

MATERIALS AND METHODS

Cell lines. MDCK cells (ATCC CCL-34) and SSF-123 primary sheep skin fibroblasts (gift from William Osborne, University of Washington, Seattle) were grown in Dulbecco's modified Eagle's medium (DMEM) with a high concentration of glucose (4.5 g/liter) and 10% fetal bovine serum at 37°C in a 10% CO_2–air atmosphere at 100% relative humidity. The transformed MDCK cells were grown under the same conditions, except that 5% fetal bovine serum was used.

Plasmids. The plasmids expressing the JSRV and ENTV Env proteins and their mutants have been previously described (14). Briefly, all Env cDNAs were cloned into pSX2neo, an expression vector in which the gene of interest is driven by the Moloney murine leukemia virus promoter. All JSRV Env constructs were tagged with identical FLAG sequences at their carboxy termini, and ENTV Env constructs were tagged with identical FLAG sequences at their amino termini. The amino-terminal FLAG tag was preceded by a preproinsulin endoplasmic reticulum signal sequence, and these sequences were added in place of the natural ENTV Env signal sequence. The retroviral vector plasmid based on pMSCV-puro that encodes human RON has been described previously (5).

Viruses and transduction. The JSRV (PJ4) (22) and ENTV (PN172) (7) packaging cell lines have been described previously. PN229 packaging cells produce vectors with a hybrid ENTV/JSRV Env which leads to higher-titer production of vectors with an ENTV host range (29). The LJeSN (7) and LAPSN (18) vectors express JSRV Env protein and human placental alkaline phosphatase (AP), respectively: expression of the cDNAs is driven from the Moloney murine leukemia virus promoter, and expression of neomycin phosphotransferase (Neo) is driven by an internal simian virus 40 (SV40) early promoter (17). Vesicular stomatitis virus G (VSV-G) pseudotypes were generated by transfection of 293/LAPSN cells with a plasmid encoding VSV-G as described previously (13). For transduction, cells were seeded at 7.5×10^4 cells per well in six-well plates on day 0 and were transduced in the presence of Polybrene (4 μg/ml) on day 1. LAPSN vector titer was determined by AP staining on day 4, and transformed foci were examined 2 weeks after transduction.

Antibodies. Antibodies against phospho-Akt and Akt were purchased from Cell Signaling Technology (Beverly, Mass.). Monoclonal anti-FLAG antibody was purchased from Sigma (Sigma, St. Louis, Mo.). Monoclonal antiphosphotyrosine antibody 4G10 and polyclonal anti-p85 antibody were purchased from Upstate Biotechnology (Lake Placid, N.Y.). Anti-RON polyclonal antibody (C-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). All primary antibodies were used at 1:1,000 dilutions unless otherwise noted. The secondary horseradish peroxidase-conjugated antimouse or antirabbit antibodies were purchased from DAKO (Glostrup, Denmark) and were used at 1:1,000 to 1:2,000 dilutions.

CoIP and IB. Cells were serum starved overnight by cultivation in DMEM without serum and were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100) in the presence of protease inhibitor cocktail (Sigma) plus 1 mM sodium orthovanadate, 20 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. For coimmunoprecipitation (CoIP), approximately 1-mg (protein) samples of cell lysates were incubated with antibody in the presence of prewashed protein A Sepharose beads at 4°C for 3 h. Following several washes, the resulting precipitates were then resuspended with lysis buffer, boiled, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblotting (IB), ~30-μg (protein) samples of cell lysates were subjected to SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech., Buckinghamshire, England) and IB using appropriate primary and secondary antibodies followed by enhanced chemiluminescence (ECL) detection (Pierce, Rockford, Ill.). For reblotting, PVDF membranes that had been previously blotted were stripped and were reblotted with an appropriate antibody.

RESULTS

MDCK cells can be transformed by JSRV and ENTV Env proteins. Transfection of MDCK cells with plasmids encoding Neo and either the JSRV or ENTV Env proteins yielded transformed colonies of rounded, loosely attached cells after ~10 days of G418 selection (Fig. 1, top panels). In contrast, G418-selected colonies of MDCK cells transfected with a control plasmid encoding Neo and the 10A1 MLV Env protein exhibited a cobblestone morphology typical of untreated MDCK cells (not shown). Transduction of MDCK cells with a retroviral vector encoding JSRV Env (LJeSN) also induced transformed foci in the cell monolayer (Fig. 1, right middle panel), while MDCK cells transduced with an otherwise identical retroviral vector encoding human placental alkaline phosphatase (LAPSN) showed a flat morphology typical of parental MDCK cells (Fig. 1, left middle panel). When plated at low density, MDCK cells transformed by JSRV Env were refractile, attached poorly to the culture dish, and grew as isolated cells (Fig. 1, lower right panel), in sharp contrast to the parental MDCK cells that grew as cobblestone-like clusters (Fig. 1, lower left panel). Quantitatively, the transforming activities of JSRV and ENTV Env proteins in MDCK cells were similar, with ~30 transformed colonies per dish transfected with 2.5 μg of DNA (Table 1).

The cytoplasmic tail of JSRV Env, but not the YXXM motifs present in the cytoplasmic tails of the JSRV and ENTV Env proteins, is essential for MDCK cell transformation. Transfection of MDCK cells with a plasmid encoding JSRV-10A1, a chimeric Env with the cytoplasmic tail of JSRV Env having been replaced by that of 10A1 Moloney leukemia virus, did not induce transformation (Table 1), indicating that the cytoplasmic tail of JSRV Env protein is necessary for MDCK cell transformation. The cytoplasmic tails of the JSRV and ENTV Env proteins both contain YXXM peptide motifs that in other proteins mediate binding to the p85 regulatory component of PI3K (27). However, mutation of the tyrosine or methionine residues within the YXXM motifs present in the JSRV or ENTV Env proteins failed to abolish their transforming activities in MDCK cells (Table 1). Notably, the Y590 mutants of JSRV Env (Jenv-Y590F-FLAG and Jenv-Y590C-FLAG) and ENTV Env (FLAG-Eenv-Y590F), as well as the triple-tyrosine mutant of ENTV Env (FLAG-Eenv-Y590,2,6F), exhibited relatively high transforming activities on MDCK cells (3 to 33% of the transforming activity of the parental Env proteins). These results are similar to our previous findings in 208F and NIH 3T3 fibroblasts, although the relative transforming activities of the YXXM mutants were higher in MDCK cells than in the fibroblast cell lines (14).

The PI3K/Akt pathway is activated in MDCK cells transformed by the JSRV or ENTV Env proteins and their YXXM mutants. We isolated MDCK cells that were fully transformed by each Env construct. Akt was phosphorylated at residue Ser473 in serum-starved MDCK cells transformed by JSRV or ENTV Env proteins, and Akt phosphorylation was completely inhibited by 10 or 25 μM LY294002 (LY), a PI3K-specific inhibitor, indicating that Akt activation in the transformed cells was PI3K dependent (Fig. 2). To check whether PI3K was important for maintaining the transformed phenotype of JSRV or ENTV Env-transformed MDCK cells, we treated the cells with 10 μM LY for 3 days. LY is unstable in culture medium, so we replaced the medium daily. LY treatment partially converted the rounded, transformed morphology to a relatively flat morphology (data not shown), indicating that PI3K activation is required for MDCK cell transformation.

We next examined whether Akt was activated in MDCK cells transformed by the JSRV and ENTV Env YXXM mutants. Similar to the results obtained with 208F rat fibroblasts (14), Akt phosphorylation was evident in MDCK cells trans-

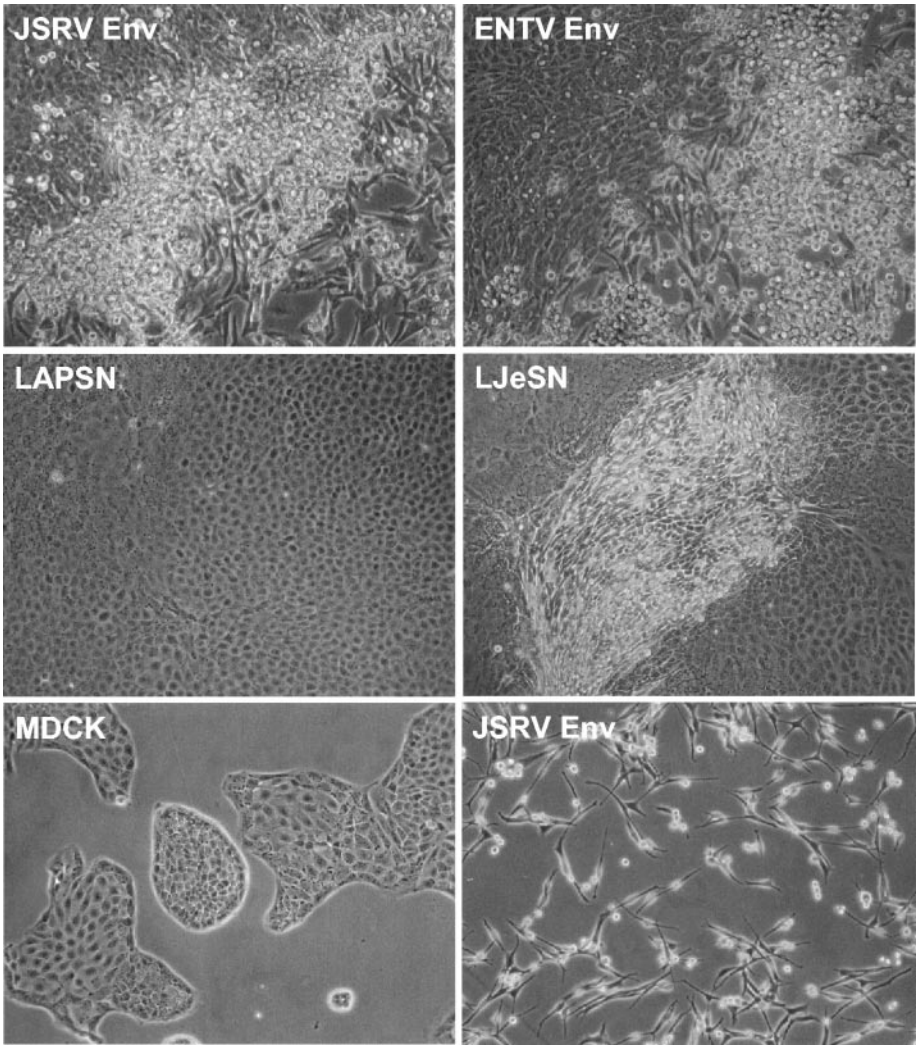

FIG. 1. Transformation of MDCK cells by JSRV and ENTV Env proteins. MDCK cells were transfected with plasmids encoding the Env proteins of JSRV (JSRV Env) or ENTV (ENTV Env) by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) or were transduced by retroviral vectors encoding AP (LAPSN) or JSRV Env (LJeSN). The next day cells were trypsinized and seeded at a 1:2 dilution into 6-cm-diameter dishes in the presence (for JSRV Env and ENTV Env) or absence (for LJeSN and LAPSN) of G418 (600-μg/ml active concentration). The culture medium was replaced every 3 days, and transformed foci were photographed 14 days after DNA transfection or viral transduction (top four panels). The lower panels show the parental MDCK cells (left) and the JSRV Env-transformed MDCK cells (right) seeded at low density and photographed 4 days later.

formed by all YXXM mutants tested (Fig. 3), and Akt activation in these cells was completely inhibited by 10 or 25 μM LY (data not shown), indicating that Akt activation in these cells was also PI3K dependent.

The Env protein of JSRV is not tyrosine phosphorylated in the transformed MDCK cells. Interaction between the YXXM motifs of receptor tyrosine kinases and the p85 regulatory subunit of PI3K is dependent on tyrosine phosphorylation of the motif (27). To test whether the YXXM motifs in the JSRV Env protein were phosphorylated, we immunoprecipitated the Env protein from lysates of the transformed cells with an anti-FLAG antibody and immunoblotted the precipitates using 4G10, a monoclonal antibody that specifically detects tyrosine-phosphorylated proteins. No tyrosine phosphorylation of JSRV Env protein was detected (Fig. 4A, lane 1). Reblotting of the same PVDF membrane showed that Env protein, especially the transmembrane (TM) subunit, was present in the immunoprecipitate (Fig. 4B, lane 1). Nor was phosphorylated Env detected in immunoblots of total cell lysates harvested from JSRV Env-transformed cells (Fig. 4A, lane 3). We also were unable to detect phosphorylation of ENTV Env, but this protein was FLAG tagged at the amino terminus, and the Env protein was barely detectable after immunoprecipitation; thus, it was difficult to draw a firm conclusion regarding the lack of ENTV Env phosphorylation (data not shown).

JSRV Env protein does not coimmunoprecipitate with PI3K in the transformed MDCK cells. To further explore a possible interaction of Env with PI3K in MDCK cells transformed by JSRV Env, we used a CoIP approach. Although JSRV Env protein could be successfully immunoprecipitated from cell lysates by using an anti-FLAG antibody (Fig. 5B, lane 1), the p85 regulatory subunit of PI3K that is abundantly present in

TABLE 1. Transforming activities of JSRV and ENTV Env proteins and their YXXM mutants in MDCK cells[a]

Env protein[b]	No. of transformed colonies/dish (2.5 μg of DNA)	
	Expt 1	Expt 2
Jenv-FLAG	27, 24	26, 24
JSRV-10A1	0, 0	0, 0
Jenv-Y590F-FLAG	6, 5	4, 6
Jenv-Y590C-FLAG	5, 9	6, 8
Jenv-M593A-FLAG	22, 25	16, 19
Jenv-M593E-FLAG	5, 8	6, 8
FLAG-Eenv	32, 28	30, 33
FLAG-Eenv-Y590F	7, 9	8, 9
FLAG-Eenv-Y590,2,6F	2, 1	1, 1
10A1 Env	0, 0	0, 0

[a] MDCK cells were seeded at 5×10^5 cells per well in six-well plates on day 0, were transfected with 5 μg of plasmids encoding the indicated Env proteins using Lipofectamine 2000 (Invitrogen) on day 1, and were trypsinized and seeded at a 1:2 dilution into 6-cm-diameter dishes on day 2 in medium containing G418 (600 μg/ml active concentration). After ≈10 days of drug selection, nontransfected MDCK cells were all dead. Transformed and G418-resistant colonies were scored approximately 2 weeks after DNA transfection. The numbers of G418-resistant colonies were comparable among all dishes (≈100 per dish) except for those transfected with 10A1 Env (≈50 per dish). Results from two independent experiments are presented.

[b] All JSRV Env constructs were tagged with FLAG sequences at their carboxy termini, while all ENTV Env constructs were FLAG tagged at their amino termini. JSRV-10A1 is a construct encoding a chimeric JSRV Env with its cytoplasmic tail being replaced by that of 10A1. For details, refer to reference 14.

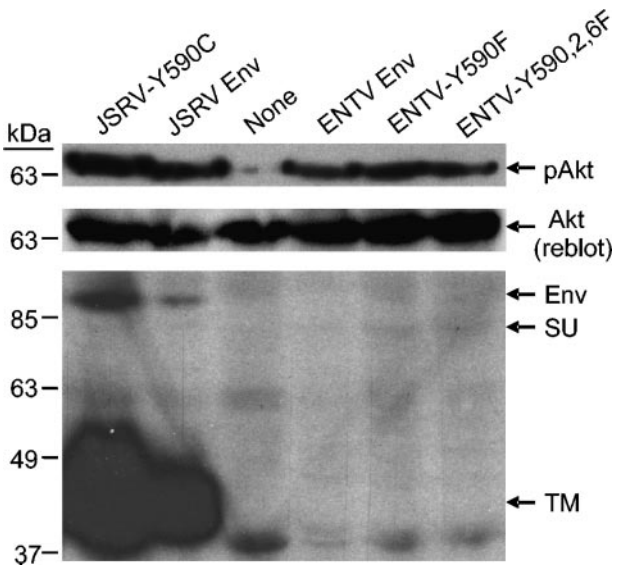

FIG. 3. Akt is activated in MDCK cells transformed by YXXM mutants of JSRV/ENTV Env. Measurement of Akt activation (pAkt) and total Akt was performed as described in the legend to Fig. 2. The lower panel shows an immunoblot using anti-FLAG antibodies to detect FLAG-tagged Env proteins. The FLAG-tagged Env protein expressed in each cell line is listed above. Note that the full-length and SU portion of ENTV Env were barely detected by these methods.

MDCK cells (Fig. 5A, lanes 3 and 4) was not detected in immunoprecipitates (Fig. 5A, lane 1), indicating that JSRV Env protein does not interact with p85 in the transformed cells, although it is possible that the proximity of the FLAG tag to the presumed PI3K binding site might prevent binding of both proteins at the same time. This result is consistent with the finding above that the YXXM motif of the JSRV Env protein was not tyrosine phosphorylated in the transformed MDCK cells and thus should not bind PI3K. Together these results and those using 208F fibroblasts (14) indicate that the Env proteins activate PI3K through as yet unidentified adaptor molecule(s).

RON is not expressed in MDCK cells, and RON expression does not enhance Env transformation. Previous studies have shown that RON is not expressed in MDCK cells, as determined by IB with antibodies against RON and by showing that the RON ligand, macrophage-stimulating protein (MSP), does not bind to or induce scattering of MDCK cells (6, 31, 32). We confirmed the immunoblotting (Fig. 6, lane 6) and scattering results (data not shown) for the MDCK cells used here. How-

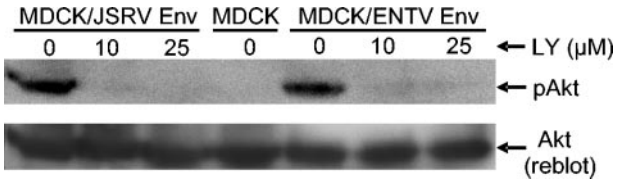

FIG. 2. Akt is activated in MDCK cells transformed by JSRV or ENTV Env proteins, and Akt activation is PI3K dependent. MDCK cells transformed by JSRV or ENTV Env protein were grown overnight in serum-free DMEM and were then treated with LY at the indicated concentrations for 1 h. Cells were lysed and were subjected to SDS-PAGE followed by IB with antibodies against phospho-Akt (upper panel). The PVDF membrane was stripped and reblotted with anti-Akt (lower panel). In both blots, the PVDF membrane was blocked with 1% nonfat milk at room temperature for 1 h, followed by incubation overnight with anti-phospho-Akt or anti-Akt antibodies diluted 1:1,000 in 5% bovine serum albumin. The ECL-Plus detection system (Amersham BioSciences) was used for antibody detection.

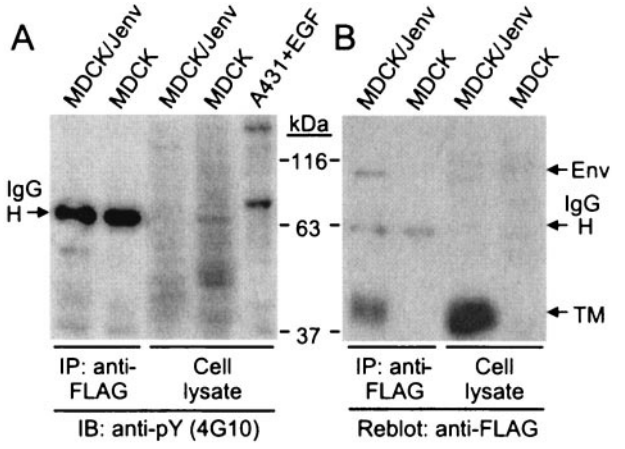

FIG. 4. JSRV Env protein is not tyrosine phosphorylated in the transformed MDCK cells. (A) MDCK cells transformed by the JSRV Env protein and the parental MDCK cells were grown in serum-free medium overnight. Cell lysates were prepared and portions were subjected to immunoprecipitation with anti-FLAG antibody (IP: anti-FLAG). Lysates and immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting, using anti-phosphotyrosine antibody 4G10. The lane labeled A431+EGF contained lysate from A431 cells that had been stimulated by epidermal growth factor (EGF; provided by Upstate Biotechnology and used as a positive control for 4G10 antibody binding). (B) The same PVDF membrane was stripped and reblotted with anti-FLAG antibody. The TM subunit and full-length JSRV Env proteins are indicated by arrows, while the SU and full-length ENTV Env proteins were not detected in this assay. IgG H indicates the IgG heavy chain.

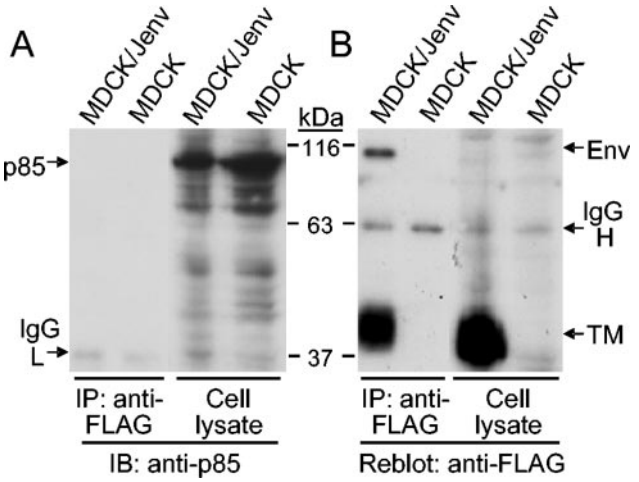

FIG. 5. JSRV Env protein does not coimmunoprecipitate with PI3K (p85) in the transformed MDCK cells. Preparation of samples was the same as described in the legend to Fig. 4. The resulting immunoprecipitates and cell lysates were subjected to SDS-PAGE, immunoblotted with anti-p85 (A), and then stripped and reblotted with anti-FLAG (B). IgG H and IgG L indicate the IgG heavy and light chains, respectively.

ever, based on the model of RON activation by JSRV Env in other cell types (5), it is possible that RON expression in MDCK cells might augment the transformation response of MDCK cells to Env. To address this possibility, we transfected MDCK cells with a retroviral vector encoding RON and puromycin phosphotransferase, selected the cells in puromycin (1 μg/ml), and isolated clonal cell lines. We screened the clones for RON expression by IB (Fig. 6, lanes 3 to 5) and by examination of scattering after treatment with MSP (data not shown). All of the selected clones exhibited RON phosphorylation in the absence of MSP stimulation (Fig. 6, lanes 3 to 5) and those that expressed the most RON exhibited high RON phosphorylation and a transformed phenotype in the absence of MSP stimulation (data not shown). We chose a relatively flat RON-expressing MDCK clone to test the effect of RON on transformation by JSRV or ENTV Env proteins. JSRV and ENTV Env induced equivalent numbers of transformed colonies in RON-expressing cells and in the parental MDCK cells

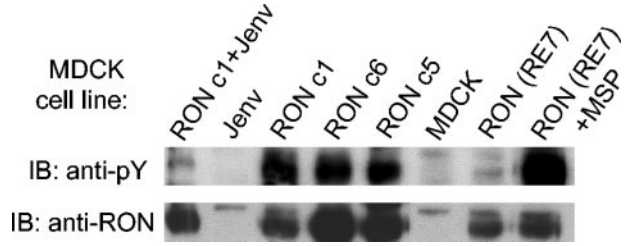

FIG. 6. RON expression and phosphorylation in MDCK cells expressing RON and/or JSRV Env (Jenv). Cell lysates were subjected to IB using 4G10 antibody (anti pY) or RON antibody (anti-RON). The proteins expressed in the MDCK cells are indicated with the clone numbers for the cells expressing RON. The last two lanes show lysates from a previously isolated cell line that expresses a small amount of RON (RE7) (32) after incubation with or without 1.1 nM MSP for 20 min.

TABLE 2. RON expression has no effect on MDCK cell transformation by JSRV and ENTV Env proteins[a]

Expressed protein	No. of transformed colonies induced by Env proteins			
	JSRV	ENTV	JSRV-10A1[b]	10A1
RON	28, 32	34, 30	0, 0	0, 0
None	24, 29	31, 35	0, 0	0, 0

[a] Transformation assays were performed as described in Table 1. Results are representative of two independent experiments.
[b] JSRV-10A1 indicates a chimeric JSRV Env with its cytoplasmic tail replaced by that of 10A1 murine leukemia virus (14).

(~30 per dish), showing that RON did not enhance MDCK cell transformation by the JSRV or ENTV Env proteins (Table 2).

We next examined the level of RON phosphorylation in MDCK cells expressing RON and/or JSRV Env to see whether Env stimulated RON activity as measured by RON phosphorylation. No band corresponding to phosphorylated RON was observed in MDCK cells, as expected, since these cells do not make RON (Fig. 6, lane 6). JSRV Env expression did not induce RON expression, nor was a band corresponding to phosphorylated RON observed (Fig. 6, lane 2). Surprisingly, JSRV Env expression in MDCK cells expressing RON caused a marked reduction in RON phosphorylation, while the level of RON expression was relatively unchanged (Fig. 6, compare lanes 1 and 3), the opposite of what was expected based on the model for Env transformation of BEAS-2B cells (5). To help explain these results, we performed a CoIP assay but failed to detect an interaction between JSRV Env and RON (data not shown). These results show that RON is not involved in transformation of MDCK cells by JSRV or ENTV Env proteins and that, even when RON is expressed in MDCK cells, its activity is not stimulated by JSRV Env expression.

Canine Hyal2 appears to play no role in MDCK cell transformation. Given the role of Hyal2 in Env transformation of BEAS-2B epithelial cells, we addressed the possibility that the Hyal2 protein present in MDCK cells might play a role in MDCK cell transformation. We first examined whether canine Hyal2 could function as a receptor for JSRV or ENTV in MDCK cells. A JSRV vector exhibited a titer of 1.7×10^3 AP focus-forming units (FFU)/ml on MDCK cells (Table 3), showing that canine Hyal2 can function as a receptor for JSRV. However, ENTV vectors made with either PN172 or PN229 retrovirus packaging cells were unable to transduce MDCK cells (titer of <10 FFU/ml), showing that canine Hyal2 does not support ENTV entry into MDCK cells, presumably due to

TABLE 3. Titers of JSRV and ENTV vectors on MDCK cells

Vector pseudotype (packaging cell line)	Vector titer (AP^+ FFU/ml) on[a]:	
	MDCK cells	SSF cells
JSRV (PJ4)	1.7×10^3	3.8×10^5
ENTV (PN172)	<10	6.0×10^3
ENTV (PN229)	<10	3.0×10^5
VSV-G	1.5×10^5	4.2×10^4

[a] Vector titers were determined by exposure of MDCK or cells (seeded in six-well plates the day before at 7.5×10^4 to 7.5×10^5 cells per well) to serial dilutions of viral stocks, followed by AP staining. The titers were representative of at least two independent experiments, and results varied by <10%.

a lack of interaction between canine Hyal2 and ENTV Env protein.

To directly measure the interaction of ENTV Env with canine Hyal2 expressed on MDCK cells, we used a fusion protein consisting of the ENTV surface subunit (SU) domain linked to a human immunoglobulin G (IgG) constant region fragment and measured binding of this protein to MDCK cells and MDCK cells expressing human Hyal2, as previously described (29). The titer of ENTV pseudotype LAPSN vector (made by using PN229 packaging cells) on MDCK cells expressing human Hyal2 was 5×10^5 AP^+ FFU/ml, showing that the human Hyal2 receptor was functional in the MDCK cells. Binding of the ENTV SU-IgG to MDCK cells was 10-fold lower than binding to MDCK cells expressing human Hyal2 (data not shown), supporting the hypothesis that ENTV Env does not interact with canine Hyal2. Since we cannot tell from these experiments whether the low level of ENTV SU-IgG binding to MDCK cells is due to nonspecific binding or to a low level of specific binding to canine Hyal2, we cannot rule out an interaction between ENTV Env and canine Hyal2. However, the fact that JSRV but not ENTV vectors can transduce MDCK cells, yet both Env proteins transform the cells at the same rate, argues that Env interaction with canine Hyal2 is not important for Env transformation of MDCK cells.

DISCUSSION

Cell transformation by the sheep retrovirus Env proteins involves two distinct pathways. One is mediated by the cytoplasmic tail of Env that indirectly activates the PI3K/Akt signaling pathway, and the viral receptor Hyal2 is not involved. Another involves the receptor tyrosine kinase RON, and Hyal2 plays a critical role. The first mechanism is operative in transformed mouse, rat, and chicken fibroblasts (2, 3, 12, 14, 20, 33), while the second mechanism is operative in immortalized BEAS-2B human lung epithelial cells transformed by JSRV Env protein (5). In this study, we show that JSRV and ENTV Env proteins can transform MDCK cells through a mechanism similar to what we found in 208F rat fibroblasts (14). First, the cytoplasmic tail of Env protein is essential for transformation of MDCK cells, as evidenced by the fact that JSRV-10A1 chimeric Env protein lacking the cytoplasmic tail of JSRV Env fails to transform MDCK cells. Second, the YXXM motifs of Env proteins are not absolutely required for MDCK cell transformation, as all YXXM mutants of JSRV/ENTV Env still transformed MDCK cells. Third, Akt is activated in MDCK cells transformed by either parental Env or their YXXM mutants, and Akt phosphorylation in the transformed cells is PI3K dependent. Fourth, the Env proteins are not tyrosine phosphorylated in the transformed cells, nor does there appear to be an interaction between Env proteins and p85, the regulatory subunit of PI3K. Fifth, RON plays no role in MDCK transformation because it is not expressed in these cells, nor does expression of RON increase transformation by Env. Last, Hyal2 appears to play no role in MDCK cell transformation. Collectively, we conclude that the mechanism of Env-mediated transformation in MDCK cells is similar to that found in rodent fibroblasts and distinct from that obtained with BEAS-2B human epithelial cells.

Results presented here and in prior studies show that the cytoplasmic tail of the JSRV Env protein is essential for transformation of MDCK epithelial cells and rodent and chicken fibroblasts (2, 14, 20). However, it is unclear whether it is sufficient for transformation. A recent study shows that the carboxy-terminal 141 amino acids of JSRV Env protein can efficiently transform 208F cells (3), but it is unknown whether the membrane-spanning domain and/or the extracellular domain of JSRV Env that are included in this construct (referred to as pMyr-JSE6770 in reference 3) contribute to the observed transforming activity. We have tested this construct in MDCK cells but failed to observe any transforming activity and only detected a minimal level of transforming activity in 208F cells (data not shown). Thus more work is needed to resolve the issue of whether the cytoplasmic tail of Env protein alone is sufficient for cell transformation.

We demonstrate in this study that Akt is activated in MDCK cells transformed by either JSRV or ENTV Env proteins or their YXXM mutants and that Akt phosphorylation is dependent on PI3K (Fig. 2 and 3). However, we found no evidence for Env tyrosine phosphorylation nor an interaction between Env and the p85 regulatory subunit of PI3K in the transformed MDCK cells (Fig. 4 and 5), indicating that Env proteins must interact with an as yet unidentified adaptor(s) to activate the PI3K/Akt signaling pathway. A similar mechanism has been previously proposed for Env-mediated cell transformation in 208F rat fibroblasts (14). Interestingly, Maeda et al. recently reported that PI3K is not required for mouse NIH 3T3 cell transformation by JSRV Env protein, although Akt phosphorylation was still detected in the transformed cells (15). While this discrepancy may result from the use of different cell types for transformation assays and/or different experimental procedures, this study does open up another possibility that the Env protein of JSRV Env, and ENTV as well, may transform cells through a PI3K-independent Akt activation pathway. In this sense, it will be important to hunt for the Env-interacting molecules in these transformed cells, study their interactions with Env and with the downstream signaling molecules, and explore their roles in cell transformation. Identification of adaptor molecules that interact with Env proteins may also reveal new signaling pathways involved in cell transformation by JSRV/ENTV Env proteins.

Although Akt activation is evident in MDCK cells transformed by JSRV or ENTV Env proteins, the intensity of Akt phosphorylation is weaker than what we observe in 208F cells. This could be due to a relatively low-level expression of total Akt in MDCK cells or to poor recognition of the canine Akt by the antibodies we used in this study. Accordingly, we have modified our routine IB procedure to increase its sensitivity for detection of the phospho- and total Akt proteins in MDCK cells (see Fig. 2 legend). It is noteworthy that the extent of Akt phosphorylation in the transformed MDCK cells correlated with the transformed phenotype of the cells, with the most highly transformed cells showing very clear Akt phosphorylation (data not shown). However, it is possible that PI3K-activated signaling pathways other than that involving Akt are also important for cell transformation.

The results reported here reinforce the notion that sheep retrovirus Env proteins can transform cells by different mechanisms in different cell types. A similar phenomenon has been previously noticed for other oncogene-mediated cell transfor-

mations. For instance, transformation of mouse NIH 3T3 cells by p21 Ras is mediated by activation of Raf-1, while transformation of primary human embryonic kidney epithelial cells by Ras is mediated by Ral-GDS (9). Sheep retroviruses specifically infect airway epithelial cells, leading to OPA or ENT; therefore, it would be important to determine if ovine epithelial cell lines, preferably the primary epithelial cells of sheep or goats, can be transformed by the JSRV or ENTV Env proteins and investigate the underlying mechanisms. Although they are difficult to grow, a technique for isolating and culturing primary sheep type II pneumocytes, the predominant target for JSRV infection, has recently been developed (J. C. DeMartini, personal communication). Ultimately, it will be important to explore which, if any, of the mechanisms revealed by in vitro cell transformation are relevant to oncogenesis in sheep or in small animal models of the sheep disease and investigate the possible implications of sheep retroviral oncogenesis in our understanding of human lung cancer, in particular bronchiolalveolar carcinoma.

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REFERENCES

1. **Alberti, A., C. Murgia, S.-L. Liu, M. Mura, C. Cousens, M. Sharp, A. D. Miller, and M. Palmarini.** 2002. Envelope-induced cell transformation by ovine betaretroviruses. J. Virol. **76:**5387–5394.
2. **Allen, T. E., K. J. Sherrill, S. M. Crispell, M. R. Perrott, J. O. Carlson, and J. C. DeMartini.** 2002. The jaagsiekte sheep retrovirus envelope gene induces transformation of the avian fibroblast cell line DF-1 but does not require a conserved SH2 binding domain. J. Gen. Virol. **83:**2733–2742.
3. **Chow, Y.-H. J., A. Alberti, M. Mura, C. Pretto, P. Murcia, L. M. Albritton, and M. Palmarini.** 2003. Transformation of rodent fibroblasts by the jaagsiekte sheep retrovirus envelope is receptor independent and does not require the surface domain. J. Virol. **77:**6341–6350.
4. **Clayton, F.** 1988. The spectrum and significance of bronchioloalveolar carcinomas. Pathol. Annu. **23:**361–394.
5. **Danilkovitch-Miagkova, A., F.-M. Duh, I. Kuzmin, D. Angeloni, S.-L. Liu, A. D. Miller, and M. I. Lerman.** 2003. Hyaluronidase 2 negatively regulates RON receptor tyrosine kinase and mediates transformation of epithelial cells by jaagsiekte sheep retrovirus. Proc. Natl. Acad. Sci. USA **100:**4580–4585.
6. **Danilkovitch-Miagkova, A., A. Miagkov, A. Skeel, N. Nakaigawa, B. Zbar, and E. J. Leonard.** 2001. Oncogenic mutants of RON and MET receptor tyrosine kinases cause activation of the β-catenin pathway. Mol. Cell. Biol. **21:**5857–5868.
7. **Dirks, C., F.-M. Duh, S. K. Rai, M. I. Lerman, and A. D. Miller.** 2002. Mechanism of cell entry and transformation by enzootic nasal tumor virus. J. Virol. **76:**2141–2149.
8. **Fan, H. (ed.).** 2003. Jaagsiekte sheep retrovirus and lung cancer. Curr. Top. Microbiol. Immunol. **275:**1–248.
9. **Hamad, N. M., J. H. Elconin, A. E. Karnoub, W. Bai, J. N. Rich, R. T. Abraham, C. J. Der, and C. M. Counter.** 2002. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. **16:**2045–2057.
10. **Kadono, Y., Y. Okada, M. Namiki, M. Seiki, and H. Sato.** 1998. Transformation of epithelial Madin-Darby canine kidney cells with p60(v-src) induces expression of membrane-type 1 matrix metalloproteinase and invasiveness. Cancer Res. **58:**2240–2244.
11. **Kim, K. R., T. Yoshizaki, H. Miyamori, K. Hasegawa, T. Horikawa, M. Furukawa, S. Harada, M. Seiki, and H. Sato.** 2000. Transformation of Madin-Darby canine kidney (MDCK) epithelial cells by Epstein-Barr virus latent membrane protein 1 (LMP1) induces expression of Ets1 and invasive growth. Oncogene **19:**1764–1771.
12. **Liu, S.-L., F.-M. Duh, M. I. Lerman, and A. D. Miller.** 2003. Role of virus receptor Hyal2 in oncogenic transformation of rodent fibroblasts by sheep betaretrovirus Env proteins. J. Virol. **77:**2850–2858.
13. **Liu, S.-L., C. L. Halbert, and A. D. Miller.** 2004. Jaagsiekte sheep retrovirus envelope efficiently pseudotypes human immunodeficiency virus type 1-based lentiviral vectors. J. Virol. **78:**2642–2647.
14. **Liu, S.-L., M. I. Lerman, and A. D. Miller.** 2003. Putative phosphatidylinositol 3-kinase (PI3K) binding motifs in ovine betaretrovirus Env proteins are not essential for rodent fibroblast transformation and PI3K/Akt activation. J. Virol. **77:**7924–7935.
15. **Maeda, N., Y. Inoshima, D. A. Fruman, S. M. Brachmann, and H. Fan.** 2003. Transformation of mouse fibroblasts by Jaagsiekte sheep retrovirus envelope does not require phosphatidylinositol 3-kinase. J. Virol. **77:**9951–9959.
16. **Maeda, N., M. Palmarini, C. Murgia, and H. Fan.** 2001. Direct transformation of rodent fibroblasts by jaagsiekte sheep retrovirus DNA. Proc. Natl. Acad. Sci. USA **98:**4449–4454.
17. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
18. **Miller, D. G., R. H. Edwards, and A. D. Miller.** 1994. Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. Proc. Natl. Acad. Sci. USA **91:**78–82.
19. **Palmarini, M., H. Fan, and J. M. Sharp.** 1997. Sheep pulmonary adenomatosis: a unique model of retrovirus-associated lung cancer. Trends Microbiol. **5:**478–483.
20. **Palmarini, M., N. Maeda, C. Murgia, C. De-Fraja, A. Hofacre, and H. Fan.** 2001. A phosphatidylinositol 3-kinase docking site in the cytoplasmic tail of the Jaagsiekte sheep retrovirus transmembrane protein is essential for envelope-induced transformation of NIH 3T3 cells. J. Virol. **75:**11002–11009.
21. **Perk, K., and I. Hod.** 1982. Sheep lung carcinoma: an endemic analogue of a sporadic human neoplasm. J. Natl. Cancer Inst. **69:**747–749.
22. **Rai, S. K., J. C. DeMartini, and A. D. Miller.** 2000. Retrovirus vectors bearing jaagsiekte sheep retrovirus Env transduce human cells by using a new receptor localized to chromosome 3p21.3. J. Virol. **74:**4698–4704.
23. **Rai, S. K., F.-M. Duh, V. Vigdorovich, A. Danilkovitch-Miagkova, M. I. Lerman, and A. D. Miller.** 2001. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. Proc. Natl. Acad. Sci. USA **98:**4443–4448.
24. **Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, J. B. Park, J. S. Rhim, and C. C. Harris.** 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. **48:**1904–1909.
25. **Sharp, J. M., and K. W. Angus.** 1990. Sheep pulmonary adenomatosis: studies on its etiology, p. 177–185. *In* G. Petursson and R. Hoff-Jogensen (ed.), Maedi-Visna and related diseases. Kluwer Academic Publishers, Boston, Mass.
26. **Sharp, J. M., K. W. Angus, E. W. Gray, and F. M. Scott.** 1983. Rapid transmission of sheep pulmonary adenomatosis (jaagsiekte) in young lambs. Brief report. Arch. Virol. **78:**89–95.
27. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider et al.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
28. **Travis, W. D., T. V. Colby, B. Corrin, Y. Shimosato, and E. Brambilla.** 1999. Histological typing of lung and pleural tumors. International histological classification of tumors, 3rd ed. World Health Organization, Geneva, Switzerland.
29. **Van Hoeven, N. S., and A. D. Miller.** 2005. Improved enzootic nasal tumor virus pseudotype packaging cell lines reveal virus entry requirements in addition to the primary receptor Hyal2. J. Virol. **79:**87–94.
30. **Vleminckx, K., L. Vakaet, Jr., M. Mareel, W. Fiers, and F. van Roy.** 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell **66:**107–119.
31. **Wang, D., Q. Shen, Y. Q. Chen, and M. H. Wang.** 2004. Collaborative activities of macrophage-stimulating protein and transforming growth factor-beta1 in induction of epithelial to mesenchymal transition: roles of the RON receptor tyrosine kinase. Oncogene **23:**1668–1680.
32. **Wang, M. H., C. Ronsin, M. C. Gesnel, L. Coupey, A. Skeel, E. J. Leonard, and R. Breathnach.** 1994. Identification of the ron gene product as the receptor for the human macrophage stimulating protein. Science **266:**117–119.
33. **Zavala, G., C. Pretto, Y. H. Chow, L. Jones, A. Alberti, E. Grego, M. De las Heras, and M. Palmarini.** 2003. Relevance of Akt phosphorylation in cell transformation induced by Jaagsiekte sheep retrovirus. Virology **312:**95–105.