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Virology 350 (2006) 347–357
VIROLOGY
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Association of RON tyrosine kinase with the Jaagsiekte sheep retrovirus envelope glycoprotein

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Received 15 December 2005; returned to author for revision 6 January 2006; accepted 26 January 2006
Available online 28 February 2006

Abstract

The envelope (Env) of Jaagsiekte sheep retrovirus (JSRV) functions as an oncoprotein. One of the mechanisms of JSRV-induced cell transformation that has been proposed for epithelial cells involves JSRV Env binding Hyaluronidase 2 (the JSRV receptor), thereby inducing its degradation and allowing the release and activation of RON tyrosine kinase which is normally suppressed by HYAL-2. In this study, we report that HYAL-2 and RON are not critical for the JSRV Env-induced transformation of the rat epithelial cell line IEC-18, while the cytoplasmic tail of the JSRV Env is critical to transform this cell line. We have also determined that RON can associate with the JSRV Env under normal and stringent conditions. In addition, the cytoplasmic tail of the JSRV and the enJS5F16 (non oncogenic JSRV-related endogenous retrovirus) Env proteins appears to have a major influence on the activation status of RON. Thus, it appears that the interaction of the JSRV Env with RON is more complex than previously thought and requires further investigation.
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Keywords: Jaagsiekte sheep retrovirus; JSRV; enJSRVs; Endogenous retroviruses; Ron tyrosine kinase; Stk; Ovine pulmonary adenocarcinoma; Retrovirus

Introduction

Jaagsiekte sheep retrovirus (JSRV) is the causative agent of a naturally occurring lung adenocarcinoma of sheep known as ovine pulmonary adenocarcinoma (OPA) (Palmarini and Fan, 2001; Palmarini et al., 1997; Sharp and DeMartini, 2003). OPA is one of the major viral diseases of sheep. In addition, many pathological features of OPA tumors are typical of some forms of human lung adenocarcinomas, indicating that OPA can be a useful (large) animal model for pulmonary neoplasms of man.

JSRV is a replication competent retrovirus, but it induces cell transformation by a different mechanism from the great majority of oncoretroviruses. The expression of the JSRV Env is sufficient to induce transformation of a variety of cell lines in vitro (Allen et al., 2002; Liu and Miller, 2005; Maeda et al., 2001; Rai et al., 2001) and to induce lung adenocarcinoma in immunocompromised mice (Wootton et al., 2005). Thus, both in vitro and in vivo, at least in immunocompromised mice, the JSRV Env can function as a powerful oncoprotein.

The mechanisms of JSRV-induced cell transformation through Env are not completely understood (Fan et al., 2003). Both receptor-independent and receptor-dependent mechanisms have been proposed. The cytoplasmic tail (CT) of the transmembrane domain (TM) is the main determinant of Env-induced transformation (Palmarini et al., 2001a, 2001b) by a receptor-independent mechanism. In particular, a tyrosine (Y590) part of an SH-2 binding domain present within the CT of the JSRV Env is critical for transformation. The influence of Y590 on JSRV Env-induced cell transformation varies and depends from the amino acid substituting Y590, the cell lines used for the transformation assays and experimental conditions (Allen et al., 2002; Hofacre and Fan, 2004; Liu and Miller, 2005; Liu et al., 2001; Palmarini et al., 2001a, 2001b; Zavala et al., 2003). Both the PI-3K/Akt and Ras-MEK-MAPK pathways have been implicated in JSRV-induced cell transformation (Hallwirth et al., 2005; Liu and Miller, 2005; Maeda et al., 2001, 2005; Palmarini et al., 2001a, 2001b), but the exact mechanisms by

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doi:10.1016/j.virol.2006.01.040

which the Env activates these pathways are not clear. Akt activation in cells expressing the JSRV Env, for example, appears to be dependent from Y590, but the latter is not phosphorylated (Liu and Miller, 2005). In addition, the JSRV Env has not been found to associate with PI-3K (Alberti et al., 2002; Liu et al., 2003b; Zavala et al., 2003).

JSRV Env receptor-dependent mechanisms have been suggested in transformation of the human BEAS-2B cell line (Danilkovitch-Miagkova et al., 2003). The cellular receptor for JSRV is Hyaluronidase 2 (HYAL-2), a glycosylphosphatidylinositol (GPI)-anchored surface protein. In man, HYAL-2 maps in the 3p21.3 chromosomal region which is often deleted in lung and other types of cancer (Lerman and Minna, 2000; Petursdottir et al., 2004), suggesting that tumor suppressor genes are present in this location. BEAS-2B cells were derived from human bronchial epithelial cells immortalized with an adenovirus-12 SV40 hybrid virus (Reddel et al., 1988). In BEAS-2B cells, it was shown that HYAL-2 binds the receptor tyrosine kinase RON leading to an inactive complex. However, when the JSRV Env is overexpressed, HYAL-2 binds the latter, inducing its degradation so that RON is free to autophosphorylate and induce activation of downstream signal transduction pathways (including PI-3K/Akt and MEK/MAPK) which eventually lead to cell transformation (Danilkovitch-Miagkova et al., 2003).

The JSRV-receptor-dependent mechanism hypothesized for BEAS-2B was proposed also as the possible mechanism for transformation of epithelial cells in general (Danilkovitch-Miagkova et al., 2003). However, subsequent studies in Madin–Darby canine kidney cells transformed by the JSRV Env (Liu and Miller, 2005) suggested that neither RON nor canine Hyal-2 was involved in the mechanisms of transformation of this epithelial cell line. In addition, HYAL-2 was not found to modulate basal or macrophage stimulating factor (MSP)-induced RON activity in 208F cells (Miller et al., 2004).

Thus, the interaction between RON, HYAL-2 and the JSRV Env is not clear and warrants additional studies. Here, using an epithelial rat cell line, we show that also in epithelial cells the JSRV Env is able to induce transformation in a receptor-independent fashion. In addition, we show that the JSRV Env can also associate with RON and that the CT of the TM domain is a main determinant of the biological response to RON–Env interaction. Thus, the interaction between the JSRV Env, RON and HYAL-2 is complex and can offer multiple interpretations.

Results

Transformation of IEC-18 cells by the JSRV Env can be achieved independently of the receptor binding domain

In BEAS-2B cells, it has been found that HYAL-2 negatively regulates RON. Thus, the interaction between the JSRV Env and HYAL-2 (and their subsequent intracytoplasmic degradation) has been deemed critical for RON activation and cell transformation (Danilkovitch-Miagkova et al., 2003). We reasoned that, if this were the case, the envelope of the JSRV-related endogenous retroviruses (enJSRVs) (DeMartini et al., 2003; Palmarini et al., 2001a, 2001b) would also induce cell transformation because they also use HYAL-2 as cellular receptor (Spencer et al., 2003). In previous studies, we have shown that the Env of enJS5F16 (one of the enJSRV loci) does not induce cell transformation due to differences between exogenous and endogenous Env proteins that we mapped in the CT (Palmarini et al., 2001a, 2001b).

We initially also used BEAS-2B for our transformation assays, but, unfortunately, these cells showed a high level of background that did not allow us to reliably distinguish the transformed from the non-transformed cells, as also found by other investigators (Maeda et al., 2005; Miller et al., 2004). We then tested the rat epithelial cell line IEC-18 (Quaroni and Isselbacher, 1981). Typical results obtained in IEC-18 transformation assays are shown in Fig. 1, and Table 1 summarizes the data obtained. Although the transformation efficiency of the JSRV Env in IEC-18 was relatively low compared to other cell lines such as 208F cells or NIH/3T3, cell transformation could clearly be achieved by the JSRV Env. Foci of elongated

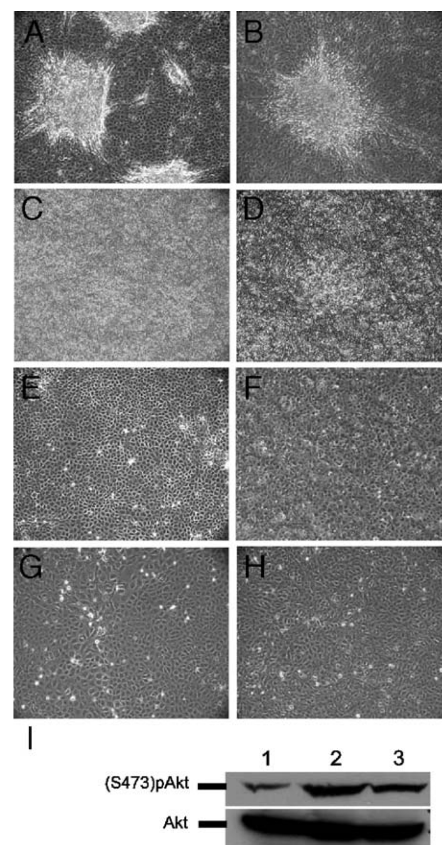

Fig. 1. Transformation assays in rat epithelial cells (IEC-18). IEC-18 cells were transfected as described in Materials and methods, and foci of transformed cells were counted 4 weeks after transfection. JSRV Env induced foci of transformed cells (A) that were expanded and resulted in cultures (IEC-18-ΔGP) of refractile cells with a fibroblast-like phenotype (C). MMuLVprr-JSE (JSRV-MMuLV chimeric Env) was also able to induce cell transformation (B). Panel D shows the resulting cultures of the expanded foci of MMuLVprr-JSE (IEC-18–23). Panels E–H show typical results obtained by transfecting IEC-18 cells respectively with JSE-En2 (enJS5F16 Env), JSEY590D (YSRV Env with Y590 mutated to D), pMyr-JSE6770 (truncated JSRV Env) and pCDNA 3.1. Panel I shows Western blotting from lysates of serum-starved IEC-18 (lane 1), IEC-18-ΔGP (lane 2) and IEC-18–23 (lane 3); an increase in Akt phosphorylation is visible in both lanes 2 and 3.

Table 1
Transformation assays in IEC-18 cells

Plasmids	Number of foci						
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
pCDNA 3.1	0	0	0	0	0	0	0
pCMV3JS21ΔGP	25	19	14	18	21	17	38
pEnvEn2	nd[a]	nd	nd	nd	nd	0	0
pMMuLVprr-JSE	3	nd	nd	4	10	nd	6
pCMV3JS21ΔGPY590D	1	0	0	nd	nd	1	0
pMyr-JSE6770	nd	0	0	nd	nd	nd	nd

[a] Not done.

refractile cells that lost the epithelial phenotype of the parental IEC-18 cells were visible 4 weeks after transfection (Fig. 1). The Env of enJS5F16 did not induce cell transformation (Fig. 1E), and the expression plasmid for the JSRV Env mutant Y590D (JSEY590D) induced only one transformed focus in two out of five assays (Fig. 1F). We were not able to induce foci of transformed cells (Fig. 1G) with pMyr-JSE6770 that contains only part of TM, the membrane spanning domain and the CT of JSRV directed to the membrane with a myristoylation signal. pMyr-JSE6770 can transform 208F cells (Chow et al., 2003), although we subsequently found its transforming efficiency to be variable and in general lower than previously described (Varela and Palmarini, unpublished data). On the other hand, chimeric Env proteins, formed by the receptor binding domain (RBD) and proline-rich region (PRR) of MMuLV with the remaining portion of the SU and TM domain from JSRV (Chow et al., 2003), induced transformation with approximately 25% efficiency compared to the JSRV Env as determined by counting the number of foci obtained. In general, however, foci of transformed cells induced by this chimeric Env were smaller in size with respect to those induced by the JSRV Env.

Foci from IEC-18 cells transformed by JSRV and the chimeric MMuLV-JSRV Env were picked and expanded. The resulting lines (called IEC-18-ΔGP and IEC-18–23; Figs. 1C and D) consisted of refractile cells with a fibroblast-like phenotype that could be easily differentiated from the parental IEC-18 cells. Both IEC-18-ΔGP and IEC-18–23 were able to form colonies in soft agar (not shown), and, in both of them, we found increased levels of phosphorylated Akt with respect to the parental IEC-18 cell line (Fig. 1I). We could not detect phosphorylated RON in both IEC-18-ΔGP and IEC-18–23, although RON expression in these cells (and in the parental IEC-18) was at the limits of detection with the available reagents (not shown).

JSRV, enJS56A1, chimeric MMuLV-JSRV and MMuLV Envs co-immunoprecipitate with RON

Next, we further investigated the nature of the JSRV Env–RON association considering that the model of Env induced transformation of BEAS-2B cells (Danilkovitch-Miagkova et al., 2003) does not fit with the mechanisms of Env transformation in other epithelial cell lines (Liu and Miller, 2005; Maeda et al., 2005).

Co-immunoprecipitation studies were performed in 293T cells by transfecting them with an expression plasmid for human RON and expression plasmids for JSRV Env, JSRV ENV mutant Y590D, enJS5F16 Env, chimeric MMuLV-JSRV Env, MMuLV Env and HYAL-2. All the expressed Env proteins have a FLAG epitope that is tagged in the carboxy terminal portion of the cytoplasmic tail. HYAL-2 (also tagged with a FLAG epitope but at its amino terminal portion) was used as a positive control, and normal rabbit serum was used as a further immunoprecipitation control. We found that all the Env proteins used in this study were able to associate with RON and co-immunoprecipitation was shown both using an anti-RON or an anti-FLAG serum (Fig. 2A). Interestingly, only the full-length Env proteins were pulled down by anti-RON antibodies, suggesting that RON–Env interaction might occur co-translationally in the Golgi and this association interferes with Env cleavage. We noted that, while JSRV, enJSRV and MMuLV Env proteins used in this study are normally processed (into the SU and TM domains), we were not able to detect a processed TM for the MMuLV-JSRV Env chimera (bottom panel, Fig. 2A). This is not entirely surprising given the fact that this is a chimeric envelope protein formed by the receptor binding domain and proline-rich region of MMuLV and the remaining portion of the SU and TM from JSRV (Chow et al., 2003). We speculate that the folding of the SU and TM in this chimeric molecule hampers the access of the cleavage site to cellular proteases considering that the SU–TM boundary in this particular chimera is identical to the JSRV Env. The MMuLV-JSRV chimera has indeed a reduced ability to transform cells (with respect to JSRV) and grossly reduced infectivity when used to pseudotype retroviral vectors (Chow et al., 2003). On the other hand, it has been shown that failure to cleave MLV Env does not necessarily preclude its ability to reach the cell surface, incorporating into virion particles and mediate infection (Zavorotinskaya and Albritton, 1999).

It is also noticeable (Fig. 2 for example) that two bands of similar molecular weight are present for the full-length JSRV, enJS5F16 and JSRV Y590D Env proteins. This phenomenon is probably due to the immunoprecipitation of partially (newly synthesized) and fully glycosylated forms of the Env proteins.

All the Env proteins described above were still able to co-immunoprecipitate with RON if Triton-X-100 rather than SDS was used in the lysis buffer (data not shown).

Next, we performed a series of experiments aimed to determine whether the co-immunoprecipitation of RON with the various Env proteins was due to a real association or to artifacts of the experimental conditions. First, we repeated the co-immunoprecipitation experiments described above, replacing RON with another receptor tyrosine kinase, the epidermal growth factor receptor (EGFR). As shown in Fig. 2B, EGFR did not co-immunoprecipitate with any of the Env proteins employed above or with HYAL-2. An additional membrane protein, CD4, did not co-immunoprecipitate with RON (data not shown).

Next, we increased the stringency of the co-immunoprecipitation of RON with the JSRV Env and other Env proteins by disrupting cells with SDS lysis buffer for 25 min at 37 °C

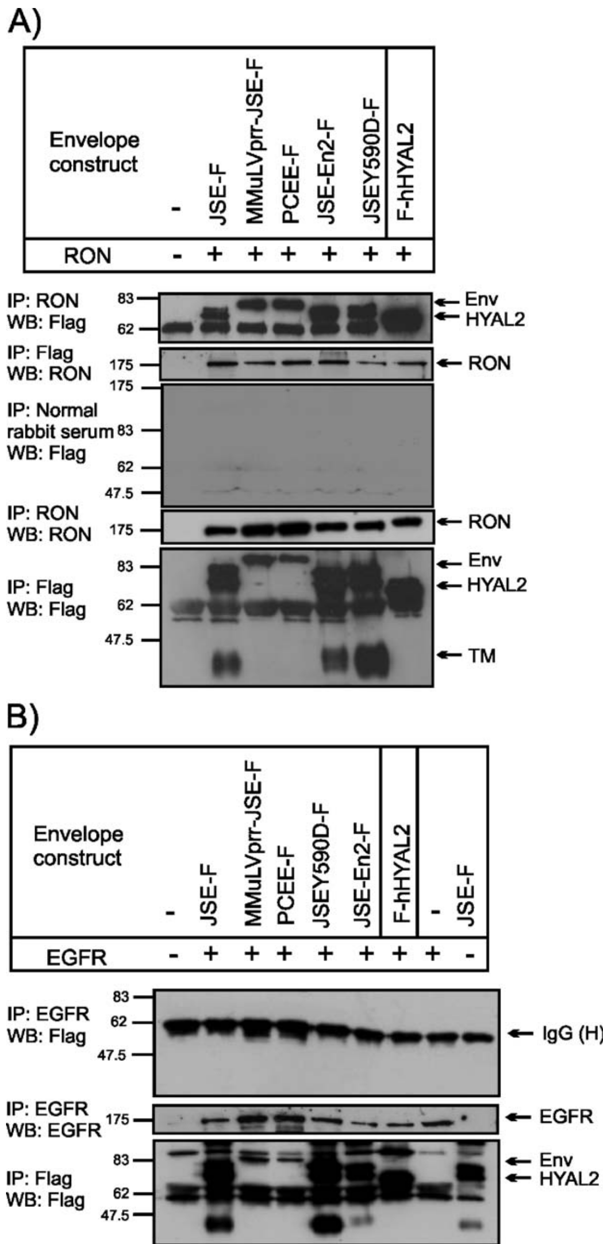

Fig. 2. Wild type, endogenous, chimeric and mutant of the JSR-Env co-immunoprecipitate with RON. 293T cells were co-transfected with expression plasmids for human RON (A) or EGFR (B) and expression plasmids for either one of the following plasmids: wild type JSRV Env (JSE-F), chimeric JSRV-MMuLV Env (MMuLVprr-JSE-F), MMuLV Env (CEE-F), enJS5F16 Env (JSE-En2-F), Y590D mutant (JSEY590D-F) and HYAL-2 (F-hHYAL-2). Forty eight hours after transfection, cells were lysed and lysates were immunoprecipitated (IP) and analyzed by SDS-PAGE and Western blotting (WB). Antisera used for IPs and Western blots are indicated beside each panel. Results are described in the text. Please note that the MMuLV Env (pCEE-F) is processed correctly, but the TM domain (p15) is not visible in the figure because the gels shown are usually run for long periods of time in order to resolve HYAL-2 from the IgG heavy chain and better visualize proteins of high molecular weight such as RON.

(Fig. 3A). The stringency of the possible RON–Env association was tested also by incubating the immunoprecipitates with increasing amounts of sodium chloride in the last washing step for 30 min at room temperature (Fig. 3B). Under both experimental conditions, all the tested proteins were able to co-immunoprecipitate with RON.

Finally, we used a JSRV Env without the FLAG epitope (JSE) in the C-terminus to determine whether the RON–JSE-F association could be specifically competed. Co-immunoprecipitations were performed from lysates of cells co-transfected with fixed amounts of RON and JSE-F and increasing amounts of JSE. As shown in Fig. 4A, JSE competes with JSE-F for RON association. We found also MMuLV Env to compete with the JSRV Env for RON association (data not shown).

We also performed co-immunoprecipitations from lysates of cells co-transfected with fixed amounts of RON tagged with the HA epitope and JSE-F and increasing amounts of non-tagged RON. Fig. 4B shows that the RON-HA/JSE-F association can be competed by untagged RON.

Many studies on the transforming properties of the JSRV Env have been performed in cell lines of mouse and rat origin. We tested whether Stk, the mouse counterpart of RON, was also able to co-immunoprecipitate with the JSRV Env (and the other Envs employed in this study) when co-expressed in the same cells. As shown in Fig. 5A, all the Env proteins employed in this study (and HYAL-2) co-immunoprecipitated with Stk. Expression from an internal promoter of Stk leads also to expression of a short form of Stk (sf-Stk) (Persons et al., 1999) that lacks most

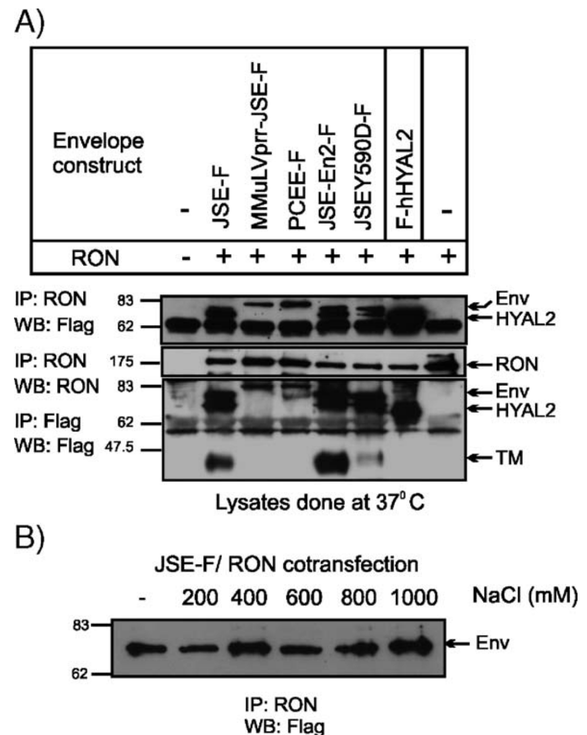

Fig. 3. Stringent conditions do not modify the association between RON and Env proteins used in this study. (A) 293T cells were co-transfected as described in Materials and methods. Forty eight hours after transfection, cells were lysed at 37 °C for 25 min; lysates were then immunoprecipitated (IP) with RON or FLAG antibodies and analyzed by Western blotting (WB) as indicated beside each panel. (B) Immunoprecipitates from cells co-transfected with RON and JSE-F (expression plasmid for the flagged JSRV Env) were washed with increasing amounts of sodium chloride for 30 min at room temperature and analyzed by WB as indicated.

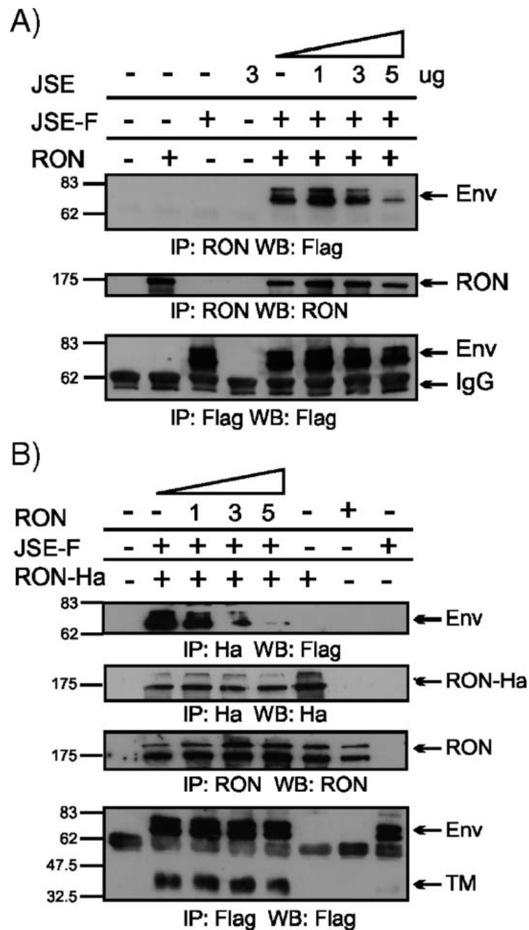

Fig. 4. Competition assays between tagged and non-tagged JSRV Env. 293T cells were co-transfected with 3 μg of RON (A) or 3 μg of RON-HA (B) expression plasmids, 1 μg of the expression plasmid for the JSRV Env tagged with the FLAG epitope (JSE-F) and increasing amounts of the non-tagged JSRV Env (JSE) (A) or non-tagged RON (B). Cells were lysed 48 h after transfection. RON and RON-HA were immunoprecipitated using RON or HA antibodies, while the tagged JSRV Env was immunoprecipitated with FLAG antibodies and analyzed by Western blotting as indicated below each panel. Note that RON-Ha cross-reacts also with anti-RON antibodies.

of the extracellular domain but retains the transmembrane and tyrosine kinase sequences. sf-Stk was unable to co-immunoprecipitate with the JSRV Env and other Env proteins (Fig. 5B), suggesting that the RON/Stk extracellular domain is necessary to associate with the JSRV Env.

All the experiments described above point to the fact that RON is a promiscuous protein and, beside binding to HYAL-2, also associates with the Envs of JSRV, enJSRVs, JSRV mutant Y590D, MMuLV and MMuLV/JSRV chimeric Env protein. However, JSRV cannot use RON as cellular receptor (data not shown) or enhance transformation in 208F cells (data not shown) similarly at what has been shown by other authors in dog MDCK cells (Liu and Miller, 2005).

Confocal microscopy

We performed confocal microscopy to determine whether the JSRV/enJSRV Env proteins and RON can co-localize when co-expressed in the same cells. In transfected HeLa cells, JSRV and enJS5F16 Env proteins showed membrane and broad cytoplasmic distribution, consistent with a protein synthesized in the endoplasmic reticulum and transported to the cell membrane. Co-localization of RON with both JSE-V5 and JSE-En2-Flag was observed at the cell membrane (Fig. 6). Co-localization between RON and Env proteins was also observed in the cytoplasm, although overexpression and the diffuse staining make it difficult to assess the biological relevance of these data.

Endogenous but not exogenous JSRV Env inhibits RON autophosphorylation

Next, we investigated whether direct Env–RON association could lead to RON activation. We performed co-transfection experiments using amounts of RON expression plasmid that with our experimental conditions would either lead to RON autophosphorylation (3 μg) or that would keep RON

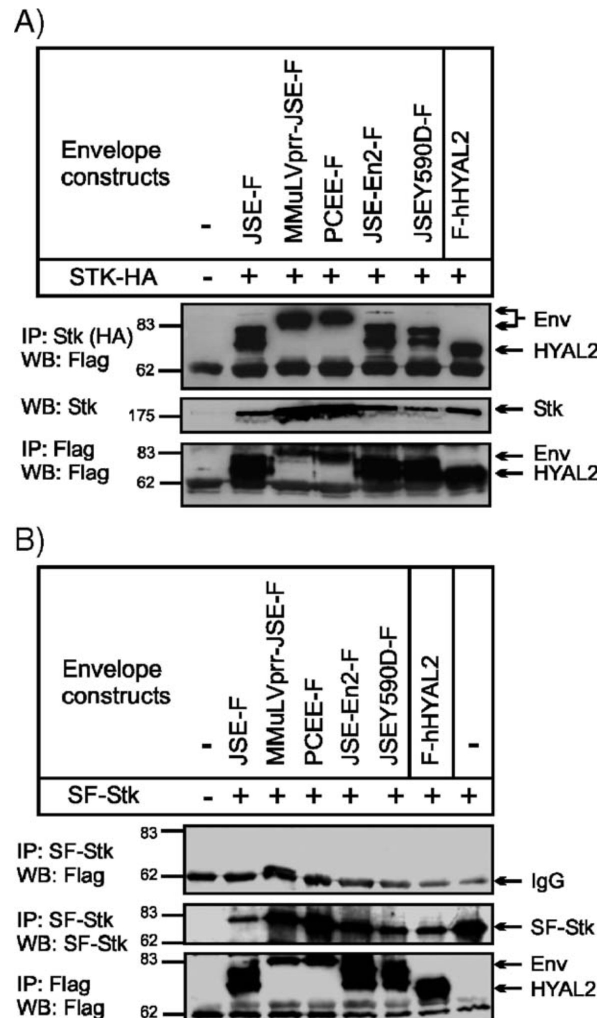

Fig. 5. Stk and not the short form of Stk (Sf-STK) co-immunoprecipitate with the various Env proteins used in this study. 293T cells were co-transfected as in Fig. 2 with the exception that expression plasmids for mouse Stk (A) or sf-Stk (B) were used instead of RON or EGFR. Cells were lysed, immunoprecipitated (IP) and analyzed by Western blotting with antibodies indicated beside each panel.

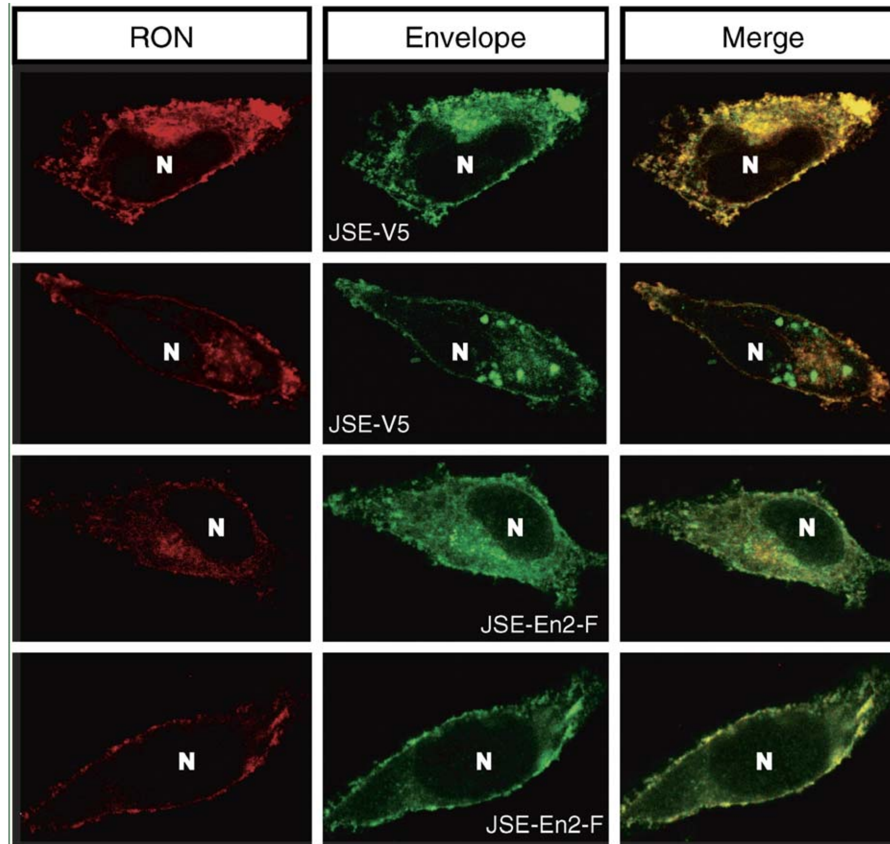

Fig. 6. Confocal microscopy of HeLa cells expressing JSRV/enJSRV Env and RON. Photomicrographs are representative examples of the major phenotypes observed in cells expressing JSE-V5 (JSRV Env tagged with a V5 epitope) or JSE-En2-F (enJS5F16 Env tagged with a FLAG epitope) and RON. Specific staining for all proteins was mainly observed at the cell surface or diffuse within the cytoplasm. Co-localization is especially observed at the cell membrane.

expression detectable but with no or limited signs of activation (100 ng–1 μg).

In 293 cells co-transfected with 1 μg of RON and increasing amounts of the various Env expression plasmids, we were not able to detect any increase of RON phosphorylation (not shown). Thus, the JSRV Env (or any other Env used in this study) was not able by itself to induce RON phosphorylation in our experimental conditions. However, by co-transfecting 293 cells with 3 μg of RON expression plasmid (e.g. an amount of DNA sufficient to induce its autophosphorylation) and increasing amounts of Env or HYAL-2 expression plasmid, we discovered a different outcome resulting from the interaction of RON with the various Env proteins (Fig. 7). The JSRV, MMuLV and chimeric JSRV-MMuLV Env did not affect RON autophosphorylation. HYAL-2 blocked RON activation as shown before by other authors (Danilkovitch-Miagkova et al., 2003). Surprisingly, enJS5F16-Env blocked RON autophosphorylation, too. We found the degree of the block of RON phosphorylation by enJS5F16 variable (as shown in Figs. 7B–C), but we could not identify any technical reason behind this variability. JSEY590D (Palmarini et al., 2001a, 2001b) had an intermediate phenotype between the JSRV and enJS5F16 Env (Fig. 7D).

These experiments suggest that the CT of the transmembrane domain of the JSRV/enJS5F16 Env (and Y590 in particular) influences the effects of the interaction with RON despite both JSRV and enJS5F16 utilize HYAL-2 as cellular receptor (Rai et al., 2001; Spencer et al., 2003). Furthermore, these experiments reinforce the concept that RON can associate with the JSRV Env despite the fact that it is not clear if this interaction occurs in vivo.

Discussion

In this study, we have shown that the interaction between the JSRV Env, HYAL-2 and RON tyrosine kinase is more complex than previously thought. We have shown that the JSRV Env can associate with RON tyrosine kinase and the cytoplasmic tail of the JSRV/enJS5F16 Env influences the biological effects of Env–RON association. Furthermore, transformation of IEC-18 cells suggests that transformation of epithelial cells by the JSRV Env is likely to occur independently of an interaction with HYAL-2.

JSRV-induced transformation of BEAS-2B cells had been proposed as a model for viral transformation of epithelial cells (Danilkovitch-Miagkova et al., 2003) where the interaction of the JSRV Env with HYAL-2 induces RON dimerization and activation due to the release of the inhibitory association of HYAL-2 with RON. Thus, in epithelial cells, it was thought that there was a receptor-dependent mechanism of JSRV-induced

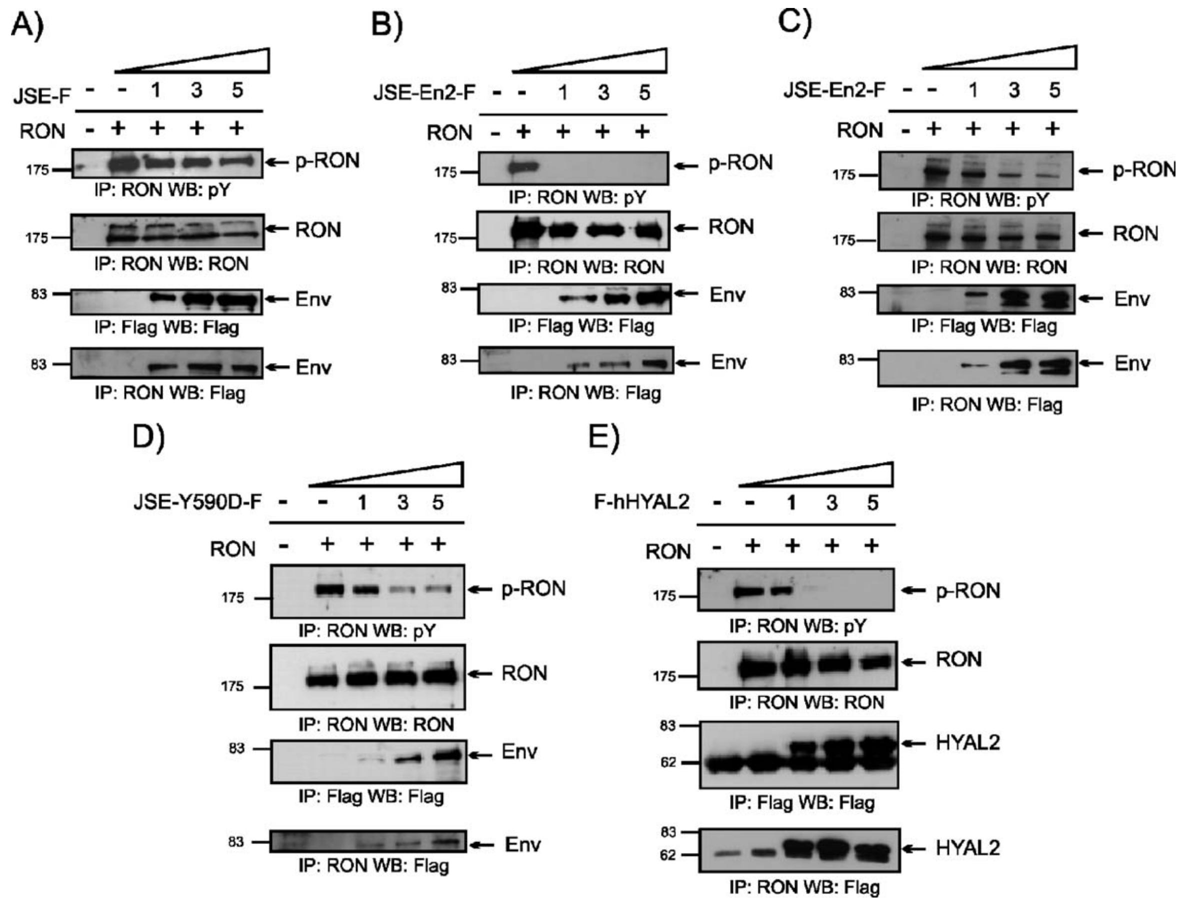

Fig. 7. RON–Env interaction is influenced by the cytoplasmic tail of the envelope. 293 cells were co-transfected with 3 μg of the RON expression plasmid and increasing amounts of expression plasmids for the tagged (with a FLAG epitope) JSRV Env (A), enJS5F16 Env (B and C), Env mutant JSE-Y590D (D) and HYAL-2 (E). Forty eight hours after transfection, cells were starved of serum overnight and lysed as described in Materials and methods. Lysates were immunoprecipitated and analyzed by Western blotting as indicated below each panel.

transformation that was distinct from the essentially receptor-independent mechanism seen in virus-induced transformation of fibroblasts. However, JSRV induced transformation also of the canine epithelial cell line MDCK (Liu and Miller, 2005) and rat epithelial RK3E cells (Maeda et al., 2005) with mechanisms similar to those observed in fibroblasts and largely dependent upon the cytoplasmic tail of the Env. In addition, adeno-associated vectors overexpressing the JSRV Env induced lung adenocarcinomas in immunocompromised mice (Wootton et al., 2005), although mouse HYAL-2 does not bind the JSRV Env (Liu et al., 2003a, 2003b; Rai et al., 2001).

In this study, using another epithelial cell line (IEC-18), we determined that interaction of the JSRV Env with HYAL-2 is likely not critical to induce cell transformation of epithelial cell lines because (i) chimeric MMuLVprr-JSE bearing the receptor binding domain of MMuLV (Chow et al., 2003) (and consequently not using HYAL-2 as cellular receptor) induced cell transformation while (ii) the Env of the JSRV-related endogenous retrovirus enJS5F16 (that uses HYAL-2 as cellular receptor) (Spencer et al., 2003) did not induce cell transformation. Thus, as a whole, it appears from the results of this and previous studies that transformation of epithelial cells is mainly HYAL-2-independent (Liu and Miller, 2005; Maeda et al., 2005; Wootton et al., 2005).

Given the apparent differences between transformation of IEC-18 cells and the BEAS-2B cells, we decided to investigate further the interaction of the JSRV Env with RON tyrosine kinase. Initially, we confirmed the results of previous studies showing that RON binds HYAL-2 and the latter negatively regulates RON autophosphorylation. However, in parallel experiments, we also found that the JSRV Env co-immunoprecipitates with RON. The envelope of M-MMuLV, another retrovirus highly divergent from JSRV, also co-immunoprecipitates with RON. We took great care in controlling for artifacts of the immunoprecipitation assays. We determined that the JSRV (and the other Env proteins described in this paper) do not bind EGFR (Schlessinger, 2004), another receptor tyrosine kinase, using the same experimental conditions used in Env–RON co-immunoprecipitations. In addition, Env–RON association is maintained if the co-immunoprecipitation is performed from cells lysed at 37 °C in SDS buffer. Furthermore, washing the immunoprecipitates with increasing concentration of salts did not disrupt Env–RON association. Confocal microscopy suggests that RON and the JSRV/enJS56A1 Env often co-localize at the cell membrane and in the cytoplasm (although overexpression can be of course a confounding factor).

Most importantly, JSRV and enJSRV Env proteins had a different biological effect on RON phosphorylation. The non-

oncogenic enJS5F16 Env blocked RON autophosphorylation, while the JSRV Env did not affect RON autophosphorylation at all in transiently transfected 293 cells. The main differences between the JSRV and enJS5F16 Env proteins are present in the cytoplasmic tail of the transmembrane domain (Palmarini et al., 2000). Indeed, JSRV Env mutant JSEY590D that contains a single amino acid mutation in the CT (Palmarini et al., 2001a, 2001b) also blocked RON autophosphorylation although to a lesser degree than the endogenous enJS5F16. These results strongly suggest that the cytoplasmic tail of the JSRV/enJSRVs Env modulates the biological effect of RON/Env interaction. It is interesting to note that RON generates downstream signals mainly through phosphorylation of two sites in its CT (Ponzetto et al., 1994); thus, a possible interaction between the CTs of RON and Env could be envisaged.

In our study, however, we have not been able to demonstrate that the JSRV Env interacts with RON in a biologically relevant system, such as in transformed cells. We failed, with the available reagents, to show RON activation in IEC-18 cells, similar to other studies in MDCK and 208F cells (Liu and Miller, 2005; Miller et al., 2004). Overexpression of proteins in a transient transfection system can lead to artifacts that have to be interpreted with caution. RON has been shown to associate with a variety of proteins including MET, EGFR, integrins and adhesion proteins (Danilkovitch-Miagkova and Leonard, 2001). Unfortunately, RON activation is quite difficult to detect in vivo, and, in most studies, RON/Stk activation is shown in cells that overexpress (either transiently or stably) this protein (Angeloni et al., 2004; Danilkovitch-Miagkova and Leonard, 2001; Follenzi et al., 2000; Miller et al., 2004; Peace et al., 2003; Penengo et al., 2003; van den Akker et al., 2004; Wang et al., 1995; Wei et al., 2005a, 2005b; Yokoyama et al., 2005). Thus, the association between JSRV Env and RON might have little biological significance. However, the experimental conditions employed in this study have also confirmed the previously described data on RON–HYAL-2 association and the down-regulation of RON by HYAL-2 (Danilkovitch-Miagkova et al., 2003). However, in side-by-side experiments, we have also showed that the envelope of a JSRV-related endogenous retrovirus can inhibit RON autophosphorylation. Thus, it appears that, as a whole, the biological relevance of RON–HYAL-2–Env association in JSRV-induced cell transformation still needs to be clarified. The association of RON with the JSRV Env is not specific for this virus, given the fact that also M-MMuLV Env co-immunoprecipitates with RON. What advantage might receive M-MLV by binding RON is not immediately apparent. Possibly, retroviruses might benefit from activating infected cells through stimulation of receptor tyrosine kinases, but the relevance of these data awaits confirmation from other biological systems.

The block of RON autophosphorylation by enJSRVs Env is striking and appears to be determined by differences between the CTs of JSRV and enJS5F16 Env. Possibly, the CT of enJS5F16 masks (directly or via other interacting proteins) RON phosphorylation sites. Further studies will be necessary to investigate the mechanisms of enJS5F16 Env-induced block of RON phosphorylation.

enJSRVs are highly expressed in a spatial and temporal regulated fashion in the genital tract of the ewe and in the binucleate cells of the conceptus trophoblast (Palmarini et al., 2004). The ability of enJSRVs to block JSRV at different time points of the replication cycle (Mura et al., 2004; Spencer et al., 2003) is one of the reasons hypothesized for their fixation in the sheep genome. Downregulation of RON could also be envisaged as a ‘protective’ mechanism towards the conceptus. RON knockout mice show compromised cell-mediated immunity (Correll et al., 1997). However, RON is a downregulator of inflammation (Correll et al., 2004) and is important in trophoblast functions (Hess et al., 2003), and, consequently, the effect of RON modulation by enJSRVs in vivo has to be carefully investigated and can only be speculated at this time.

The association of the JSRV Env with RON might have some effect on the overall pathogenesis of JSRV infection in sheep. We have recently demonstrated that clinical disease is not the most common outcome of JSRV infection during the commercial lifespan of sheep (Caporale et al., 2005). Thus, the association of the JSRV Env with RON might not be critical for transformation but might have some influence on JSRV pathogenesis. For example, JSRV has been found to infect adhering cells/macrophages (Holland et al., 1999) whose activation is also known to be mediated by RON (Correll et al., 2004). Further studies will be necessary to investigate this point.

Materials and methods

Plasmids

pCMV3JS21ΔGP, the expression plasmid for the $JSRV_{21}$ envelope, has been previously described (Maeda et al., 2001) and it will be called JSE in this paper for simplicity. pJSE-V5 expresses wild type JSRV Env fused with the V5 epitope in the carboxy terminal of the TM domain. pMMuLVprr-JSE, which contains the MMuLV receptor binding domain (RBD) and proline-rich region of Moloney murine leukemia virus (MMuLV) and the remaining portion of the envelope from the JSRV Env, has been previously described (Chow et al., 2003). MMuLVprr-JSE-F derives from MMuLVprr-JSE and contains a FLAG epitope in the carboxy terminal of the TM domain (Chow et al., 2003). pEnvEn2, the expression plasmid for the Env of enJS5F16, has been described before (Palmarini et al., 2001a, 2001b). JSEY590D-F and JSE-En2-Flag express respectively the JSRV Env with the point mutation Y590D (Palmarini et al., 2001a, 2001b) in the CT and the Env of enJS5F16 (Palmarini et al., 2000) both fused with the FLAG epitope in their carboxy terminal. pCEE-F, expressing the MMuLV Env with a C-terminal FLAG epitope, was derived from pCEE+ (MacKrell et al., 1996) which was kindly provided by Paula Cannon.

$pCMV_1$-F-hHyal2 is an expression plasmid for the human HYAL-2 gene containing a preprotrypsin leader sequence fused at the N-terminal with the FLAG epitope and was constructed by introducing the human HYAL-2 cDNA sequence (MGC-1922) into pFLAG-CMV-1 (Sigma). pC1-neo-hRON, the expression plasmid for the human RON gene, was kindly

provided by Pam Correll. pRK5-HERc, the expression plasmid for epidermal growth factor receptor, was provided by Silvia Gartner. pCDNA 3.1-STK-HA, pCDNA 3.1-Ron-Ha and MSV-Neo-MYC-SFSTK were kindly provided by Pam Correll.

Cell culture

Human 293, 293T and HeLa cells were grown in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere and 95% humidity. Rat epithelial IEC-18 cells (ATCC CRL-1589) were grown with the same media and conditions except that 5% fetal bovine serum was used and the media was enriched with 0.1 units/ml of bovine insulin.

Transformation assays

IEC-18 rat epithelial cells were seeded at 2.5×10^5 per 6 cm diameter plate. Each transformation assay consisted in transfecting four plates of IEC-18 cells with 10.4 μg of plasmid DNA each using the Calphos Mammalian transfection kit from Clontech following the manufacturer's instructions. The day after the transfection, cells were washed three times with phosphate-buffered saline (PBS) and split into 4 × 10 cm plates. Media was replaced every other day for 4 weeks when foci were counted. Results are shown as the total number of foci in 4 plates. Transformed foci were picked up and expanded to give rise to transformed cell lines.

Colony assays

Colony growth assays in soft agar were carried out to test anchorage independence essentially as already described (Macpherson and Montagnier, 1964). Petri dishes (6× cm) were coated with 2 ml of a base layer formed by 0.5% agar, 1× D-MEM, 20% FBS and 10% tryptase phosphate buffer (TPB). Cells (2×10^4) were 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 then incubated for 2 weeks (37 °C, 5% CO_2, 95% humidity). Two milliliters of incubation media was added to the cells every 3–4 days.

Antibodies

RON C-20 polyclonal antibody against the β chain of RON was purchased from Santa Cruz Biotechnology. Monoclonal anti-Flag M2 and anti-Flag polyclonal antibodies were purchased from Sigma. Anti-HA monoclonal and polyclonal antibodies were purchased from Covance and Abcam, respectively. Monoclonal anti-phosphotyrosine antibody clone G410 and Anti-EGF Receptor clone LA22 were purchased from Upstate Biotechnology. Anti-MYC antibody was purchased from Cell Signaling. Anti-V5 antibody was purchased from Invitrogen. Secondary anti-rabbit IgG peroxidase linked F(ab′) fragment from donkey was purchased from Amersham Biosciences. Peroxidase-conjugated goat anti-mouse antibody was purchased from Jackson Research. Goat anti-rabbit IgG labeled with Alexa-488 and donkey anti-mouse IgG labeled with Alexa-594 were purchased from Molecular Probes.

Cell lysates

For the evaluation of AKT phosphorylation, transformed and parental IEC-18 cells were grown up to 80% confluency, serum starved overnight and lysed with Triton lysis buffer (Cell Signaling) with the addition of a protease inhibitors cocktail (Complete—Roche) and 1 mM PMSF. To test RON phosphorylation, human 293 cells were transfected with the appropriate plasmids (see Results section) using the Calphos Mammalian transfection kit (Clontech); 48 h after transfection cells were serum-starved for 12–16 h and lysed as described above.

Co-immunoprecipitation assays were performed in 293T cells transfected with the appropriate plasmids as above. Forty eight hours after transfection, cells were lysed with SDS-NP40 lysis buffer [0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5% NP-40, 50 mM TRIS, 150 mM NaCl] with the addition of a protease inhibitors cocktail (Complete—Roche) and 1 mM PMSF. Lysates were sonicated and then centrifuged for 20 min at 10,000 rpm to remove insoluble material. For the evaluation of protein–protein interactions, 200 μg of whole cell extracts was rocked with 20 μl of protein A agarose beads (Santa Cruz Biotechnology) and primary antibody at 4 °C for 3 h. After three washes with lysis buffer, beads were resuspended in SDS loading buffer, boiled for 5 min and subjected to SDS-PAGE and Western blotting using standard protocols (Ausbel et al., 2000). Detection was achieved by using the appropriate secondary antibodies labeled with horseradish peroxidase followed by enhanced chemiluminescence (ECL) detection with SuperSignal West Pico chemiluminescent reagent (Pierce). When necessary membranes were stripped with restoring buffer (Pierce) and used again with another antibody. Each experiment has been repeated at least two times independently.

Confocal microscopy

HeLa cells were grown in chamber slides and transfected with the indicated plasmids using lipofectamine (Invitrogen). Forty eight hours after transfection, cells were fixed with 3% paraformaldehyde and processed as previously described (Sfakianos et al., 2003). Slides were analyzed by using a Leica GMIR2 confocal microscope.

Acknowledgments

We would like to thank Lorraine Albritton and Os Jarrett for useful suggestions, Pam Correll and Silvia Gartner for suggestions and reagents and Brendon Sumic for technical help with some of the assays performed in this study. This work was supported from grant CA95706-01 from the National Cancer Institute of the National Institutes of Health. MP is a Wolfson-Royal Society research merit awardee.

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