**E1a Gene Expression Blocks the ERK1/2 Signaling Pathway by Promoting Nuclear Localization and MKP Up-regulation**

**IMPLICATION IN v-H-Ras-INDUCED SENESCENCE**

Juan L. Callejas-Valera1, Juan Guinea-Viniegra1,2, Carmen Ramirez-Castillejo1, Juan A. Recio5, Eva Galan-Moya4, Natalia Martinez3, Jose M. Rojas4, Santiago Ramón y Cajal3, and Ricardo Sánchez-Prieto1,6

From the 1Centro Regional Investigaciones Biomédicas/Facultad de Medicina, Universidad de Castilla la Mancha, Albacete 02006, the 2Programa de Oncología Médica, Institut de Recerca Vall d’Hebron Hospital, Universitario Vall d’Hebron, Barcelona 08035, the 3Unidad de Biología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid 28220, and the 4Servicio de Anatomía Patológica, Hospital Universitario Vall d’Hebron, Barcelona 08035, Spain

In response to oncogenic signals, cells have developed safe mechanisms to avoid transformation through activation of a senescence program. Upon v-H-Ras overexpression, normal cells undergo senescence through several cellular processes, including activation of the ERK1/2 pathway. Interestingly, the E1a gene from adenovirus 5 has been shown to rescue cells from senescence by a yet unknown mechanism. We investigated whether E1a was able to interfere with the ERK1/2 signaling pathway to rescue cells from v-H-Ras-mediated senescence. Our results show that, E1a overexpression blocks v-H-Ras-mediated ERK1/2 activation by two different and concomitant mechanisms. E1a through its ability to interfere with PKB/Akt activation induces the down-regulation of the PEA15 protein, an ERK1/2 nuclear export factor, leading to nuclear accumulation of ERK1/2. In addition to this, we show that E1a increases the expression of the inducible ERK1/2 nuclear phosphatases (MAPK phosphatases) MKP1/DUSP1 and DUSP5, which leads to ERK1/2 dephosphorylation. We confirmed our observations in the human normal diploid fibroblasts IMR90, in which we could also show that an E1a mutant, unable to bind retinoblastoma protein (pRb), cannot rescue cells from v-H-Ras-induced senescence. In conclusion, E1a is able to rescue from Ras-induced senescence by affecting ERK1/2 localization and phosphorylation.

Oncogenic activation of RAS genes has been clearly connected with cancer (1), however, the effect of oncogenic Ras could be different depending on the cellular context, as it has been shown for K-Ras (2). In this sense, it is known that v-H-Ras transformation in normal human fibroblasts (10). Interestingly, E1a is able to interfere with PKB/Akt, referred as Akt, and p38 MAPK signaling pathways (11, 12), that have been also involved in cellular senescence (13, 14).

Therefore, we decided to study the possibility of E1a interfering with the ERK1/2 pathway to be rescued from v-H-Ras-induced senescence. Our results demonstrate that E1a is able to block induction of ERK1/2 in response to v-H-Ras overexpression and serum stimulation. This effect is mediated by promoting the nuclear accumulation of ERK1/2 through decreasing the expression levels of the ERK1/2 nuclear export factor, PEA15. In addition to this, we have observed a coordinated up-regulation of the inducible ERK1/2 phosphatases MKP1/DUSP1 and DUSP5 leading to nuclear accumulation of the unphosphorylated form of ERK1/2.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Plasmid, Transfections, and Retrovirus Infection—**
Cells were maintained in 5% CO2 and 37°C. The IMR90 and COS7 cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, glutamine plus antibiotics (BioWhittaker). pLPCE1a, GFP, and pLPCE1a-ERK1/2, which expresses a constitutively active form of ERK1/2, were kindly provided by Dr. J. Guillemin (INSERM Unité 170, Lab Slrl, Villejuif, France). The retroviral vector pLPCE1a was kindly provided by Dr. E. Filletti (National Cancer Institute, Bethesda, MD).

**Antibodies**—
The following antibodies were used: anti-ERK1/2 (Biosource), anti-pERK1/2 (Cell Signaling Technology), anti-phospho-p38 MAPK (Cell Signaling Technology), anti-p38 MAPK (Cell Signaling Technology), anti-pRb, anti-PEA15 (Transduction Laboratories), anti-caspase-3 (Cell Signaling Technology), anti-GFP (Abcam), and anti-HA (Sigma).

**Immunofluorescence Microscopy**—
Cells were plated on覆盖面 glass coverslips (Nunc, Denmark) and fixed in 4% paraformaldehyde containing 0.3% Triton X-100 for 10 min. Following washing in PBS, cells were blocked in 3% normal goat serum for 1 h and incubated overnight at 4°C with the primary antibodies against Rb (Cell Signaling Technology), ERK1/2 (PharMingen, San Diego, CA), PML bodies (Novagen, Madison, WI), or PEA15 (Transduction Laboratories). After washing, cells were incubated for 1 h with a secondary antibody and stained with DAPI, 4,6-diamidino-2-phenylindole.
v-H-Ras have been previously described (12). Expression vectors for HA-ERK2, active B-Raf, MEK1 active (MEEEK), or dominant-negative (MEKEA) were kindly provided by Dr. J. S. Gutkind (NIDCR, National Institutes of Health, Bethesda, MD). Mutant ΔCR2 in pLPC was kindly provided by Dr. S. W. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). GST-HA-ERK2 and v-H-Ras in PW2ZL were kindly provided by Dr. A. Chiloeches (Universidad de Alcalá de Henares, Madrid) and Dr. M. Serrano (CNIO, Madrid Spain), respectively. HA-Akt hyperactive and inactive forms in PCMV5 were kindly provided by Dr. D. Alessi (MRC, Dundee, UK). HA-MKP1 in pRK5 expression vector was a generous gift of Drs. R. Pulido and R. Rocío Cejudo-Marín (CIPF, Valencia, Spain). GST fusion protein purification was performed in Healthcare. GST fusion protein for E1a was generated by PCR using standard techniques. The following primers were used: forward 5'-GATCCATGAGACATATTATCT GCC-3' and reverse 5'-CTGTTGAGACATATTATCT GCC-3'. The sequence of the primers used for PEA15 was 5'-GACATTATCGGCGACGGGAAGGAGGAGGCTTCTACG-3'. DNA mutation was confirmed by automatic sequence analysis in an automatic sequencer ABI Prism 7000.

Real Time PCR Analysis—Total RNA was obtained with the Qiagen RNA isolation kit, following the manufacturer’s instructions. Reverse transcription was performed using 1 μg of DNase-treated RNA in a 20-μl reaction volume. Primers for real time PCR was designed using the ‘primer express’ program. The sequence of the primers used for PEA15 was 5'-TAGCTTCCGTGAGGGAGGCACACACC-3' and 5'-CTTCTGCAGCACCGGGGCTTCTTCG-3'; for MKP1, 5'-AGCCACATCTGCTTTGTTA-3' and 5'-CTGGCCCATGAAGCTGAAGTTCTTGGGCTTTAC-3'. Primers were cloned into the BamHI and EcoRI sites of PGX4T3 (GE Healthcare). GST fusion protein purification was performed in BL21 bacteria as previously described (15).

Direct Mutagenesis—To generate the mutant PEA15 in serum 116, the Direct Mutagenesis kit from Stratagene was used following the manufacturer’s instructions, the primer selected was 5'-GACATTATCGGCGACGGGAAGGAGGAGGCTTCTACG-3'. DNA mutation was confirmed by automatic sequence analysis in an automatic sequencer ABI Prism 7000.

Real Time PCR Analysis—Total RNA was obtained with the Qiagen RNA isolation kit, following the manufacturer’s instructions. Reverse transcription was performed using 1 μg of DNase-treated RNA in a 20-μl reaction volume. Primers for real time PCR was designed using the ‘primer express’ program. The sequence of the primers used for PEA15 was 5'-TAGCTTCCGTGAGGGAGGCACACACC-3' and 5'-CTTCTGCAGCACCGGGGCTTCTTCG-3'; for MKP1, 5'-AGCCACATCTGCTTTGTTA-3' and 5'-CTGGCCCATGAAGCTGAAGTTCTTGGGCTTTAC-3'. DNA mutation was confirmed by automatic sequence analysis in an automatic sequencer ABI Prism 7000.

Western Blotting and Immunoprecipitation Procedures—Cells were treated and collected in lysis buffer (20 mM HEPES, pH 7.5, 0.3 mM NaCl, 2.5 mM MgCl2, 10 mM EGTA, 1% Nonidet P-40, 40 mM β-glycerophosphate, 1 mM dithiothreitol) plus protease and phosphatase inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na3VO4). Then, the indicated amount was loaded onto 8–15% SDS-PAGE and blotted against the different antibodies. In the case of the immunoprecipitation assays, extracts were pre-cleared and then, the soluble fraction was incubated with the indicated antibody (1 μg/sample for 2 h), incubated for 45 min in the presence of protein G (Gamma Bind-Sepharose, Amersham Biosciences) and then washed 3 times in the same lysis buffer. Antibody detection was achieved by enhanced chemiluminescence (ECL, Amersham Biosciences). Protein quantification was performed using the BCA Protein Assay Kit (Pierce) following the manufacturer’s instructions. Results show a representative blot of three.

In Vitro Kinase Assay—COS7 cells transfected either with empty vector or with MEEEK were starved 18 h and collected in lysis buffer (25 mM HEPES, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholic acid) plus protease and phosphatase inhibitors. Each extract (same total protein amount) was incubated with 1 μg of anti-MEK for 2 h, and 2 h more with protein G. The immune complexes were washed three times: first with 1% Nonidet P-40 and 2 mM Na3VO4 in phosphate-buffered saline, then with Tris, pH 7.5, and finally with kinase buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM Na3VO4). Immunocomplexes were incubated in kinase buffer with GST-E1a wild type or GST-HA-ERK2 (10 μg each) and [γ-32P]ATP for 30 min at 37°C. The kinase reactions were loaded onto 10% SDS-PAGE and the gel was exposed to x-ray film.

Immunofluorescence—COS7 cells were grown onto glass coverslips. 24 h after transfection cells were starved for 9–12 h and then samples were fixed in paraformaldehyde (4%) at 4°C for 5 min and then incubated in phosphate-buffered saline containing 10% normal goat serum and 0.3% Triton X-100 (blocking buffer). Samples were then incubated with mouse anti-HA for 2 h at room temperature and, after extensive washing, incubated 45 min with Alexa Fluor 546-conjugated anti-mouse antibodies (Molecular Probes) counterstained with DAPI (Sigma) and mounted with Fluorosave (Calbiochem). Positive immunofluorescence was detected in a Leica-DMRXA pho-
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tomicroscope. The same approach was performed using the IMR90 cell line but without transient transfection.

RNA Interference Assays—shRNA against MKP1 was purchased from Sigma (catalog number SHDNA-004417 mission RNA interference). We selected the best performing clone shRNA for further analysis, as judged for functional interference with endogenous MKP1 by Western blotting and/or RT-PCR assays.

RESULTS

E1a Blocks v-H-Ras-induced ERK1/2 Activation and Promotes Its Nuclear Localization—To explore the mechanisms by which E1a bypasses v-H-Ras-induced senescence we decided to evaluate the effect of E1a in ERK1/2 activation mediated by v-H-Ras. Initially, we expressed v-H-Ras and HA-tagged ERK2 (HA-ERK2) in COS7 cells in the presence or absence of E1a. As shown in Fig. 1A, E1a almost completely blocks HA-ERK2 activation mediated by v-H-Ras in a dose-dependent fashion (data not shown). Next, we evaluated if E1a was specifically interfering with any upstream molecules of the Ras signaling pathway. Therefore, HA-ERK2 plus B-Raf, or constitutively active MEK1 (MEKEE), was transfected in the presence or absence of E1a. As shown in Fig. 1B, E1a blocks HA-ERK2 activation regardless of the upstream molecule used. Furthermore, we also excluded other mechanisms such as competition between ERK1/2 and E1a for MEK1 kinase activity. In vitro kinase assay, using the glutathione S-transferase (GST) fusion protein of E1a and ERK2 in the presence or absence of MEKEE was performed. As shown in Fig. 1C, MEKEE was unable to phosphorylate GST-E1a, whereas GST-ERK2 induced a marked phosphorylation. Therefore, we decided to explore if E1a was affecting ERK1/2 localization. HA-ERK2 localization was evaluated in the presence or absence of E1a in COS7 cells. E1a expressing cells showed a robust nuclear localization for HA-ERK2 similar to that observed in v-H-Ras expressing cells (more than 50%), although in control cells localization was only around 15% (fig. 1D). To further characterize this observation we decided to evaluate if ERK1/2 activation is a requisite for the prominent nuclear localization induced by E1a. Therefore, COS cells were transfected with HA-ERK plus E1a and incubated for 4 h in the presence of a specific inhibitor for MEK1/2. As shown in Fig. 1E, middle panel) no effect was detected by the presence of the MEK inhibitor. Furthermore, the same approach was performed using a dominant negative MEK1 (MEKAA) showing the same results (Fig. 1E, right panel). Interestingly, MEKAA or treatment with UO126 were able to block nuclear localization induced by v-H-ras (Fig. 1F). Importantly, the inhibitory effects of both MEKAA and UO126 were proved by biochemical approaches (supplementary materials Fig. S1). In summary, this set of experiments demonstrates that E1a blocks ERK1/2 activation mediated by v-H-ras and induces its nuclear accumulation independently of the activation status.

E1a Up-regulates Inducible Nuclear Phosphatases for MAPK—Our previous data suggested that E1a is affecting the ERK1/2 signaling pathway in at least two ways, one related to the ERK1/2 phosphorylation state and the other through interference with its subcellular localization. Then, we investigated the lack of HA-ERK2 phosphorylation detected in the presence of E1a. In this sense, it has been recently reported that the catalytic subunit of protein phosphatase 2A is up-regulated by E1a (16) and has also been reported to modulate ERK1/2 phosphorylation (17). Taking advantage of the specific protein phosphatase 2A inhibitor, okadaic acid, we evaluated the effect of this phosphatase on ERK2 activation in the presence of E1a. We detected no effect in HA-ERK2 activation mediated by v-H-Ras, whereas p38 MAPK, an endogenous internal control, undergoes a marked increase in its phosphorylation in the presence of okadaic acid (Fig. 2A). However, considering the prominent nuclear localization induced by E1a, we decided to assess the role of a subgroup of the MKPs, the nuclear inducible MKPs that dephosphorylate ERK1/2 specifically in the nucleus (18).

One of the best characterized members of this subgroup of phosphatases is MKP1, also known as CL100 or DUSP1 (19). First, we evaluated the effect of E1a expression on exogenous HA-tagged MKP1 (HA-MKP1). As shown in Fig. 2B, overexpression of E1a did not modify the protein levels of HA-MKP1. This data indicates that E1a could be affecting to MKP1 at the transcriptional level. Therefore we switched our experimental model of IMR90 cells in which we performed quantitative real time-PCR (qRT-PCR) studies for MKP1. A marked increase in RNA levels were detected for MKP1 in E1a expressing cells (Fig. 2C, histogram), which was confirmed at the protein level by Western blotting (Fig. 2C, right panel). To further support the role of MKP1 we decided to knock down this particular MKP using RNA interference approaches. Based on the high homology of mRNA sequences between human and monkey (identity >95%) we screened 5 different shRNA for human MKP1 in 293 T cell (see supplementary materials Fig. S2). The sequence of the best performing clone was blasted against the mRNA sequence of monkey showing a 100% homology (supplementary materials Fig. S3). The selected clone was stably transfected in COS7 cells and endogenous MKP1 expression was challenged (see supplementary materials Fig. S3). The same selected pools of COS7 cells with shRNA against MKP1 or GFP were transiently transfected with HA-ERK2 and the indicated combinations of v-H-ras and E1a. As shown, the knock down of MKP1 allows HA-ERK2 activation mediated by v-H-ras in the presence of E1a up to 60%, whereas in control cells the activation was less than 10% (Fig. 2D).

Furthermore, other members of this subfamily of MKPs were also tested by Western blotting. Whereas DUSP5 showed a clear increase in IMR90 E1a expressing cells, confirmed by RT-PCR (supplementary materials Fig. S5), no effect was detected in DUSP2/PAC1 (Fig. 2E). Finally, in DUSP4/MKP2, which has recently been reported to have a critical role in replicative senescence (20), no protein was detected regardless of the expression of E1a. As a positive control for MKP2 expression, HeLa cells were UV irradiated (supplementary materials Fig. S4), showing the expected increased. This observation supports previous observations that demonstrates a marked expression of MKP2 in old human normal fibroblasts but not in early doubling time populations (21).

E1a Decreases PEA15 Expression through Inhibition of Akt Kinase Activity—However, although the up-regulation of DUSP1/MKP1 and DUSP5 could explain the lack of activation for ERK1/2 in the presence of E1a, the mechanism leading to
**FIGURE 1.** E1a blocks activation of HA-ERK2 mediated by v-H-Ras and promotes its nuclear localization. 

**A**, COS7 cells were transfected with HA-ERK2 (0.5 μg) plus 2 μg of pLPC v-H-Ras with or without 2 μg of pLPCE1a. Total amounts of DNA were normalized with pLPCGFP. The 24-h transfection cells were starved for 12 h and immunoprecipitated against HA. Then immune complexes were resuspended in loading buffer and blotted against phospho-ERK1/2. E1a and v-H-Ras expression was determined using total cell lysate (TCL).

**B**, COS7 cells were transfected and processed as in A, but using the indicated expression vector.

**C**, in vitro kinase for the constitutively active form of MEK1 using as a substrate GST fusion protein for E1a or ERK2. The figure shows a representative image of three independent experiments. Equal levels of each GST fusion protein and MEKEE were checked by Coomassie staining and Western blotting, respectively (data not shown).

**D**, COS7 cells were transfected as in A with HA-ERK2 (0.5 μg) alone or with 3 μg of pLPC v-H-Ras or pLPCE1a. Cells were starved 12 h and then processed for immunocytochemistry assays. At least 100 cells positive for HA were counted in each experiment. Nuclei were counterstained by DAPI. Images showed a representative experiment of at least three. Histograms are the average of three independent experiments.

**E**, COS7 cells were transfected as in A with HA-ERK2 (0.5 μg) plus with 2.5 μg of pLPCE1a alone or with pCEFL MEK AA (5 μg). Cells were starved 12 h and then processed for immunocytochemistry assays. In the case of treatment with UO126, cells were incubated after starving during 4 h with 10 μM. At least 100 cells positive for HA were counted in each experiment. Nuclei were counterstained by DAPI. Images showed a representative experiment of at least three. Histograms are the average of three independent experiments.

**F**, same as in E but using v-H-Ras. At least 100 cells positive for HA were counted in each experiment. Nuclei were counterstained by DAPI. Images show a representative experiment of at least three. Histograms are the average of three independent experiments.
nuclear localization of ERK1/2 remains unknown. Interestingly, it has been reported that E1a is able to modulate PEA15, although with contradictory results (9, 22). This nuclear export factor for ERK1/2, PEA15, is a protein critical for ERK1/2 localization (23). To elucidate if E1a was interfering at the transcriptional level qRT-PCR studies were performed. No differences were detected due to E1a expression in GFP-tagged PEA15 (GFP-PEA15) or in endogenous PEA15 (Fig. 3A). Thus, Western blotting assays for GFP-PEA15 or control vector were transiently transduced and processed as described in the legend to Fig. 1A. TCL were blotted against tubulin or E1a. The panels show a representative image of three independent experiments with nearly identical results. E1a was unable to affect the expression level of GFP-PEA15 to Asp (HA-Akt DD). In the presence of this hyperactive Akt, the expected decrease in GFP-PEA15 by E1a. To further support the role of Ser116 PEA15 in down-regulation mediated by E1a, we decided to engineer a mutant form that mimics Akt phosphorylation by changing Ser116 to Asp, rather than inhibition of the Akt-mediated phosphorylation by changing to Ala. In fact, it has been reported that changes to Gly induce lower stability up to 3-fold less that the wild type and subsequently low expression levels (24). Therefore, COS7 cells were transfected with the mutant form of PEA15 in which Ser116 was changed to Asp. As expected this mutant form was not down-regulated by E1a expression (Fig. 3D). In summary, E1a is able to inhibit phosphorylation on Ser116 through down-regulation of the Akt signaling pathway, leading to a less stable PEA15.

Down-modulation of ERK1/2 Signaling Pathway by E1a Requires Binding to pRb Protein—The data above clearly show that E1a is interfering with the ERK1/2 signaling pathway. To evaluate the effect in terms of v-H-Ras-induced senescence, we used a mutant form of E1a (ΔCR2) lacking the binding site to pRb, which is critical for most of the biological properties of E1a (for a review, see Ref. 27), including the ability to overcome v-H-Ras-induced senescence (28). First, we evaluated the effect of this mutant in ERK1/2 activation and localization using COS7 cells, showing no effect in HA-ERK2 (Fig. 4, A and B). Furthermore, localization correlated again with normal expression levels of PEA15 (Fig. 4C). Next, we decided to extrapolate our observations to the experimental system of IMR90 cells. We then asked if the changes observed in the nuclear localization observed was also applicable in IMR90 cells. As expected, the nuclear accumulation was only detected in cells expressing E1a and not in those expressing ΔCR2 (Fig. 5A). Therefore, the same pools were re-infected with virus carrying v-H-Ras, selected with hygromycin, and 6–8 days later 3-β-galactosidase stained. The E1a pRb-binding motif was found to be critical to bypass Ras-induced senescence, as judged by β-galactosidase staining (Fig. 5A). In the presence of an E1a form unable to bind pRb, protein expression levels of endogenous PEA15, MKP1, and DUSP5 were comparable with controls (Fig. 5B). Finally, the ability to inactivate Akt was also measured in E1a and ΔCR2 mutant expressing cells (Fig. 5C). Akt activation was only found to be impaired in the presence of an E1a able to bind pRb and supporting the critical role of Akt activity in PEA15 down-regulation and subsequent ERK1/2 nuclear localization.
E1a Expression Blocks ERK1/2 Activation by Serum—To explore whether the interference of E1a onto the ERK1/2 pathway could be a broader mechanism, not only restricted to v-H-Ras expression, we evaluated the effects of E1a in the activation of ERK1/2 mediated by serum. As shown in Fig. 6A, E1a expression in IMR90 cells blocks the ERK1/2 activation mediated by serum, whereas cells overexpressing GFP or mutant ΔCR2 showed clear ERK1/2 activation. Furthermore, we also analyzed the localization of endogenous ERK1/2 in response to serum. As shown in Fig. 6B, cells with E1a expression showed a prominent nuclear localization in basal conditions (0.5% serum) that was almost unaffected by the addition of serum, whereas GFP or ΔCR2 cells increased the nuclear localization in response to serum. Furthermore, we evaluated the phosphorylation status in this experimental system by immunocytochemistry (Fig. 6C). As expected, active forms were almost undetectable in basal conditions. In response to serum, GFP or ΔCR2 cells showed a marked increase in the active form that localizes in the nucleus and cytoplasm. In summary, the proposed effect of E1a onto ERK1/2 signaling seems to be a general mechanism that probably affects a wide variety of stimuli as we demonstrate by using serum.

DISCUSSION

In this report we demonstrate that E1a down-modulates the activation of the ERK1/2 signaling pathway in response to v-H-Ras or serum. This effect involves changes in the subcellular localization of ERK1/2 and the induction of inducible nuclear MKPs that can affect ERK1/2 phosphorylation status, leading to a mislocalized and inactive ERK1/2.

Regarding the nuclear localization of ERK1/2, our data propose a close relationship between its nuclear accumulation and the down-regulation of PEA15. We show here a novel mechanism, independent of ERK1/2 activation by MEK1/2, by which down-modulation of Akt signaling by E1a mediates down-regulation of PEA15 by interfering with Ser116 phosphorylation, which has a critical role in PEA15 stability. Recently, another report did not support the down-regulation...
of PEA15 by E1a (22). It is noteworthy that the down-regulation of PEA15 is described in IMR90 cells (Ref. 9 and this report), whereas in that report ovarian cancer cells were used. Nonetheless, we demonstrate down-regulation at the protein level onto both exogenous and endogenous PEA15, suggesting that E1a is affecting PEA15 stability. The apparent contradictory results indicate, once more, the relevance of the cellular context in driving the E1a gene-elicited biological responses. One clear example is the distinct biological response to E1a expression observed in normal cells versus transformed cells (for a review, see Ref. 29). In fact, the different status of the Akt signaling pathway between normal and tumor-derived cell lines, as well as other mechanisms, could explain the differences observed between both models. Nevertheless, PEA15 seems to be an excellent convergence point for ERK1/2 and Akt signaling pathways that needs to be fully addressed. This fact is even more interesting if we consider the implication of PEA15 in biological processes such as response to angiotensin (25) or cell motility and invasion (30, 31).

But the interference of E1a with the ERK1/2 signaling pathway is also due to the up-regulation of the...
nuclear inducible MKPs. Interestingly, in contrast to PEA15, MKPs are regulated at the transcriptional level. The up-regulation of the MKPs could be extremely important to abrogate ERK1/2 signaling, considering the prominent nuclear localization of ERK1/2 induced by E1a and that most of the biological substrates of ERK1/2 are in the nucleus. Our data with RNA interference approaches demonstrate a role for MKP1/DUSP1 in the E1a-mediated blockage of ERK1/2 activation. Interestingly the knockdown of MKP renders a clear restoration of ERK1/2 activation, although not complete. However, several issues should be considered, first the RNA interference approach is not able to totally knock down MKP1/DUSP1 expression; in fact, there is still almost a 25% expression of endogenous MKP1/DUSP1 in our best performing clone (supplementary materials Figs. S2 and S3) and second, the possibility of compensatory mechanisms such as the up-regulation of DUSP5. In this regard, further studies are necessary to fully address the role of this particular MKP and DUSP5 in E1a-mediated inhibition of ERK1/2.

Regarding the ability of E1a to bypass v-H-Ras-induced senescence, our data demonstrate that two signaling pathways, implicated in oncogene-induced senescence (OIS), Akt and ERK1/2, are affected by E1a. Interestingly recent evidence suggest that down-modulation of the Akt and ERK1/2 signaling pathways as well as the up-regulation of molecules such as MKP1/DUSP1 or DUSP5 are required for oncogene-induced senescence in IMR90 cells (32). Our results could be considered contradictory with this previous report due to the fact that ERK1/2, Akt, MKP1/DUSP1, and DUSP5 seem to be regulated in the same way by E1a but obtaining different biological effects. However, several differences should be considered. First, in this previous report the knock down of NF-1 or oncogenic B-Raf was used to induce senescence, whereas here we are using overexpression of v-H-Ras. Second and more important, we are studying the effect of v-H-Ras in the context of already E1a expressing cells, in which key molecules for oncogene-induced senescence, such as pRb, are annulled prior to v-H-Ras overexpression. In fact, one of the conclusions of this study is the critical role of the E1a Rb-binding motif in the transforming activity of E1a, supporting previous reports (28). It is noteworthy that the ability of E1a to escape from v-H-ras-associated senescence is not related to pRb exclusively, binding to other cellular proteins, such as p300, seems also to be critical (28). Nevertheless, our data indicate that E1a modifies both signaling pathways regardless of the oncogenic insult, supported by the data obtained in basal conditions and corroborated by the use of the ΔCR2 mutant. Nonetheless, the role of E1a in oncogene-induced senescence needs to be fully clarified, considering the importance of this biological process in cancer (33). It is also important to mention the critical role of ERK1/2 localization in v-H-Ras-induced senescence. In this regard, the effect of E1a expression renders the same biological effect as RNA interference against PEA15, reported to block v-H-Ras-induced senescence (9), indicating the importance of this nuclear export factor.

Finally, the lack of ERK1/2 activation by serum in the presence of E1a led us to conclude that the mechanism by which E1a targets the ERK1/2 signaling pathway is a general mechanism and not only specific to the activation by v-H-Ras. In fact, our observations could explain the pro-apoptotic ability of E1a in low serum or in response to ionizing radiation as previously reported (34, 35), considering the critical role the ERK1/2 signaling pathway plays in cellular response to both stimulus (36, 37).

In summary, our data provide evidence for a specific down-modulation of the ERK1/2 signaling pathway mediated by E1a. This effect is mediated through changes in nuclear localization, in which the effect of E1a onto PEA15 through the inhibition of PKB/Akt is a critical step, as well as the up-regulation of MKP1 and Dusp5 at the transcriptional level. These findings could explain some of the biological properties given to E1a, such as the ability to rescue from v-H-Ras-induced senescence. Whether this model is applicable or not to other contexts is currently being studied, as it is the identification of other possible key players, modulated by E1a, in the rescue from v-H-Ras-induced senescence.

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