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Modulation of Cellular Migration and Survival by c-Myc through the Downregulation of Urokinase (uPA) and uPA Receptor▽†

Daniela Alfano,[1]‡§ Giuseppina Votta,[1]‡ Almut Schulze,[2] Julian Downward,[3] Mario Caputi,[4] Maria Patrizia Stoppelli,[1] and Ingram Iaccarino[1]*

Institute of Genetics and Biophysics Adriano Buzzati-Traverso, Consiglio Nazionale delle Ricerche (CNR), Via P. Castellino 111, 80131 Naples, Italy[1]; Gene Expression Laboratory, Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom[2]; Signal Transduction Laboratory, Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom[3]; and Department of Cardio-Thoracic Sciences, Second University of Naples, Naples, Italy[4]

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It has been proposed that c-Myc proapoptotic activity accounts for most of its restraint of tumor formation. We established a telomerase-immortalized human epithelial cell line expressing an activatable c-Myc protein. We found that c-Myc activation induces, in addition to increased sensitivity to apoptosis, reductions in cell motility and invasiveness. Transcriptome analysis revealed that urokinase (uPA) and uPA receptor (uPAR) were strongly downregulated by c-Myc. Evidence is provided that the repression of uPA and uPAR may account for most of the antimigratory and proapoptotic activities of c-Myc. c-Myc is known to cooperate with Ras in cellular transformation. We therefore investigated if this cooperation could converge in the control of uPA/uPAR expression. We found that Ras is able to block the effects of c-Myc activation on apoptosis and cellular motility but not on cell invasiveness. Accordingly, the activation of c-Myc in the context of Ras expression had only minor influence on uPAR expression but still had a profound repressive effect on uPA expression. Thus, the differential regulation of uPA and uPAR by c-Myc and Ras correlates with the effects of these two oncoproteins on cell motility, invasiveness, and survival. In conclusion, we have discovered a novel link between c-Myc and uPA/uPAR. We propose that reductions of cell motility and invasiveness could contribute to the inhibition of tumorigenesis by c-Myc and that the regulation of uPA and uPAR expression may be a component of the ability of c-Myc to reduce motility and invasiveness.

The proto-oncogene c-*myc* is involved in a wide range of cellular processes, including proliferation, growth control, differentiation, and apoptosis (13). As a result of either chromosomal translocations or constitutive activation of signal transduction pathways, c-Myc is found to be overexpressed in many tumors, ranging from B-cell lymphoma to colon and breast carcinomas (33). c-Myc is a pleiotropic transcription factor belonging to the basic helix-loop-helix zipper (bHLHZ) family. A series of studies have shown that c-Myc is able to modulate the transcription of a very large number of genes, up to 15% of all genes (11). In most cases, however, the link between the functions of c-Myc target genes and the ability of the proto-oncogene to contribute to cellular transformation remains elusive.

Recent advances in transcription analysis have validated the knowledge that c-Myc not only is a transactivator but also is able to directly repress transcription (10, 13). The mechanism of c-Myc-mediated gene repression is still unclear but in most cases seems to rely on the ability of c-Myc to form inhibitory complexes with other transcription factors like Miz-1 (Myc-interacting zinc finger protein 1) (36) and Sp1/Sp3 (19).

Activation of c-Myc has been shown to be necessary but not sufficient for full transformation in several cellular and animal models of tumorigenesis. In primary rodent fibroblasts, c-*myc* requires the presence of an active variant of the oncogene *ras* in order to induce colony formation in soft agar and tumor formation in nude mice (27). A similar observation for lymphoid tumors, in which c-Myc requires the expression of the antiapoptotic protein Bcl-2 to induce transformation, has been made previously (16, 42). Cooperation with antiapoptotic oncogenes is possibly required to counteract the intrinsic proapoptotic ability of c-Myc, especially in cells depleted of survival factors or subjected to stress (14, 35). This possibility has led to the speculation that c-Myc has embedded tumor suppressor activity and that full oncogenic exploitation of c-Myc requires the inactivation of its tumor suppressor activity. In this model, cooperation with oncogenes like those for Bcl-2 or Bcl-x_L or with the loss of p53 function can be explained by the notion that the apoptotic program engaged by c-Myc is dependent at least in part on the p53 tumor suppressor pathway (46) and proceeds through the permeabilization of the outer mitochondrial membrane, a process regulated by members of the Bcl-2 family of proteins (24, 25). Similarly, Ras has been shown to suppress c-Myc-induced apoptosis through the activation of protein kinase B (PKB)/Akt (26), although the mechanism of cooperation between c-Myc and Ras may be more complex and not exclusively involve cell protection from apoptosis.

The correlation between the phenotypic changes induced by

* Corresponding author. Mailing address: Institute of Genetics and Biophysics Adriano Buzzati-Traverso, Consiglio Nazionale delle Ricerche (CNR), Via P. Castellino 111, 80131 Naples, Italy. Phone: 39-081-6132449. Fax: 39-081-6132720. E-mail: ingram@igb.cnr.it.

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‡ These authors contributed equally to the work.

§ Present address: Department of Chemistry, University of Salerno, via Ponte don Melillo 1, 84084 Fisciano, Salerno, Italy.

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c-Myc and changes in gene expression has been the focus of several reports. c-Myc activation of genes involved in protein biosynthesis, energy metabolism, and cell cycle regulation is mostly consistent with its ability to drive cell growth and proliferation. Interestingly, the ability of c-Myc to induce apoptosis in human fibroblasts in response to serum withdrawal has been suggested to rely entirely on its inhibitory effect on Miz-1 (34). Thus, c-Myc target genes responsible for the ability of c-Myc to induce apoptosis are more likely to be among the transcriptionally repressed genes.

c-Myc is known to repress the expression of a number of genes involved in the regulation of cell adhesion and the cytoskeleton (20). In primary keratinocytes, modulation of this set of genes has been associated with decreased cell motility observed following c-Myc activation (18). The possibility that reduction of cell motility may be a consequence of c-Myc transcriptional repression and may be related to cancer was indirectly suggested by the observation that c-Myc silencing in established cancer cell lines can induce an increase in cell motility (6). The authors of the study provide evidence that Miz-1-mediated transcriptional repression of the CCL5 chemokine is responsible for the c-Myc-induced decrease in motility in MCF-7 cells. Transcriptional repression by c-Myc may therefore be correlated with its ability to induce apoptosis and reduce cell motility.

Among the genes promoting cell migration and survival are those encoding the serine protease urokinase (uPA) and the cognate uPA receptor (uPAR). These proteins play a central role in tumor development through their ability to control cytoskeleton dynamics, cell adhesion, and extracellular matrix (ECM) integrity (4). High levels of uPA and uPAR have been found in many human malignancies, and this finding strongly correlates with poor prognosis (8, 21). uPA is a multidomain protein which contains an amino-terminal growth factor-like domain (GFD) linked to a catalytic moiety spanning residues 158 to 411. uPA converts the proenzyme plasminogen to plasmin, thus leading to the degradation of fibrin and of most ECM components. High-affinity binding of uPA to uPAR occurs through the GFD and neither involves nor impairs the catalytic activity of the uPA protease domain (40, 43). uPAR is a glycosylphosphatidylinositol-anchored protein with a high affinity for uPA (9). Ligand engagement of uPAR induces profound rearrangements of the cell cytoskeleton and modulates migration, adhesion, and growth (4). Recent evidence shows that uPAR expression also promotes the survival of epithelial cells (1). In most cases, these effects are independent of the proteolytic activity of uPA but require uPAR-mediated signaling through the physical and functional interaction of uPAR with transmembrane receptors, such as the integrins, FPRL1, and the epidermal growth factor (EGF) receptor (37, 38, 44).

We have established a nontransformed, telomerase-immortalized human epithelial cell line expressing an activatable version of the c-Myc protein (MycER) and discovered a profound effect of c-Myc activation on cell death, motility, and invasiveness. By transcriptome analysis, we found that c-Myc activation caused strong downregulation of uPA and uPAR. Our results provide evidence that downregulation of the uPA/uPAR system accounts for most of the proapoptotic and antimigratory effects exerted by c-Myc activation and that subsequent introduction of a constitutively active form of the *ras* oncogene can override both the proapoptotic and antimigratory tumor suppressor-like activities. Our results therefore suggest that the effects of c-Myc and Ras activation may be modulated by the control of uPA and uPAR expression.

MATERIALS AND METHODS

Reagents. Polyclonal anti-uPA antibody was a gift of P. A. Andreasen, Aarhus, Denmark. Monoclonal anti-uPAR R4 antibody was a gift of G. Hoyer-Hansen, Finsen Institute, Copenhagen, Denmark. Polyclonal anti-Ras and anti-phospho-MEK1/MEK2 antibodies were from Cell Signaling Technology (Austin, TX). Polyclonal antibodies antitubulin and antiactin, monoclonal anti-Myc clone 9E10 antibody, EGF, collagen type VI, fibronectin, 4-hydroxytamoxifen (4-OHT), and rhodamine-conjugated phalloidin were from Sigma (St. Louis, MO). Monoclonal antibody anti-p53 was from Santa Cruz Biotechnology (Heidelberg, Germany). zVAD-FMK (benzyloxycarbonyl-Val-Ala-Aspfluoromethylketone) was from Bachem (Switzerland). The enhanced chemiluminescence detection system was from Millipore Corporation (Billerica, MA). All cell culture reagents were purchased from Lonza (Verviers, Belgium). PolyFect and HiPerFect transfection reagents were from Qiagen (Hilden, Germany). The protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). Restriction enzymes were from New England Biolabs (Beverly, MA). The C-terminally histidine-tagged uPA human variant (His-uPA) and the untagged uPA 1-158 variant (corresponding to the first 158 amino acids of human uPA) were expressed as secreted products in the methylotrophic yeast *Pichia pastoris* and purified by 5B4-agarose chromatography, as described previously (40).

Plasmids. The pBabe-Neo-IRES-EGFP vector was kindly provided by Luis Miguel Martins (MRC Toxicology Unit, Leicester, United Kingdom). To obtain the pBabe-Neo-IRES-EGFP vector expressing MycER, the region of the pBabe-puro-MycER plasmid (29) encoding MycER was excised with EcoRI and ligated into the pBabe-Neo-IRES-EGFP vector upstream of the internal ribosome entry site (IRES)-enhanced green fluorescent protein (EGFP) cassette, yielding pBabe-Neo-MycER-IRES-EGFP. The pBabe-Puro-V12Ras vector was a gift of Barbara Nicke (Bayer, Berlin).

Gene silencing. All small interfering RNA (siRNA) oligonucleotides were either from Qiagen or from Sigma. The sequences used were as follows (only sense sequences are given): for uPA, 5′-GCAUGACUUUGACUGGAAU-3′ or 5′-CUGUCUAAAUGGAGGAACA-3′; for uPAR, 5′-GCCGUUACCUCGAAUGCAU-3′ or 5′-GGACUGGCUUGAAGAUCAC-3′; for Miz-1, 5′-GCCUUACCUGUGUGAUAAG-3′ or 5′-GCCUCAUCAGCCUGCUGAATT-3′; and for c-Myc, 5′-ACAUCAUCAUCCAGGACUG-3′. Cells were transfected with siRNA oligonucleotides by the "fast-forward" protocol using HiPerFect (Qiagen) as the transfection reagent. Briefly, 2×10^5 cells/well were seeded into six-well plates and incubated with the transfection complexes (50 nM siRNA and 12 μl of HiPerFect in cell growth medium). After 48 h, the cells were processed.

Cell culture. Human telomerase reverse transcriptase-immortalized retinal pigment epithelial (hT-RPE) cells (Clontech) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum (FBS; pH 7.2 to 7.4) in a humidified atmosphere containing 5% CO_2 at 37°C. To obtain stable transfectants, 10^5 cells were seeded into 35-mm dishes the day before being transfected with 1 μg of DNA and 12 μl of PolyFect transfection reagent according to the protocol of the reagent manufacturer (Qiagen). After 48 h, cells were selected with the neomycin analogue G418 (800 μg/ml) or with puromycin (12 μg/ml). Cells were transfected with pBabe-Neo-MycER-IRES-EGFP, and following 2 weeks of selection, green fluorescent protein (GFP)-expressing cells were isolated using a FACSAria cell-sorting system (BD Bioscience). To induce MycER activation, the synthetic hormone 4-OHT at a 150 nM concentration was added to the culture medium in the presence or absence of 10% FBS.

Western blot analysis. Cells treated as specified in the figure legends were lysed for 1 h on ice in a buffer containing 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 2 mM EDTA, and 0.5% Triton X-100 supplemented with 1 mM phenylmethylsulfonyl fluoride and 1× Complete protease inhibitor cocktail (Roche). The debris was then removed by centrifugation at 10,000 × *g* for 20 min at 4°C, and the protein content was assessed by the Bradford protein assay with a kit from Bio-Rad. Aliquots of 20 μg of protein per sample were separated by SDS–10% PAGE and transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membrane was subsequently incubated for 2 h at room temperature in TBST buffer (125 mM Tris-HCl [pH 8.0], 625 mM NaCl, 0.1% Tween 20) containing 5% skim milk and further incubated with the primary antibodies.

Caspase assay. Caspase 3 and 7 activities were measured by using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI). Experiments were carried out by following the manufacturer's recommended procedures. Briefly, 5×10^3 cells/well were plated into white-walled 96-well plates and incubated for 8 h under normal growth conditions. Subsequently, the medium was replaced with new, serum-free medium with or without 4-OHT, and the plates were incubated for 48 h. After incubation, the Caspase-Glo 3/7 reagents were added to the wells, and 1 h later, luminescence was measured with a plate-reading luminometer (GloMax 96; Promega). The data are expressed as relative light units (RLU; 10^{-4}).

Analysis of the cytoskeleton. To evaluate alterations in cytoskeletal organization, 3×10^4 cells/well were plated onto clean glass slides lodged in six-well plates and incubated for 8 h under normal growth conditions. Subsequently, the medium was replaced with new, serum-free medium with or without 4-OHT, and the plates were incubated for 24 h under normal growth conditions. Cells were then fixed with 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and incubated with 0.1 μg/ml rhodamine-phalloidin for 30 min. After being washed extensively with phosphate-buffered saline, the glass slides were examined with a confocal microscope (Leica Microsystems, Milan, Italy) at a magnification of ×63 to obtain images. Data presented are the means of results from three independent experiments performed in triplicate with a minimum of 20 spots per sample observed.

Chemotaxis and invasion assays. Chemotaxis and invasion assays were performed with Boyden chambers by using 8-μm-pore-size polyvinylpyrrolidone-free filters (insert area, 0.33 cm^2) coated with collagen type VI (50 μg/ml) overnight at 4°C, with an additional layer of Matrigel (50 μg/ml) for the invasion assay, according to the methods of Carriero et al. (7) with minor modifications. Briefly, cells grown with or without 4-OHT for 24 h were detached by mild trypsinization and inoculated into the upper compartments of Boyden chambers. Chemoattractants were diluted in DMEM with 0.1% bovine serum albumin (BSA) and added to the lower compartments. After incubation at 37°C for the times specified in the figure legends, the cells on the upper side of the membrane were removed by scraping and cells on the lower side of the filters were stained with Mayer's hematoxylin (Daco) and counted.

Time-lapse video microscopy. Cells (10^4/well) were seeded into six-well plates and incubated with or without 4-OHT under normal growth conditions. After 24 h, the medium was replaced with 1% serum medium containing or not containing 4-OHT. The dynamics of cell movement at 37°C and 5% CO_2 was monitored by time-lapse cinematography using a specifically designed microscope incubator chamber placed on a motorized stage with *x*, *y*, and *z* axes (Prior) on an inverted optical microscope (Leica). Three fields from each dish were selected and scanned sequentially every 4 min for 24 h at a magnification of ×10.

RNA preparation and array hybridization. Total RNA was prepared using Trizol reagent according to the protocol of the manufacturer (Gibco BRL). Total RNAs from control and experimental samples were labeled by oligo $(dT)_{17}$-primed first-strand cDNA synthesis in the presence of Cy3-dUTP and Cy5-dUTP (Amersham), respectively. Labeled cDNA was purified using AutoSeq G-50 columns and hybridized for 16 h to human cDNA microarrays (Sanger human microarray version 1.2.1, comprising 10,000 probes representing 6,000 human genes). All hybridizations were performed in quadruplicate using duplicate RNA samples with opposite Cy3/Cy5 labeling patterns. For detailed description of the cDNA clones, preparation of the microarrays, and protocols used in sample preparation, array hybridization, washing, and handling, see http://www.igb.cnr.it/iaccarino/protocols.

Data analysis. Images were quantified using the adaptive circle method of GSI Lumonics QuantaRay software. Intensity values were imported into Silicon Genetics Genespring software, and per-spot and per-chip normalization was performed using the Lowess method. Intensity ratios were subsequently normalized with respect to those from a control hybridization, which represented a self-to-self comparison of the control sample.

Genes significantly regulated in response to MycER activation were identified by applying the criterion of a 1.5-fold change in three of four replicate experiments. To rule out effects of tamoxifen, RNA from RPE cells transfected with an empty vector and treated with 150 nM 4-OHT was used as a control (data not shown).

Probes were annotated according to the Hver1.2.1mfg30 annotation provided by the Sanger microarray facility (for more information, see http://www.igb.cnr.it/iaccarino/protocols).

qPCR. Total RNA was extracted using Qiazol reagent according to the instructions of the manufacturer (Qiagen). One microgram of total RNA was reverse transcribed using a QuantiTect reverse transcription kit according to the instructions of the manufacturer (Qiagen). One microliter of the reverse transcription reaction mixture was analyzed by real-time quantitative PCR (qPCR) with a DNA Engine Opticon 2 system (MJ Research, Boston, MA) using FluoCycle SYBR green mix for qPCR (Euroclone, Pero, Italy). The mRNAs measured were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and used at 0.25 μM. Sequences are as follows: uPA forward primer, 5′-ACAGCATTTTG GTGGTGACT-3′, and uPA reverse primer 5′-GCCATCCCGGACTATACAG A-3′; uPAR forward primer, 5′-CATGTCTGATGAGCCACAGG-3′, and uPAR reverse primer, 5′-CTGGAGCTGGTGGAGAAAAG-3′; Miz-1 forward primer, 5′-GTGGTGGACGGTGTTCACTT-3′, and Miz-1 reverse primer, 5′-GCCACGGCCAGCACATCAT-3′; GAPDH forward primer, 5′-ACATGTTC CAATATGATTCCA-3′, and GAPDH reverse primer, 5′-TGGACTCCACGA CGTACTCAG-3′; and c-Myc forward primer, 5′-CCAGCAGCGACTCTGAG G-3′, and c-Myc reverse primer, 5′-CCAAGACGTTGTGTGTTC-3′. The relative level of expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistics. Data are expressed as means ± standard deviations and represent one of at least three separate experiments undertaken in triplicate, unless stated otherwise. Differences between data sets were determined by the Student *t* test. Differences described as significant in the text correspond to P values of <0.05.

RESULTS

Activation of c-Myc in hT-RPE cells increases cellular sensitivity to apoptosis. To investigate the effects of c-Myc activation in the context of a human nontransformed epithelial cell line, we established the hT-RPE cell line expressing the 4-OHT-inducible MycER chimera (29). The MycER chimera was expressed by bicistronic mRNA together with the marker EGFP. This approach enabled us to use fluorescence-activated cell sorting (FACS) to generate a population of cells stably expressing MycER (hT-RPE-MycER cells), which was subsequently analyzed. hT-RPE-MycER cells were first tested by Western blotting for the expression of MycER. Figure 1A shows that the MycER expression level was four- to fivefold higher than the endogenous c-Myc level but comparable to the amount of c-Myc expressed by the NCI-H292 lung carcinoma cell line. We therefore tested if the activation of c-Myc in hT-RPE-MycER cells could influence cellular sensitivity to apoptosis, as shown previously in other systems (15). Cells were grown in serum-free medium in the presence or absence of 4-OHT for 48 h and then tested for cellular caspase activity. As shown in Fig. 1B, the activation of c-Myc with 4-OHT induced a marked increase in caspase 3/caspase 7 activity. No increase in caspase activity was observed in cells transfected with an empty vector and treated with 4-OHT for the same amount of time, indicating that the increase in caspase activity was dependent on c-Myc activation. Furthermore, similar to that in other nontransformed cell systems (12, 46), c-Myc activation resulted in strong induction of p53 and its target gene expressing p21 (Fig. 1C). We observed no induction of p53 and p21 in RPE cells transfected with an empty vector, indicating that induction was not the result of 4-OHT treatment. Figure 1D shows representative photographs of hT-RPE-MycER cells treated with 4-OHT for 48 h in the absence of serum with and without the pancaspase inhibitor zVAD-FMK or EGF. It is evident that the activation of c-Myc in the absence of serum causes an increasing number of cells to detach from the tissue culture plate. FACS analysis revealed that c-Myc activation causes an increase in the proportion of propidium iodide-positive cells, from 2% of control cells to 15% of 4-OHT-treated cells. The propidium iodide-positive phenotype was completely inhibited by the presence of zVAD, as expected based on the observed induction of caspase activation (Fig. 1B). Cell detachment was also reduced by the presence of EGF

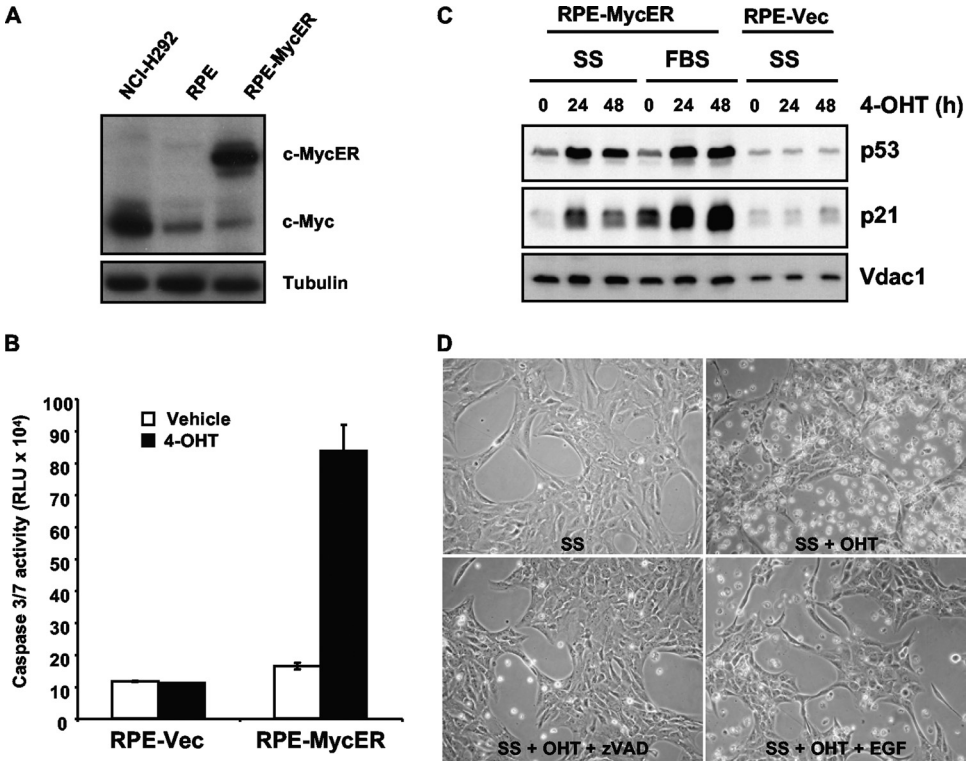

FIG. 1. Activation of c-Myc in hT-RPE cells increases p53 and p21 protein levels and sensitizes cells to apoptosis. (A) Western blot analysis of c-Myc expression in the lung carcinoma cell line NCI-H292 and in hT-RPE and hT-RPE-MycER cells. (B) Analysis of caspase 3/caspase 7 activities in hT-RPE cells transfected with an empty vector (hT-RPE-Vec cells) and hT-RPE-MycER cells following 4-OHT treatment. Five thousand cells were plated into serum-free culture medium in 96-well plates for 48 h in the absence or presence of 4-OHT. Caspase activity was quantified using a luminometric assay that measures the amount of active caspases 3 and 7 (Promega). (C) Analysis of p53 and p21 protein levels following c-Myc activation. hT-RPE cells stably transfected with MycER or an empty vector and grown in the absence or presence of FBS in the culture medium were treated or not treated with 150 nM 4-OHT for the indicated times, and total cell lysates were analyzed by immunoblotting for the expression of p53 and p21 proteins. Vdac1 was used as a loading control. SS, serum starvation. (D) c-Myc-induced apoptosis is inhibited by zVAD and EGF. hT-RPE-MycER cells were serum starved for 48 h in the absence (SS) or presence (SS+OHT) of 4-OHT and in the presence of 4-OHT supplemented with the pancaspase inhibitor zVAD-FMK (SS + OHT + zVAD) or 50 ng/ml of EGF (SS + OHT + EGF). Representative phase-contrast pictures are shown.

in the medium, in line with previous evidence that c-Myc-induced apoptosis is prevented by the presence of growth factors (22). Therefore, c-Myc activation in hT-RPE cells results in growth factor-sensitive induction of apoptosis following serum deprivation, similar to previously described effects in rodent fibroblasts *in vitro* (15, 22, 24) and in epithelial β cells *in vivo* (35).

Activation of c-Myc in hT-RPE cells reduces their abilities to migrate, invade, and produce chemokines. The data presented above are the results of c-Myc activation under serum-free conditions. Next, we investigated the effect of c-Myc activation in the presence of serum. Representative photographs of hT-RPE-MycER cells treated with 4-OHT for 48 h in the presence of serum are shown in Fig. 2A. Interestingly, we noticed that in the presence of FBS, c-Myc activation resulted in dramatic alterations of cellular morphology. Cells were more round and compact and grew in tighter clusters. These changes were accompanied by significant rearrangement of cytoskeletal architecture, as shown by phalloidin staining of filamentous actin (Fig. 2A, bottom). No changes in cellular morphology were observed after 4-OHT treatment of hT-RPE cells transfected with an empty vector (data not shown).

Changes in cytoskeletal architecture can influence the ability of cells to adhere to their substrate and also affect cell migration. In order to investigate if activation of c-Myc can influence the ability of hT-RPE cells to migrate, we measured the directional migration of cells exposed to 4-OHT for 24 h. Cells were layered onto collagen-coated 8-μm filters in Boyden chambers. EGF or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was used as a chemoattractant and included in the lower compartment. As shown in Fig. 2B, we observed that c-Myc activation caused strong reduction in the ability of hT-RPE-MycER cells to migrate toward both chemoattractants, suggesting the impairment of a basic mechanism sustaining cell migration. Once again, the changes in migratory behavior required the expression of MycER, because no difference could be observed after treating cells transfected with an empty vector with 4-OHT (compare RPE/Vec bars with RPE/Myc bars in Fig. 2B). We also analyzed the effect of c-Myc activation on hT-RPE cell motility by using time-lapse video microscopy. Movies of hT-RPE-MycER cells grown in medium containing 1% FBS for 24 h in the absence or presence of 4-OHT are provided in the supplemental material. Interestingly, cells in which c-Myc has been activated show a dramatic increase in lamellipodium dy-

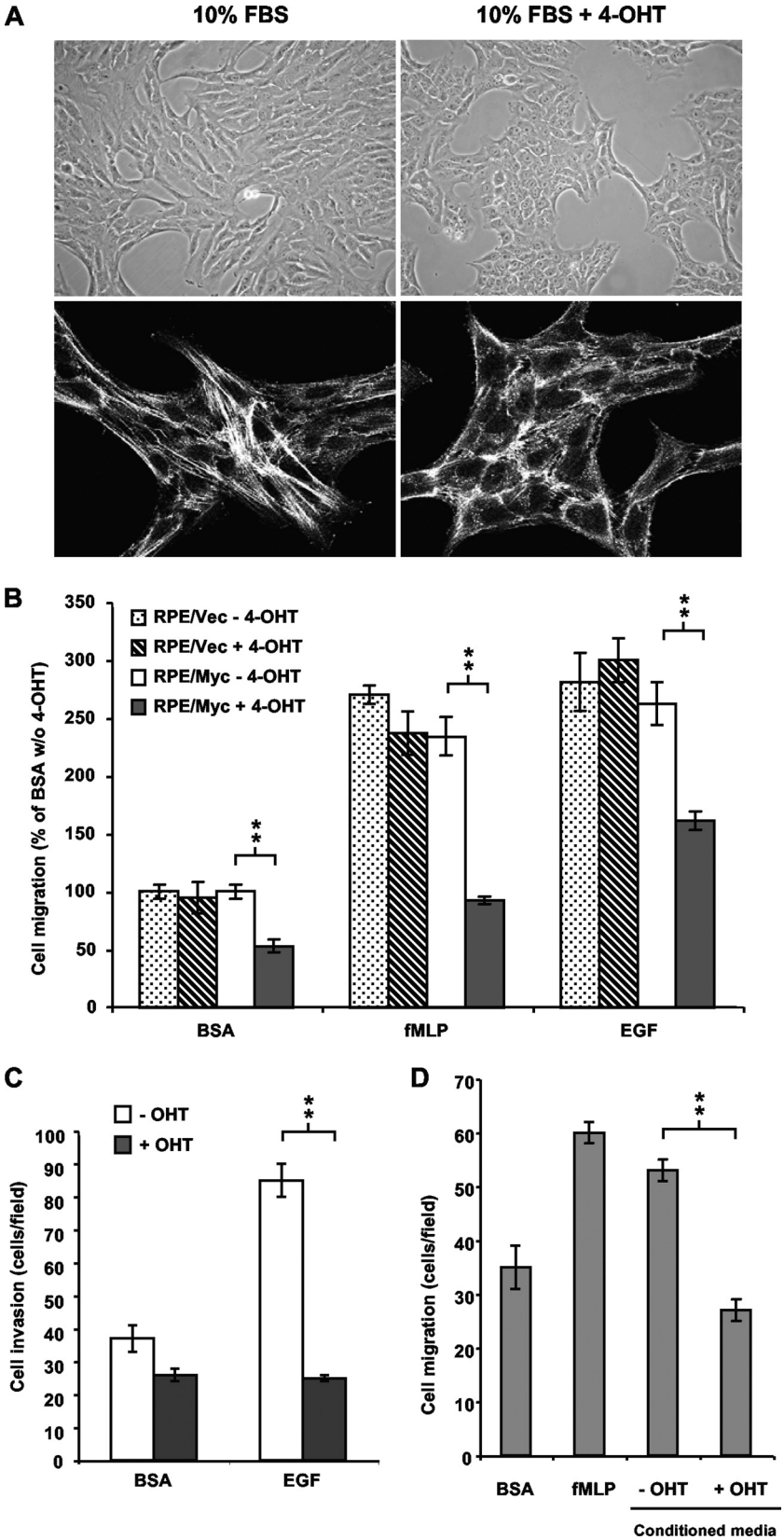

FIG. 2. Activation of c-Myc in hT-RPE-MycER cells induces reductions in cellular motility and invasiveness. (A) c-Myc activation in cells grown in complete culture medium induces changes in cellular morphology and cytoskeletal rearrangements. Phase-contrast pictures of hT-RPE-MycER cells grown in complete culture medium for 48 h in the absence or presence of 150 mM 4-OHT (top) and the same cells observed by confocal fluorescence microscopy after being stained with rhodamine-phalloidin (bottom) are shown. (B) c-Myc activation induces a reduction of basal and

namics, with a concomitant decrease in the spread area as well as reduced random migration (see Movies S1 and S2 in the supplemental material).

We next investigated whether c-Myc activation would affect the ability of cells to invade the ECM by using Matrigel-coated filters and EGF as a chemoattractant. As shown in Fig. 2C, c-Myc activation strongly impaired the ability of hT-RPE cells to invade the ECM. We exclude the possibility that the observed decrease in migration and invasion could be due to increased apoptosis following c-Myc activation, because no apoptosis could be observed in the short time frames of the experiments (data not shown).

The abilities of a cell to migrate and invade can be influenced by the production of cytokines and growth factors acting in an autocrine fashion. Interestingly, c-Myc has been shown previously to modulate the expression of the chemokine CCL5 in a way that influences cellular motility (6). To test whether hT-RPE-MycER cells secrete autocrine factors in response to c-Myc activation, we tested culture media conditioned by cells exposed to 4-OHT for 48 h for their ability to induce directional migration of parental RPE cells. As shown in Fig. 2D, we found that media conditioned by hT-RPE-MycER cells in the absence of 4-OHT served as chemoattractants to the same extent as fMLP. In contrast, the activation of c-Myc completely abolished the chemoattractant ability of hT-RPE-MycER cell-conditioned medium. Overall, these data suggest that the activation of c-Myc in hT-RPE cells induces decreases in cellular motility and invasiveness by influencing cytoskeletal architecture as well as the repertoire of secreted chemokines.

Repression of the survival/chemotactic factors uPA and uPAR is part of the transcriptional program induced by c-Myc activation. To gain insights into the mechanism underlying these profound effects of c-Myc on cellular physiology, we analyzed c-Myc-induced changes in gene expression by performing comparative hybridizations of RNAs prepared from 4-OHT-treated cells and ethanol-treated controls to cDNA microarrays. Since the presence of extracellular growth factors can influence the effect of c-Myc activation on cell sensitization to apoptosis and, possibly, on transcriptional activity, we compared changes in gene expression following the activation of MycER for 4 and 16 h in the presence and absence of EGF. To eliminate c-Myc-independent, 4-OHT-induced effects, the same experiment was performed with cells transfected with an empty vector (data not shown). Among the 10,000 cDNA clones represented on the array, 240 from cells expressing MycER but not those from cells transfected with an empty vector consistently exhibited changes in response to 4-OHT treatment either in the presence or in the absence of EGF in four replicate experiments (see Table S1 in the supplemental material). About half of them showed more than 1.5-fold reduction in expression following c-Myc activation in the presence of EGF. The percentage of genes downregulated following c-Myc activation in the absence of EGF was even higher (60%), although the total number of genes regulated more than 1.5-fold by c-Myc was limited to 136. Comparing the changes in gene expression in the absence and presence of EGF, we found only 16 genes to be differentially regulated (most of them downregulated in the absence of EGF and upregulated in the presence of EGF). Table S1 in the supplemental material shows that among the total number of c-Myc-regulated genes, 44 could be defined as known Myc target genes, being listed in the Myc target gene database (http://www.myc-cancer-gene.org). Interestingly, gene annotation analysis shows that the biological pathways most significantly associated with c-Myc activation are those linked to cytoskeletal remodeling and cell adhesion (data not shown).

As shown in Table S1 in the supplemental material, the mRNA for the urokinase-type plasminogen activator (uPA or PLAU) was among the most strongly downregulated genes both in the presence and in the absence of EGF. The expression of uPA mRNA had decreased 2.5-fold already after 4 h of 4-OHT treatment. We also found that, in agreement with the results of O'Connell et al. (32), expression of the receptor for uPA (uPAR/PLAUR) was downregulated following c-Myc activation. Downregulation of uPA and uPAR in response to c-Myc activation was confirmed by qPCR and Western blotting (Fig. 3A and B). Furthermore, considering that the transcriptional program engaged by c-Myc may be cell type specific, we investigated if c-Myc-mediated uPA and uPAR downregulation was limited to RPE cells or was a more general effect of c-Myc activation. Therefore, we established a nontransformed mammary epithelial cell line expressing MycER (MCF10A-MycER). As shown in Fig. 3C, c-Myc was able to downregulate uPA and uPAR expression in MCF10A-MycER mammary epithelial cells also. Interestingly, uPA downregulation is part of the c-Myc gene expression signature that was identified in human mammary epithelial cells (HMECs) following adenovirus-mediated c-Myc expression (http://www.oncomine.org/). Finally, we asked if the repression of uPA and/or uPAR by c-Myc occurred also in a cancer cell line, in which c-Myc overexpression is the result of oncogenic transformation. We chose MCF-7 breast carcinoma cells, because they are known to express relatively low levels of uPA/uPAR (23). Interestingly, as shown in Fig. 3D, uPA and uPAR expression in-

directional cell migration. After 24 h of 4-OHT treatment, hT-RPE-Vec or hT-RPE-MycER cells (1.5×10^5/sample) were layered onto collagen-coated 8-μm filters in Boyden chambers for 3 h at 37°C to assay directional migration toward 30 ng/ml EGF or 2 μM fMLP. BSA (0.1%) was used to measure nondirectional basal migration. The ability to migrate was measured by counting the cells on the lower-chamber side of the filter. (C) c-Myc activation induces a reduction in cellular invasiveness. Cellular invasiveness was assayed by coating filters treated as described above with an additional layer of Matrigel (50 μg/ml). After 24 h of 4-OHT treatment, hT-RPE-MycER cells (1.5×10^5/sample) were layered into the upper compartment of a Boyden chamber and incubated for 6 h at 37°C. The ability to invade toward EGF (50 ng/ml) was measured by counting the cells on the lower-chamber side of the filter. (D) c-Myc activation reduces the ability of conditioned media to drive parental hT-RPE cell migration. hT-RPE-MycER cells were grown in serum-free medium for 24 h in the absence or presence of 4-OHT. Conditioned media were collected and used as chemoattractants in the lower compartments of Boyden chambers. Parental hT-RPE cells (1.5×10^5/sample) were layered into the upper chambers and incubated for 3 h at 37°C. Cell migration was quantified by counting the cells on the lower-chamber side of the filter. Significance was calculated using the Student *t* test (** indicates a *P* value of <0.001).

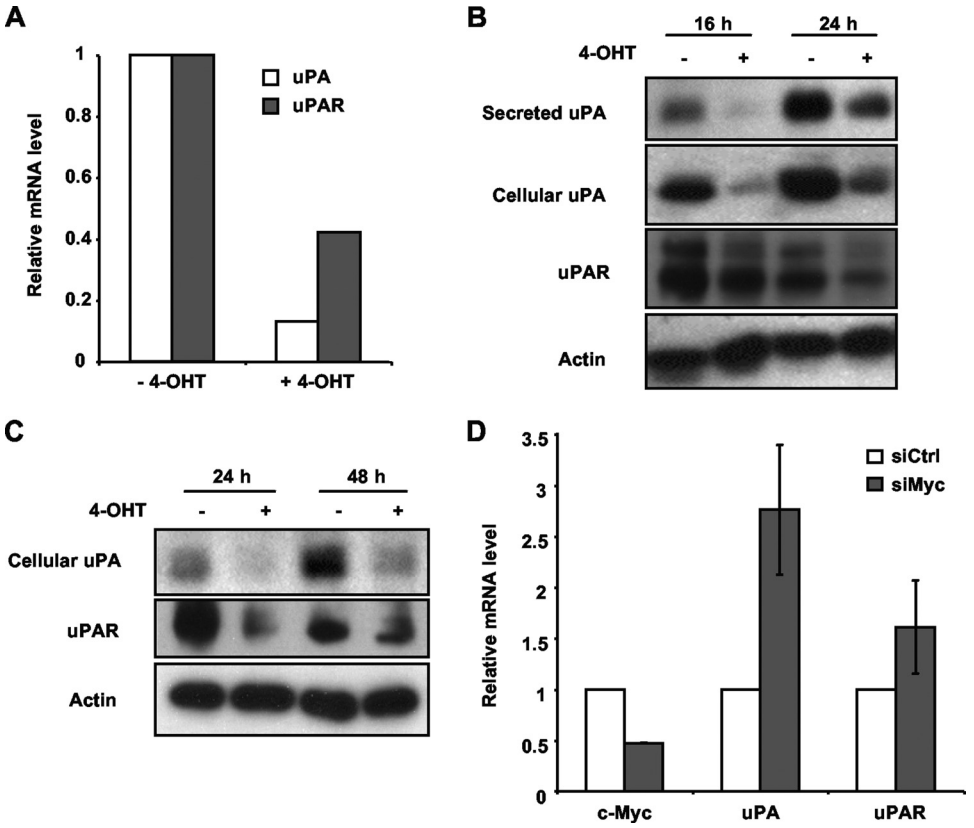

FIG. 3. Activation of c-Myc represses uPA and uPAR expression. (A) qPCR analysis of uPA and uPAR mRNA abundance in hT-RPE-MycER cells following c-Myc activation. Total RNAs from hT-RPE-MycER cells cultured in the absence or in the presence of 4-OHT for 24 h were extracted and reverse transcribed. Relative levels of uPA and uPAR mRNAs were quantified by real-time PCR using the GAPDH gene as an internal control. (B) Analysis of uPA and uPAR protein levels in hT-RPE-MycER cells following c-Myc activation. Cells were treated or not treated with 4-OHT for the indicated times, and total cell lysates were analyzed by immunoblotting to determine the amounts of uPA and uPAR in the lysates. The amount of uPA secreted was determined by concentrating equal volumes of conditioned media from 4-OHT-treated and untreated cells. Actin was used as a loading control. (C) Analysis of uPA and uPAR protein levels in MCF10A-MycER cells following c-Myc activation. Cells were treated or not treated with 4-OHT for the indicated times, and total cell lysates were analyzed by immunoblotting to determine the amounts of uPA and uPAR in the lysates. Actin was used as a loading control. (D) Analysis of uPA and uPAR mRNA levels in MCF-7 breast carcinoma cells following c-Myc silencing. MCF-7 cells were transfected with control siRNA (siCtrl) or c-Myc-targeting siRNAs (siMyc). After 48 h, RNA was extracted and relative levels of c-Myc, uPA, and uPAR mRNAs were determined by real-time qPCR using actin mRNA as an internal control. Levels of expression in cells transfected with c-Myc-targeting siRNA are reported as changes (*n*-fold) with respect to the amounts of c-Myc, uPA, and uPAR mRNAs expressed in cells transfected with the control siRNA.

creased following siRNA-mediated c-Myc silencing in MCF-7 cells.

Transcriptional repression by c-Myc appears to involve multiple, distinct mechanisms (5, 10, 13). The best characterized of these mechanisms is based on the ability of c-Myc to directly bind and inhibit the transcriptional activator Miz-1 (36). We therefore asked if uPA and uPAR transcriptional repression by c-Myc could be mediated by Miz-1. hT-RPE-MycER cells were transfected with control or Miz-1-targeting siRNAs and then treated or not treated with 4-OHT for 24 h. Levels of uPA and uPAR mRNAs in the presence and absence of c-Myc activation were then analyzed by qPCR. As shown in Fig. 4, Miz-1 knockdown abolished the ability of c-Myc to repress uPAR mRNA and partially reduced the effect of c-Myc activation on uPA mRNA, suggesting a requirement for Miz-1 in c-Myc-dependent repression of uPAR and uPA. Silencing of p53, instead, had no effect on the ability of c-Myc to repress uPA and uPAR mRNA expression (data not shown).

Repression of uPAR accounts for most of c-Myc antimigratory and proapoptotic activities. We have shown previously that the uPA/uPAR system is able to induce a survival signal in RPE cells (1). In particular, we found that uPAR expression levels positively correlated with resistance to detachment-induced apoptosis (anoikis), UV irradiation, and serum starvation. Therefore, we analyzed whether reduced uPAR expression *per se* could sensitize hT-RPE-MycER cells to apoptosis induced by serum deprivation in the absence of c-Myc induction. Expression of uPAR in hT-RPE-MycER cells was silenced by siRNA-mediated RNA interference (RNAi) to a degree similar to that achieved by c-Myc activation (Fig. 5A). Indeed, treatment of hT-RPE-MycER cells with siRNAs targeting uPAR increased their sensitivity to serum starvation by approximately twofold (Fig. 5B). However, the level of caspase activation caused by c-Myc activation is still higher than that achieved by uPAR silencing, suggesting that additional pathways contribute to c-Myc-induced sensitization to apoptosis.

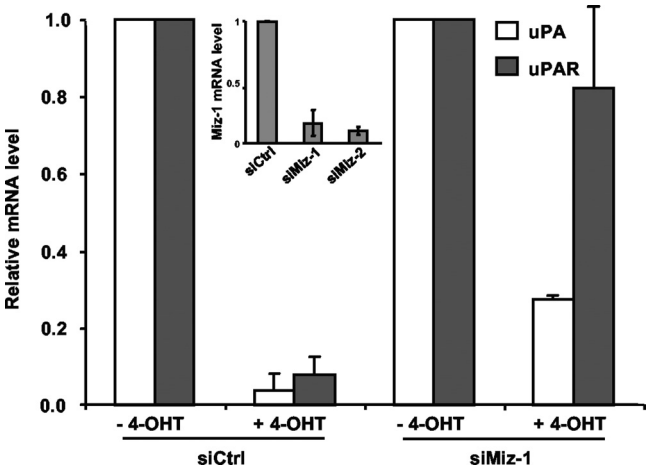

FIG. 4. Downregulation of uPAR and uPA by c-Myc requires Miz-1. uPA and uPAR mRNA abundance in hT-RPE-MycER cells following Miz-1 silencing was analyzed. Cells were transfected with control siRNA (siCtrl) or Miz-1-targeting siRNAs (siMiz-1) and, after 48 h, treated or not treated with 4-OHT for 24 h. Relative levels of uPA and uPAR mRNAs were determined by real-time qPCR using the GAPDH gene as an internal control. Levels of expression are reported as changes (*n*-fold) with respect to the amounts of uPA and uPAR mRNAs expressed by hT-RPE-MycER cells in the absence of 4-OHT. The insert shows the silencing efficiency of the two siRNAs used to reduce Miz-1 expression.

It is well established that the uPA/uPAR system plays a crucial role in regulating motility and adhesion in many cell types (4). We therefore asked if the repression of uPAR expression by c-Myc could also be responsible for the reduced motility of hT-RPE-MycER cells observed upon c-Myc activation. In order to answer this question, we used RNAi to reduce uPAR expression to levels similar to those achieved by c-Myc activation. After RNAi by control and uPAR-targeting siRNAs, cells were tested for their ability to perform directional migration as described above. Silencing of uPAR expression significantly impaired the ability of hT-RPE-MycER cells to migrate toward EGF in the absence of c-Myc activation (Fig. 5C). In addition, activation of c-Myc did not further reduce motility when uPAR expression had been silenced, suggesting a central role for uPAR in the regulation of cell migration by c-Myc.

Repression of uPA accounts for most of the reduction in the chemotactic potential of media conditioned by cells with active c-Myc. The data presented in Fig. 2D suggest that c-Myc activation is able to influence the production of chemotactic factors in the cell culture medium. It is well established that, in cells expressing uPAR, uPA can behave as a chemotactic molecule in either a paracrine or an autocrine fashion (8, 41). To analyze whether uPA is a major chemotactic factor in conditioned medium of hT-RPE-MycER cells, we silenced uPA expression in hT-RPE-MycER cells and tested the chemotactic potential of the conditioned medium. As shown in Fig. 6A, transfection with uPA-targeting siRNAs resulted in a strong reduction of the amount of uPA in the cell culture medium, to an extent similar to that observed following c-Myc activation. Accordingly, the chemotactic potential of medium conditioned by hT-RPE-MycER cells

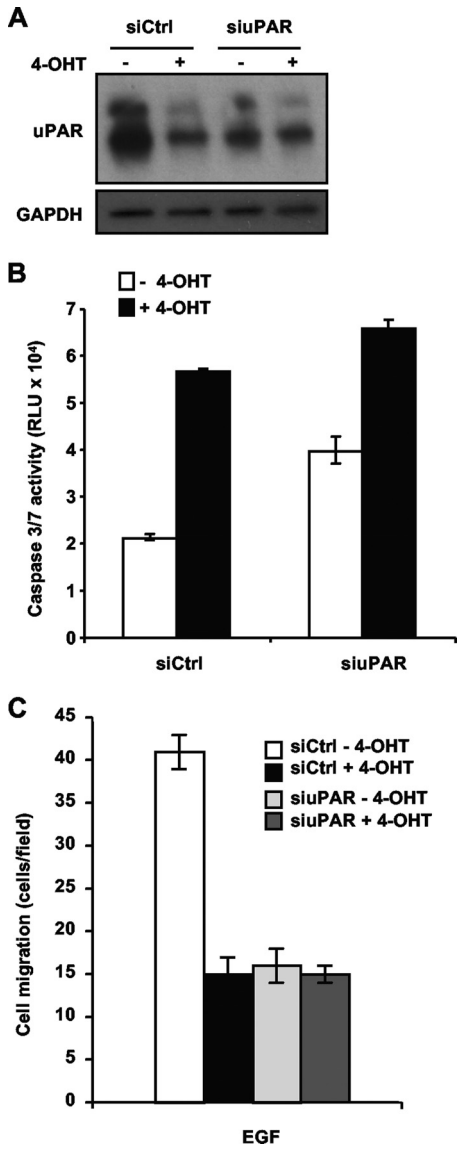

FIG. 5. uPAR silencing in hT-RPE-MycER cells recapitulates the effects of c-Myc activation on apoptosis and cell migration. (A) Analysis of uPAR protein expression in hT-RPE-MycER cells following RNAi-mediated uPAR silencing. Cells were transfected with control siRNA (siCtrl) or uPAR-targeting siRNAs (siuPAR) and, after 24 h, treated or not treated with 4-OHT for 48 h. Total cell lysates were analyzed by immunoblotting to determine the level of uPAR expression. GAPDH was used as a loading control. (B) Analysis of caspase 3/caspase 7 activity in hT-RPE-MycER cells following RNAi-mediated uPAR silencing. hT-RPE-MycER cells were transfected with control or uPAR-targeting siRNAs and, after 24 h, plated into serum-free culture medium in 96-well plates for 48 h in the absence or presence of 4-OHT. Caspase activity was quantified using a luminometric assay that measures the amounts of active caspases 3 and 7 (Promega). (C) Analysis of hT-RPE-MycER cell migration following RNAi-mediated uPAR silencing. hT-RPE-MycER cells were transfected with control or uPAR-targeting siRNAs and, after 48 h, treated or not treated with 4-OHT for an additional 24 h. After incubation, 1.5×10^5 cells/sample were layered onto collagen-coated 8-μm filters in Boyden chambers for 3 h at 37°C to assay directional migration toward EGF (30 ng/ml). The ability to migrate was measured by counting the cells on the lower-chamber side of the filter.

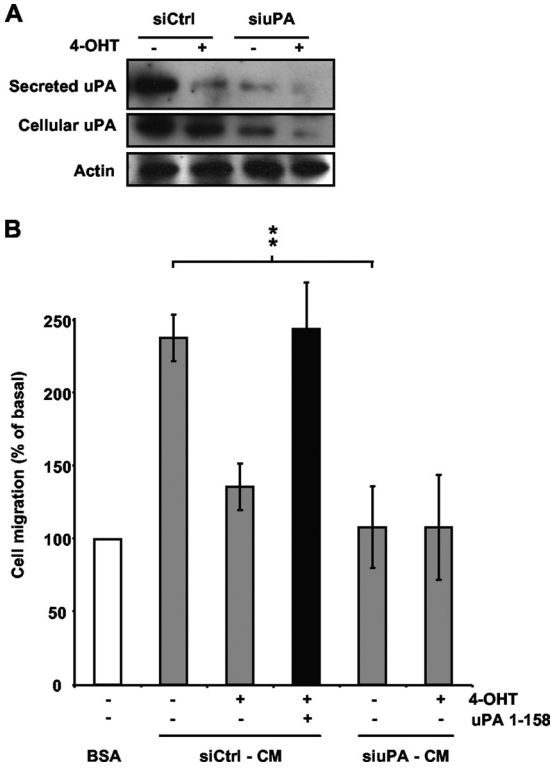

FIG. 6. uPA silencing in hT-RPE-MycER cells reduces the chemoattractive potency of conditioned medium. (A) Analysis of uPA protein expression in hT-RPE-MycER cells following RNAi-mediated uPA silencing. Cells were transfected with control or uPA-targeting siRNAs and, after 24 h, treated or not treated with 4-OHT for 48 h. Total cell lysates and conditioned media were analyzed by immunoblotting to quantify the level of uPA expression. Actin was used as a loading control. (B) uPA silencing reduces the ability of conditioned medium (CM) to drive parental hT-RPE cell migration. hT-RPE-MycER cells were transfected with control or uPA-targeting siRNAs. After 48 h, cells were shifted into serum-free medium for 24 h in the absence or presence of 4-OHT. Conditioned media were collected and used as chemoattractants in the lower compartments of Boyden chambers. Where indicated, 0.4 pmol of the 1–158 uPA fragment was added to the conditioned medium. Parental hT-RPE cells (1.5×10^5/sample) were layered into the upper compartments and incubated for 3 h at 37°C. Cell migration was quantified by counting the cells on the lower-chamber side of the filter. Significance was calculated using the Student *t* test (** indicates a *P* value of <0.001).

after uPA silencing was similar to that of medium conditioned by control siRNA-expressing cells after c-Myc activation (Fig. 6B). Furthermore, the activation of c-Myc in cells with uPA silenced did not further decrease the chemotactic potential of conditioned medium. Finally, the addition of physiological concentrations of recombinant uPA to the conditioned medium of cells in which c-Myc had been activated by the addition of 4-OHT completely eliminated the chemotactic defect (Fig. 6B). The same result was observed using an siRNA directed against another region of uPA mRNA (data not shown). These data strongly suggest that the repression of uPA expression by c-Myc accounts for most of the reduction in the chemotactic potential of media conditioned by cells in which c-Myc had been activated.

Expression of V12Ras abolishes c-Myc's antimigratory and proapototic activities. The observed reductions in cell motility and invasiveness in response to c-Myc activation seem difficult to reconcile with the pleiotropic oncogenic activity of c-Myc. However, it has been recognized previously that c-Myc has an embedded tumor suppressor activity that limits its oncogenic potential (30). A reasonable consideration is that the ability to inhibit cell motility and invasion, together with the ability to induce apoptosis, may be part of this tumor suppressor activity. Cooperation with a second oncoprotein that overcomes the effects on migration and survival could then be required for full transformation. c-Myc is known to cooperate with oncogenic Ras to induce cell transformation (27). We therefore investigated whether Ras could interfere with the reduction in cell motility and the increase in sensitivity to apoptosis in response to c-Myc activation. We also asked if the transcriptional regulation of the uPA/uPAR system could be involved.

To this end, we generated hT-RPE-MycER cells stably expressing a constitutively active form of Ras (V12Ras) and designated them hT-RPE-MycER/V12Ras cells. First, we analyzed if serum starvation-induced apoptosis was affected by the presence of V12Ras. As expected, Ras reduced caspase activation both in the absence and in the presence of activated c-Myc (Fig. 7A). Interestingly, c-Myc retains some proapoptotic activity in the presence of V12Ras. We next compared directional motilities of hT-RPE-MycER/V12Ras cells exposed or not exposed to 4-OHT. In contrast to what we observed with parental hT-RPE-MycER cells (Fig. 2A) or cells transfected with an empty vector (data not shown), the activation of c-Myc in cells expressing V12Ras caused a significant increase in cell motility (Fig. 7B). The presence of activated Ras was therefore able to reverse the effects of c-Myc activation on cell motility and apoptosis.

Expression of V12Ras does not influence the abilities of c-Myc to reduce invasion and modulate the expression of autocrine factors. We next asked whether V12Ras could also affect the ability of c-Myc to modulate the expression of autocrine factors and/or the cellular invasive potential. We tested media conditioned by hT-RPE-MycER/V12Ras cells grown in the presence or absence of 4-OHT for their ability to induce directional migration of parental RPE cells. Although the introduction of V12Ras made conditioned media from hT-RPE cells better chemoattractants (data not shown), we found that the activation of c-Myc still reduced the chemotactic potential of the conditioned media (Fig. 7C). Similarly, even though the introduction of V12Ras caused an overall increase in invasion (data not shown), activation of c-Myc caused a significant reduction in Matrigel invasion (Fig. 7D). Therefore, we observed no cooperation between Ras and c-Myc at the levels of chemokine production and Matrigel invasion.

The differential regulation of uPA and uPAR by c-Myc and Ras correlates with the effects of c-Myc and Ras on cell motility and invasiveness. It has been reported previously that Ras can regulate the expression of uPA and uPAR (28, 31). We therefore analyzed the levels of uPA and uPAR mRNAs and proteins following c-Myc activation in hT-RPE-MycER/V12Ras cells. We found that both uPA and uPAR levels in V12Ras-expressing cells were upregulated compared to the levels in cells carrying the control vector. However, while the expression of uPA was strongly downregulated in response to c-Myc activation in the presence of V12Ras, uPAR expression, although marginally reduced by c-Myc

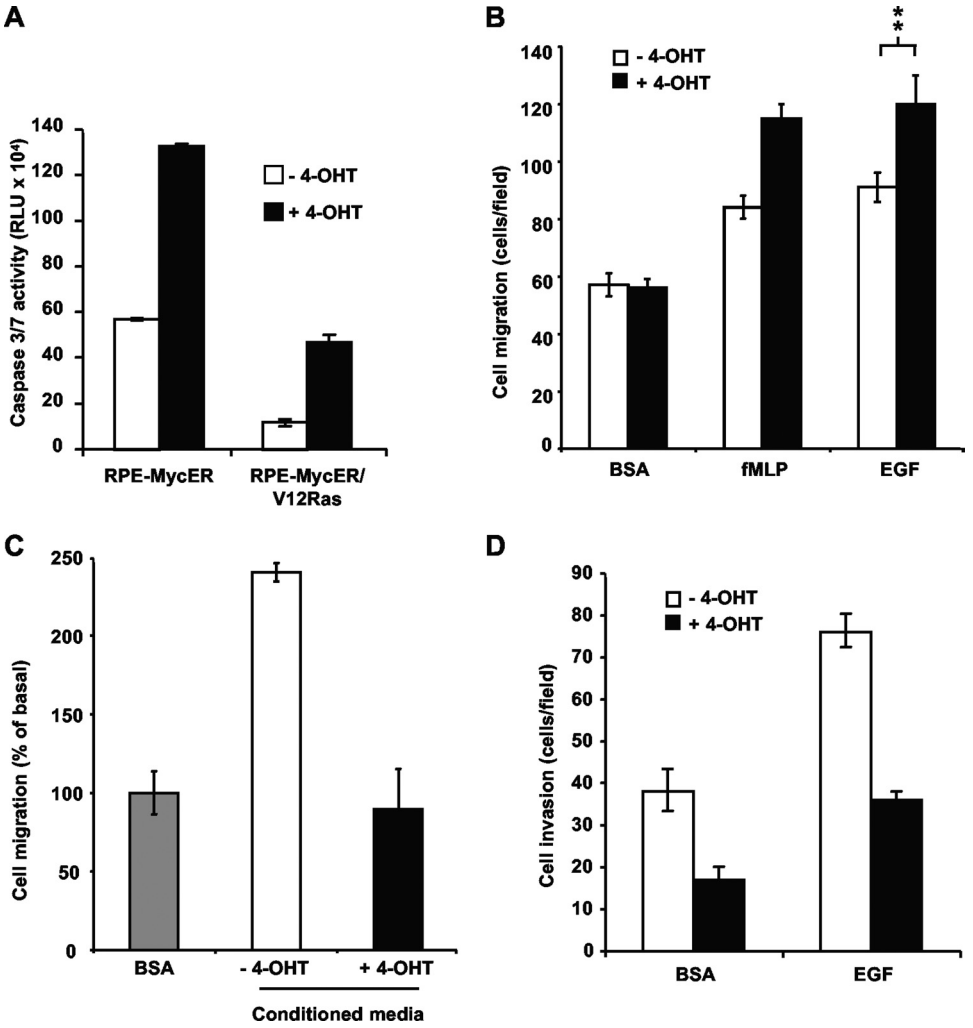

FIG. 7. V12Ras expression antagonizes the effects of c-Myc activation on hT-RPE apoptosis and cell motility but not invasiveness. (A) Effects of c-Myc activation on caspase 3/caspase 7 activity in hT-RPE-MycER cells in the absence or presence of V12Ras. Five thousand hT-RPE-MycER or hT-RPE-MycER/V12Ras cells were incubated in serum-free culture medium in 96-well plates for 48 h in the absence or presence of 4-OHT. Caspase activity was quantified using a luminometric assay that measures the amounts of active caspases 3 and 7 (Promega). (B) Effects of c-Myc activation on the motility of hT-RPE-MycER/V12Ras cells. After 24 h of 4-OHT treatment, hT-RPE-MycER/V12Ras cells (10^5/sample) were layered onto collagen-coated 8-μm filters in Boyden chambers for 2 h at 37°C to assay directional migration toward EGF (30 ng/ml) or fMLP (2 μM). BSA (0.1%) was used to measure nondirectional basal migration. The ability to migrate was measured by counting the cells on the lower-chamber side of the filter. (C) Analysis of the chemoattractive activity of medium conditioned by hT-RPE-MycER/V12Ras cells in the presence or absence of active c-Myc. Conditioned media from hT-RPE-MycER/V12Ras cells grown for 24 h in serum-free medium supplemented or not with 4-OHT were collected and used as chemoattractants in the lower compartments of Boyden chambers. Parental hT-RPE cells (10^5/sample) were layered into the upper compartments and incubated for 2 h at 37°C. Cell migration was quantified by counting the cells on the lower-chamber side of the filter. (D) Analysis of the effect of c-Myc activation on the invasiveness of hT-RPE-MycER/V12Ras cells. Cellular invasiveness was assayed by coating filters treated as described above with an additional layer of Matrigel (50 μg/ml). After 24 h of 4-OHT treatment, hT-RPE-MycER/V12Ras cells (10^5/sample) were layered into the upper compartments of Boyden chambers and incubated for 3 h at 37°C. The ability to invade toward EGF (50 ng/ml) was measured by counting the cells on the lower-chamber side of the filter. Significance was calculated using the Student *t* test (** indicates a *P* value of <0.001).

activation, remained above the level observed in the absence of Ras (Fig. 8). It is interesting that the increase of MEK phosphorylation induced by V12Ras expression was also reduced upon c-Myc activation, suggesting that regulation of the mitogen-activated protein kinase (MAPK) pathway by c-Myc may account for residual uPA and/or uPAR repression in V12Ras-expressing cells.

The differential regulation of uPA and uPAR following c-Myc activation in the presence and absence of Ras may explain the lack of cooperation between Ras and c-Myc in regulating Matrigel invasion. We reasoned that uPA's proteolytic activity could play an important role in the degradation of the Matrigel layer in the invasion assay. We therefore investigated the contribution of uPA expression to invasion by hT-RPE-MycER/V12Ras cells. Figure 9A shows that RNAi-mediated silencing of uPA in the absence of c-Myc activation strongly reduces the ability of hT-RPE-MycER/V12Ras cells to invade Matrigel in response to EGF, suggesting a strong requirement for uPA in

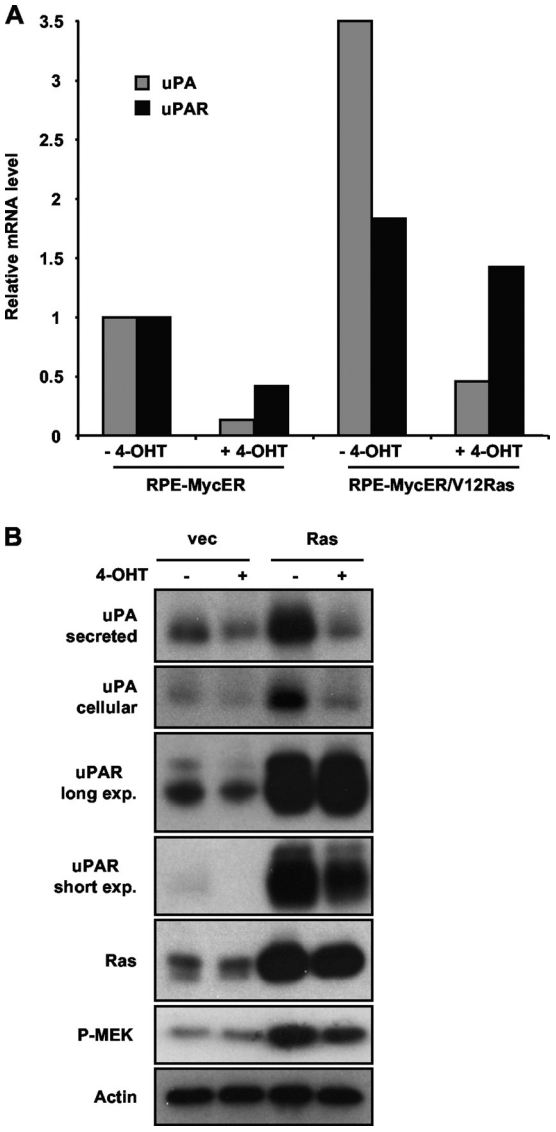

FIG. 8. Expression of uPA and uPAR is strongly increased by V12Ras, but uPA is still repressed by c-Myc activation. (A) qPCR analysis of uPA and uPAR mRNA abundance in hT-RPE-MycER and hT-RPE-MycER/V12Ras cells following c-Myc activation. Total RNAs from hT-RPE-MycER and hT-RPE-MycER/V12Ras cells cultured in the absence or in the presence of 4-OHT for 24 h were extracted and reverse transcribed. Relative levels of uPA and uPAR mRNAs were determined by real-time qPCR using the GAPDH gene as an internal control. Levels of expression are reported as the degree of change (*n*-fold) with respect to the amounts of uPA and uPAR mRNAs expressed by hT-RPE-MycER cells in the absence of 4-OHT. (B) Analysis of uPA and uPAR protein levels in hT-RPE-MycER and hT-RPE-MycER/V12Ras cells following c-Myc activation. Cells were treated or not treated with 4-OHT for 24 h, and total cell lysates were analyzed by immunoblotting to quantify the amounts of uPA and uPAR in the lysates. The amount of uPA secreted was quantified by concentrating equal volumes of conditioned media from 4-OHT-treated and untreated cells. The amount and activity of V12Ras were investigated by probing the immunoblot with antibodies against Ras and phospho-MEK1/MEK2 (P-MEK). Actin was used as a loading control. vec, cells transfected with an empty vector. Long exp. film exposed for 5 min; short exp. film exposed for 30 s.

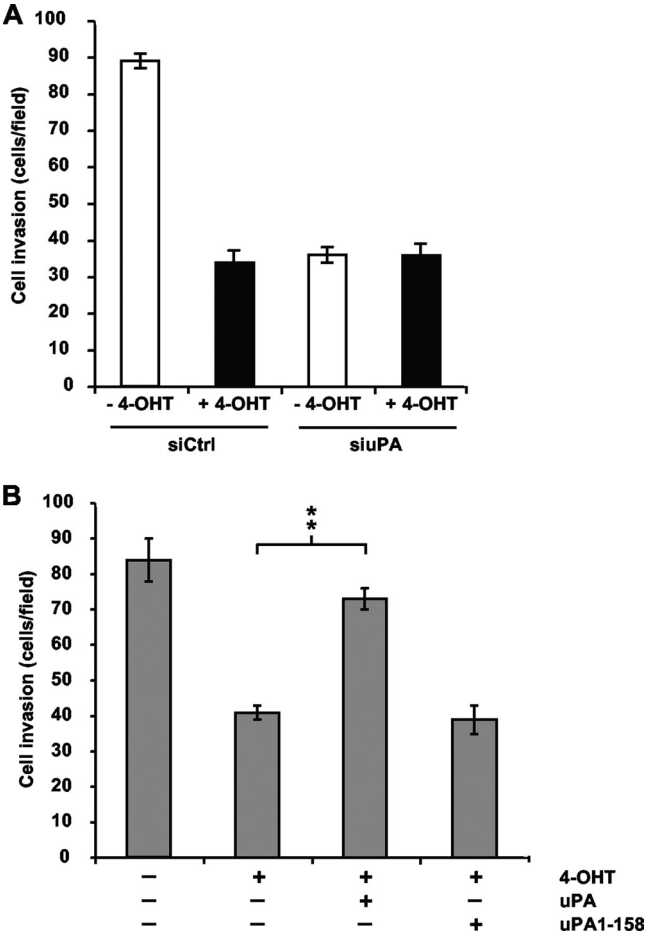

FIG. 9. hT-RPE-MycER/V12Ras cells rely on uPA's proteolytic activity for invasiveness. (A) uPA silencing reduces the ability of hT-RPE-MycER/V12Ras cells to invade Matrigel. hT-RPE-MycER/V12Ras cells were transfected with control or uPA-targeting siRNAs. After 48 h of 4-OHT treatment, hT-RPE-MycER/V12Ras cells (10^5/sample) were incubated in the upper compartments of Boyden chambers with an additional layer of Matrigel (50 μg/ml) over collagen-coated 8-μm filters. After incubation for 3 h at 37°C, the ability to invade toward EGF (50 ng/ml) was measured by counting the cells on the lower-chamber side of the filter. (B) Only proteolytically active uPA can restore the ability of hT-RPE-MycER/V12Ras cells to invade Matrigel after c-Myc activation. As described above, hT-RPE-MycER/V12Ras cells were treated or not treated with 4-OHT and assayed in Boyden chambers with a Matrigel layer. Where indicated, cells were preincubated for 2 h with either full-length uPA or an uPA variant with the catalytic domain deleted (uPA1-158). Significance was calculated using the Student *t* test (** indicates a *P* value of <0.001).

ECM invasion. We next asked if uPA's proteolytic activity was required for Matrigel invasion: hT-RPE-MycER/V12Ras cells were treated with 4-OHT in order to activate c-Myc and then incubated for 2 h with recombinant full-length uPA or with the uPA variant 1–158, in which the serine protease domain is deleted. As shown in Fig. 9B, only the proteolytically active full-length uPA could restore cellular ability to invade Matrigel in the presence of c-Myc activity.

Thus, the differential regulation of uPA and uPAR by c-Myc and Ras seems to correlate well with the distinct effects of these two oncoproteins on cell motility and invasiveness.

DISCUSSION

Deregulation of the proto-oncogene protein c-Myc in cells has been shown to confer the ability to enter the cell cycle in the absence of mitogenic factors and increased sensitivity to apoptosis (15). Given the difficulties of reconciling the proapoptotic effect of c-Myc activation with the ability of c-Myc to sustain oncogenesis, it has been proposed that c-Myc has an embedded tumor suppressor activity that limits its oncogenic potential (30). To gain insights into c-Myc tumor-suppressing activity, we took advantage of nontransformed human epithelial cells expressing MycER, an activatable form of c-Myc. Upon c-Myc activation, we noticed increased sensitization to apoptosis induced by serum withdrawal, together with cytoskeletal rearrangements and marked reductions in cellular motility and invasiveness. The analysis of transcriptional changes induced by c-Myc revealed strong downregulation of uPA and uPAR.

uPA is a complex, multifunctional factor with serine protease activity and is involved in plasminogen activation and matrix degradation. It has also a GFD responsible for uPAR binding and intracellular signaling. We have demonstrated previously that uPA signaling through its receptor protects against apoptosis induced by detachment or by chemotherapeutic agents by increasing Bcl-x_L expression levels (1). Accordingly, we observe that uPAR silencing sensitizes RPE cells to serum withdrawal-induced apoptosis to an extent that recapitulates c-Myc activation. Therefore, downregulation of the uPA/uPAR system by c-Myc appears to be a component of the increased sensitivity to serum starvation-induced apoptosis observed after c-Myc activation.

We also observed that activation of c-Myc was accompanied by a reduction in cellular motility and invasiveness. Reduction of cellular motility following c-Myc activation has already been documented. When specifically activated in the epidermis, c-Myc has been shown previously to inhibit cell motility and wound healing in transgenic mouse models (18, 45), causing the depletion of the stem cell compartment. Microarray analysis revealed a concomitant decrease in the expression of a large number of cytoskeleton proteins and components of the ECM. An effect of c-Myc on cellular motility has been reported also by Cappellen et al. (6). Following RNAi-mediated c-Myc silencing, the authors analyzed changes in the motility of three different cancer cell lines: two breast carcinoma cell lines, MCF-7 and MDA-MB231, and the cervix carcinoma cell line HeLa. Interestingly, the outcome was variable, with a strong increase in the motility of MCF-7 cells, a reduction in the motility of MDA-MB231 cells, and no change in the motility of HeLa cells.

The observation that c-Myc modulates motility in opposite ways in two different breast carcinoma cell lines implies that the consequence of c-Myc activation for cell motility may not depend merely on the cell type context but may also reflect the transformation status of the cell line. In line with this hypothesis, we found that in hT-RPE-MycER cells, the introduction of V12Ras had a strong impact on the effects of c-Myc activation. In the presence of constitutively active Ras, c-Myc activity on cell motility is reversed from negative to positive. This effect is probably correlated with the strong increase in uPAR expression induced by Ras, only marginally reduced by c-Myc activation. It is tempting to speculate that in MCF-7 cells, derived from a noninvasive breast carcinoma, the negative effect of c-Myc activation on cellular motility may still be dominant and that in MDA-MB231 cells, derived from a very aggressive breast carcinoma, further oncogenic lesions have either redirected the c-Myc transcriptional program or prevailed on c-Myc action. In accordance with this hypothesis, both uPA and uPAR protein expression levels are low in MCF-7 cells and high in MDA-MB231 cells (23). Given that MCF-7 and MDA-MB231 cells have been shown previously to have low and high Ras signatures, respectively (3), we propose that the low levels of uPA and uPAR expression in MCF-7 cells are dependent on c-Myc repression. In line with this hypothesis, when we analyzed the expression levels of uPA and uPAR in MCF-7 cells after c-Myc RNAi, we found them to be strongly increased (Fig. 3D).

In contrast to the effects of Ras on cell motility, the presence of constitutively active Ras did not reverse the negative effect of c-Myc activation on cellular ability to invade the ECM. Our mechanistic analysis showed that although the introduction of V12Ras increased the expression level of uPA to a great extent, c-Myc still had a profound effect on uPA repression. The inability of V12Ras to cooperate with c-Myc in invasiveness may therefore depend on the inability of V12Ras to sustain uPA expression in the presence of active c-Myc. This hypothesis is reinforced by the observation that only a proteolytically active recombinant form of uPA could complement the decrease in invasiveness due to c-Myc activation. Our data therefore imply that invasiveness is strongly dependent on uPA's proteolytic activity for proper matrix degradation.

It is widely accepted that tumorigenesis is a multistep process of somatic evolution, in which a genetic or epigenetic change has to provide a growth or survival advantage in the context of the tumor microenvironment. The increased uPAR levels in cells carrying active c-Myc and V12Ras greatly contribute to the enhancement of migration and protection from apoptosis. In contrast, uPA expression in hT-RPE cells is repressed by c-Myc regardless of V12Ras expression, suggesting either that a further mutation is needed or that high levels of uPA are not necessary for tumor growth and survival. Alternatively, we can speculate that low levels of uPA confer a secondary advantage that, perhaps, can be appreciated only *in vivo*. In this view, modulation of uPA by c-Myc may influence the interactions between the tumor and its microenvironment such that the decrease in invasive potential would be balanced with reduced infiltration by cells of the immune system. It has been reported, for instance, that N-Myc, inhibiting the expression of the chemokine CCL2, reduces natural killer T (NKT)-cell infiltration into the site of disease in neuroblastoma (39).

Transcriptional repression of CCL2 by N-Myc is therefore suggested to be part of a mechanism of tumor immune escape in neuroblastoma. Interestingly, together with the uPA gene, the CCL2 gene was among the genes most strongly downregulated by c-Myc in our microarray analysis.

An alternative possibility is that the expression of uPA by the tumor may not be selected during the somatic evolution process because uPA is provided to the invading tumor by the surrounding stroma. Accordingly, in human breast cancer *in vivo*, uPA has been found to be expressed also by stromal fibroblast-like cells and, in turn, bound to tumor epithelial cells, which

bear surface-associated uPA (2, 8). More recently, uPA has also been found to be part of a stroma-related gene signature that predicts resistance to neoadjuvant chemotherapy in breast cancer (17). Decreased expression of uPA by the tumor itself may even be favorable for tissue invasion, because it keeps the receptor on the tumor cell surface "unloaded" and therefore more responsive to the uPA produced from the stoma.

In conclusion, we have uncovered a negative regulatory link between c-Myc activation and the uPA/uPAR system that appears to explain the increased susceptibility to apoptosis and the reduction of cellular motility and invasiveness observed after c-Myc activation. We speculate that, together with sensitization to apoptosis, reductions in cellular motility and invasiveness may be part of the tumor-suppressing activity embedded in c-Myc activation. We propose that mutations in oncogenes like *ras* may be selected during tumor formation for their abilities to abolish the proapoptotic and antimigratory activities of c-Myc.

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