

Urokinase Signaling through Its Receptor Protects against Anoikis by Increasing BCL-xL Expression Levels*

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The acquired capabilities of resistance to apoptotic cell death and tissue invasion are considered to be obligate steps in tumor progression. The binding of the serine protease urokinase (uPA) to its receptor (uPAR) plays a central role in the molecular events coordinating tumor cell adhesion, migration, and invasion. Here we investigate whether uPAR signaling may also prevent apoptosis following loss of anchorage (anoikis) or DNA damage. If nontransformed human retinal pigment epithelial cells are pre-exposed to uPA or to its noncatalytic amino-terminal region (residues 1–135), they exhibit a markedly reduced susceptibility to anoikis as well as to UV-induced apoptosis. This anti-apoptotic effect is retained by a uPA-derived synthetic peptide corresponding to the receptor binding domain and is inhibited by anti-uPAR polyclonal antibodies. Furthermore, the stable reduction of uPA or uPAR expression by RNA interference leads to an increased susceptibility to UV-, cisplatin-, and detachment-induced apoptosis. In particular, the level of uPAR expression positively correlates with cell resistance to anoikis. The protective ability of uPA is prevented by UO126, LY294002, by an MAPK targeting small interference RNA, and by a dominant negative Akt variant. Accordingly, incubation of retinal pigment epithelial cells with uPA elicits a time-dependent enhancement of MAPK and phosphatidylinositol 3-kinase activities as well as the transcriptional activation of Bcl-xL anti-apoptotic factor. Vice versa, the silencing of Bcl-xL expression prevents uPA protection from anoikis. In conclusion, the data show that ligand engagement of uPAR promotes cell survival by activating Bcl-xL transcription through the MEK/ERK- and phosphatidylinositol 3-kinase/Akt-dependent pathways.

Oncogenic cell transformation is currently viewed as a multistep process in which a series of genetic lesions change cellular physiology leading to the acquisition of new capabilities, such as an enhanced ability to proliferate, migrate, and escape apoptotic cell death (1). Apoptosis can be viewed as a safe-lock mechanism that could prevent the establishment of a fully transformed phenotype. For instance, it is currently accepted that uncontrolled proliferation could by itself prime the transforming cell to apoptotic cell death (2, 3).

Similarly, the ability to migrate and invade through the basement membrane into surrounding tissues is one of the essential hallmarks of cancer and a prerequisite for both local tumor progression and metastatic spread, but it can also lead to apoptosis if not counterbalanced by

survival signals (1). In particular, apoptosis induced by loss of anchorage, a phenomenon also known as anoikis (from the Greek for "homelessness"), has been shown to limit the spread of epithelial cells outside of the tissue environment. Therefore, successful cellular transformation requires an increased cell resistance to death through the overexpression of anti-apoptotic factors or the activation of survival kinases (4, 5). Many cell types derived from human malignancies, such as gastric cancers, mammary tumors, colon cancers, osteosarcomas, and lung carcinomas, are resistant to anoikis (6, 7).

Among the many genes involved in the regulation of cell spreading and migration, those coding for the serine protease urokinase (uPA)² and its cognate receptor (uPAR) play a central role in tumor development because of their clear-cut ability to regulate cytoskeleton dynamics, cell adhesion, and matrix integrity (8). High levels of uPA and uPAR have been found in many human malignant tumors, and they strongly correlate with poor prognosis and unfavorable clinical outcome (9, 10). Urokinase converts the pro-enzyme plasminogen into plasmin, a wide spectrum serine protease able to degrade most of the extracellular matrix (ECM) components and activate latent collagenases. The uPA is secreted in the pro-enzyme form (pro-uPA), which can be activated in the extracellular milieu by a single proteolytic cleavage occurring between Lys¹⁵⁸ and Ile¹⁵⁹, thus generating a two-chain enzyme. The uPA is a multidomain protein that includes an amino-terminal "growth factor-like" domain (GFD, residues 1–49) followed by a "kringle" region (residues 50–131) linked by the "connecting peptide" (residues 135–158) to a large catalytic moiety (residues 158–411). High affinity binding to uPAR occurs through the GFD of uPA and neither involves nor prevents the protease catalytic activity (11, 12). The urokinase-type plasminogen activator receptor is a three-domain (D1, D2, and D3) glycosylphosphatidylinositol-anchored protein with a high affinity for uPA and the amino-terminal fragment ATF (residues 1–135), mainly interacting with the external D1 domain (13). Enzymatic cleavage of uPAR between D1 and D2 un masks a region, corresponding to residues 88–92 of the human sequence (SRSRY), that is able to elicit intracellular signaling, even as a synthetic peptide (14, 15). Mechanistically, signal transduction is activated through the physical and functional interaction of uPAR with transmembrane receptors, such as the integrins, FPRL1, or the EGF receptor (16–18). Considerable effort has been directed to the analysis of uPAR-dependent signaling effects together with the relative responses, mainly related to cell motility, adhesion, and cytoskeletal status (19). In most cases, these effects are clearly proteo-

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² The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; ATF, amino-terminal fragment (residues 1–135); GFD, uPA growth factor-like domain (residues 1–49); GFDp, peptide corresponding to uPA residues 12–32; RPE, retinal pigment epithelial cells; HEK, human embryonic kidney cells; ECM, extracellular matrix; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; poly-HEMA, poly (2-hydroxyethyl methacrylate); MEK, MAPK/ERK kinase; siRNA, small interference RNA; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FAK, focal adhesion kinase; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline.

lytically independent but uPAR-dependent. One example is provided by the uPA-dependent regulation of the ratio between cell motility and adhesion through the control of p56/59 *hck* activity in U937 monocyte-like cells (20). In the HEp3 human carcinoma cells, association of uPA to uPAR regulates the ERK1/2/p38 activity ratio, thus modulating the balance between tumor cell proliferation and dormancy (21).

To date, increasing evidence supports a role for uPA also in the regulation of cell proliferation (22). First of all, endogenous uPA is an autocrine mitogen for the human melanoma cell line GUBSB, as inhibition of receptor binding by anti-uPA antibodies in the culture medium causes a strong reduction of cell proliferation (23). If the expression of uPA and uPAR is reduced by antisense or interference RNA, prostate cancer tumorigenicity and invasion is prevented (24). Moreover, unlike the wild-type mice, the uPA^{-/-} animals do not allow progression of chemically induced melanocytic neoplasms to melanomas, indicating that uPA contributes to malignant growth (25). Furthermore, T241 fibrosarcoma cells implanted in uPA^{-/-} mice exhibit decreased proliferation and increased apoptosis, suggesting that alterations in host expression of uPA may affect the balance between tumor cell death and proliferation (26). Interestingly, uPAR-deficient HEp3 human carcinoma cells inoculated in the modified chorioallantoic membrane enter a state of dormancy in which cells survive but do not proliferate (27). Recent findings indicate that a decreased uPAR expression may promote apoptosis. This is the case of SNB19 glioblastoma cells expressing antisense uPAR constructs that are less invasive than parental cells when injected *in vivo* and undergo loss of mitochondrial transmembrane potential, release of cytochrome *c*, caspase-9 activation, and apoptosis (28). Furthermore, glioma cells bearing a reduced uPAR number are more susceptible to tumor necrosis factor- α -related apoptosis-inducing ligand-induced apoptosis than parental cells (29). Limited information about the pathways involved in the uPA-dependent control of cell growth and survival is available to date; in MDA-MB-231 breast cancer cells cultured in the presence of anti-uPA blocking antibodies, the level of phosphorylated ERK decreases substantially and apoptosis is promoted, showing that endogenous uPA is a major determinant of ERK activation and protection from apoptosis (30). Furthermore, the ability of the uPA/uPAR interaction to stimulate PI3K/Akt signaling through β 5 integrin has been described, although it seems to be dispensable for chemotaxis (31).

The aim of this study is to investigate whether uPA, in addition to its ability to degrade ECM, to drive migration, and to elicit proliferation, may contribute to tumorigenesis by preventing apoptotic cell death. In particular, we analyzed the effects of uPA on detachment-induced apoptosis of retinal pigment epithelial (RPE) cells immortalized with human TERT. These cells undergo anoikis, as well as UV light- or cisplatin-induced apoptosis, unless they are pre-exposed to uPA or to its noncatalytic amino-terminal region. The relevance of the uPA/uPAR system to the RPE survival is further indicated by the finding that the reduction of uPA or uPAR by an RNAi approach enhances cell sensitivity to anoikis. Finally, the present work sheds light on the uPAR-dependent anti-apoptotic mediators, indicating the involvement of PI3K/Akt and MEK/ERK-dependent pathways impinging on the transcriptional activation of Bcl-xL anti-apoptotic factor.

EXPERIMENTAL PROCEDURES

Reagents—Cell death detection ELISA kit, FuGENE 6 transfection reagent, and CompleteTM protease inhibitor mixture were from Roche Applied Science. All cell culture reagents were purchased from Invitrogen. RNAi human/mouse starter kit was from Qiagen (Valencia, CA). The poly(2-hydroxyethyl methacrylate) (poly-HEMA), MEK inhibitors

PD98059 and UO126, the PI3K inhibitors LY294002, cycloheximide, cisplatin, 12-*O*-tetradecanoylphorbol-13-acetate, and EGF were from Sigma. The Src kinase inhibitor PP2 was from Calbiochem. 399 rabbit (399R) anti-uPAR polyclonal antibody was from American Diagnostica (Greenwich, CT). The 5B4 anti-uPA monoclonal antibody was a gift of M. L. Nolli, Areta International, Milan, Italy. The anti- α β 5 monoclonal antibody was from Chemicon (Temecula, CA). The methylcellulose was from Stem Cell Technologies (Vancouver, British Columbia, Canada). The pPIC9 vector and *Pichia* strain GS115 were obtained from Invitrogen. The two uPA variants, uPA 1–158 or ATF (corresponding to the first 158 amino acids or 135 amino acids of human uPA, respectively), have been expressed as secreted products in the methylotrophic yeast *Pichia pastoris* and purified by 5B4-agarose chromatography, as described (12). The GFDp (DCLNGGTAVSNKYFSNIHWCN) and the 5Ala-GFDp (DCLNGGTAVSAAAAANIHWCN) peptides are a gift of P. Grieco, Naples, Italy.

Plasmids—To obtain the pPIC9 vector expressing ATF or uPA 1–158, the region encoding residues 1–130 of human uPA, included in the 1414-bp *SacI*/*FspI* fragment, was excised from pPIC9-His-uPA. This fragment was either ligated into the *SacI*/*EcoRI* sites of the pPIC9 multicloning site, together with a double strand oligonucleotide coding for residues 131–135 followed by three stop codons (5'-gcagatggaaaataatgatcaagtactg) to encode ATF, or ligated to a double strand oligonucleotide coding for residues 131–158 (5'-gcagatggaaaaaacctctctctcgcggaagaattaaaatttcagtggtggccaaaagactctgagcctcgctttaagttag) to encode uPA 1–158. The uPAR expression vector pcDNA3.1-uPAR contains the entire human uPAR cDNA (17). The pUSE/Akt^{K179M} was from Upstate (Charlottesville, VA).

Gene Silencing—The mammalian expression vector, pSUPER (Oligo-Engine, Seattle, WA), was used for siRNA expression in RPE cells. The gene-specific insert includes a 19-nucleotide sequence corresponding to nucleotides 1124–1142 downstream of the transcription start site (tgactgttgaagctgat) of uPA or to nucleotides 565–583 (gccgttacctcgaatgat) of uPAR. In both cases, this insert is separated by a 9-nucleotide noncomplementary spacer (tctcttgaa) from the reverse complement of the same 19-nucleotide sequence. These constructs are referred to as pSUPER/uPAi or pSUPER/uPARi. A control vector (pSUPER/GFPi) was constructed using a 19-nucleotide sequence (gagcgcaccatcttctca) with no significant homology to any mammalian gene sequence and based on the gene sequence of the green fluorescent protein (GFP) gene. These sequences were inserted into the pSUPER vector digested with *Bgl*III and *Hind*III (New England Biolabs). The siRNA oligonucleotides against MAPK1 and Bcl-xL were from Qiagen.

Cell Culture and Generation of Stable and Transient Transfectants—hTert-RPE (Clontech) and HEK-293 were cultured in Dulbecco's modified Eagle's medium supplemented with 100 μ g/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum (FBS) (pH 7.2–7.4) in a humidified atmosphere containing 5% CO₂ at 37 °C. For stable RPE transfectants, a semi-confluent 10-cm dish was incubated with 18 μ l of FuGENE 6 and 6 μ g of DNA according to the manufacturer's protocol. The RPE cells were then diluted, passaged, and selected with the neomycin analogue G418 (800 μ g/ml). Stable HEK-293 transfectants were obtained by electroporating 1 \times 10⁷ subconfluent HEK-293 cells with 80 μ g of plasmid DNA, in 0.9 ml of culture medium. The expression of uPAR by HEK-293/uPAR clones was quantitated by Western blotting with anti-uPAR RII antibody from total cell lysates. For transient RPE transfections, 1 \times 10⁵ cells were incubated in serum-free DMEM with 3 μ l of FuGENE 6 transfection reagent with 1 μ g of the relevant plasmids. A transfection efficiency of 60% was obtained. Transient transfectants were analyzed after 48 h. For the transfection of siRNA oligonucleo-

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tides, we used the “fast-forward” protocol; 3.5×10^4 cells/well were seeded in a 24-well plates and incubated with the transfection complexes (5 nM siRNA with 3 μ l of HiPerFect Transfection Reagent), according to the manufacturer’s protocols. Transfection efficiency (nearly 90%) and functional specificity were monitored with the RNAi starter kit by using an Alexa Fluor 488 labeled nonsilencing siRNA (Qiagen).

Western Blot Analysis—Cells, treated as specified in the figure legends, were lysed ($1.5 \times 10^5/100 \mu$ l) for 30 min on ice in a buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 100 mM NaF, 1% Triton X-100, supplemented with 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 \times CompleteTM protease inhibitor mixture. The debris was then removed by centrifugation at $10,000 \times g$ for 10 min at 4 °C, and protein content was assessed by the Bradford protein assay. Equal protein amounts were separated on 10 or 12.5% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore Corp., Billerica, MA). The membrane was subsequently incubated for 1 h at room temperature in a TBST buffer (125 mM Tris-HCl (pH 8.0), 625 mM NaCl, 0.5% Tween 20) containing 4% skim milk and further incubated with 1 μ g/ml of the following antibodies: anti-uPAR RII (a gift of G. Hoyer-Hansen, Finsen Institute, Copenhagen, Denmark); anti-uPA polyclonal (kindly provided by P.A. Andreasen, Aarhus, Denmark); anti-Bcl-x (BD Biosciences); anti- β -actin (Sigma); anti-phospho-p44/p42 MAPK, anti-phospho-Akt (Ser-473), anti-Akt, and anti-PARP (Cell Signaling, Beverly, MA). Reaction was detected using the chemiluminescence system ECL Plus (Amersham Biosciences).

Cell Sensitivity to Detachment-induced Apoptosis—RPE cells were grown to subconfluence in 24-well plates and serum-starved for 24 h. To remove membrane-bound ligands, cells were treated with an acidic buffer (50 mM glycine, 100 mM NaCl (pH 3)) for 2 min (32), washed with DMEM, and then incubated in serum-free medium with uPA-related proteins or EGF at the indicated concentrations for 8 h. When indicated, cells were pretreated with 399R anti-uPAR antibody (10 μ g/ml) or with different pharmacological inhibitors (20 μ M LY294002, 50 μ M PD98059, 10 μ M UO126, or 10 μ M PP2) or with diluents for 30 min; treated cells were then collected by mild trypsinization and seeded onto 24-well plates coated or not with poly-HEMA in the presence of the indicated molecules for 8 h. To coat 24-well culture plates, 0.5 ml of a 10 mg/ml solution of poly-HEMA in ethanol was applied twice to each dish, dried, and extensively washed with PBS. To avoid potential anti-apoptotic effects caused by clumping, cells were seeded at 5×10^4 cells/ml in DMEM, 10% FBS in the presence of 0.6% methylcellulose and plated on poly-HEMA-coated 24-well plates. After 8 h, cells were collected, washed with PBS, and then analyzed for the extent of apoptosis by the cell death ELISA, according to the manufacturer’s instructions.

Induction of UV- and Cisplatin-induced Apoptosis—RPE/Vec, RPE/SiuPA, or RPE/SiuPAR cells (5×10^4 cells/sample) were grown in 24-well plates for 16 h, cultured for 24 h with or without 10% serum, and then UV-irradiated with 20 J/cm² using a Stratelinker 2400 (Stratagene) in PBS. Immediately after UV irradiation, PBS was removed, and the original medium was put back into plates. When indicated, cells were treated with an acidic buffer for 2 min, washed with DMEM, and then exposed, in serum-free conditions, to the indicated effectors or antibodies for 30 min prior to UV irradiation. After 24 h, both attached and floating cells were collected and subjected to cell death ELISA. A sample of cells not exposed to UV light was included as negative control. For cisplatin-induced apoptosis, RPE/Vec, RPE/SiuPA, or RPE/SiuPAR cells (5×10^4 cells/sample) were plated into 24-well plates and after 16 h were serum-starved or not, and after 24 h the cell culture medium was

replaced with fresh DMEM, 10% FBS either containing 100 μ M cisplatin or diluents for 24 h.

mRNA Quantification by Real Time PCR—RPE cells were grown to subconfluence and, 16 h later, serum-starved for 24 h; 4×10^6 cells were incubated for 5 h with or without 10 nM uPA 1–158 and washed with phosphate-buffered saline; the total RNA was isolated by acid-phenol extraction using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. 1 μ g of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, MI), and 2 μ l of a 1:10 dilution of reverse transcription reaction were analyzed by quantitative real time PCR with a DNA Engine Opticon 2 System (MJ Research, Boston), using DyNAmo HS SYBR Green qPCR kit (Finnzyme). *Bcl-xL* mRNA quantitation was normalized to the internal glyceraldehyde-3-phosphate dehydrogenase mRNA. Primers, designed using Primer3 software and used at 0.25 μ M, were as follows: for *Bcl-xL* amplification, 198-bp product, forward primer 5′-AAG-GATACAGCTGGAGTCAG-3′ and reverse primer 5′-GAGTTCAT-TCACTACCTGTTC-3′; for glyceraldehyde-3-phosphate dehydrogenase amplification, 161-bp product, forward primer 5′-ACATGTTCCAA-TATGATTCCA-3′ and reverse primer 5′-TGGACTCCACGACGT-ACTCAG-3′. The sample from cells not exposed to uPA 1–158 was chosen as reference value. The relative level of expression was calculated with the formula $2^{-\Delta\Delta Ct}$.

Statistics—Data are shown as mean \pm S.D. and represent one of at least three separate experiments undertaken in triplicate, unless stated otherwise. Differences between data sets were determined by the Student’s *t* test. Differences described as significant in the text correspond to $p < 0.05$.

RESULTS

The Amino-terminal Region of uPA Protects Retinal Pigment Epithelial Cells from Anoikis—The catalytically independent association of urokinase (uPA) with its receptor (uPAR) has an established role in cell mobilization. Because several motogens have been shown to exert an intrinsic survival activity on cells deprived of ECM attachment (33), we sought to investigate if uPAR engagement with uPA could have a similar protective effect. To study the effect of uPA on detachment-induced apoptosis (anoikis), we made use of a telomerase-immortalized, non-transformed retinal epithelial cell line (hTert-RPE or RPE). Nontransformed epithelial cells can undergo anoikis if seeded in dishes coated with poly-HEMA, a compound that inhibits cell adhesion by preventing matrix deposition (33). In agreement with pre-existing literature, we observed that as early as 4–6 h after plating RPE cells onto poly-HEMA-coated dishes, a significant fraction (~40%) of cells become positive to annexin V and subsequently exhibit caspase-3 and PARP activation (not shown) (34). To test the effect of human uPA on cells undergoing anoikis, recombinant pro-uPA and catalytically inactive uPA-related products, including the ATF (residues 1–135), uPA 1–158, and GFD peptide (GFDp, DCLNGGTAVSNKYFSNIHWCN) were employed (Fig. 1A). In agreement with Elner *et al.* (35), we found that RPE express about 8,000 surface receptors per cell.³ When RPE cells were plated onto poly-HEMA-coated dishes, the extent of anoikis increased by 10-fold with respect to cells seeded in uncoated dishes, as assessed by quantitating the cytosolic DNA-histone complexes (Fig. 1B). However, if RPE cells deprived of cell attachment were incubated with recombinant pro-urokinase for 8 h, the extent of anoikis was reduced by ~50%. To exclude any effect due to catalytic activity, cells were exposed to ATF under the same conditions. As a result, the protective ability of full-

³ P. Franco, D. Alfano, and M. P. Stoppelli, unpublished data.

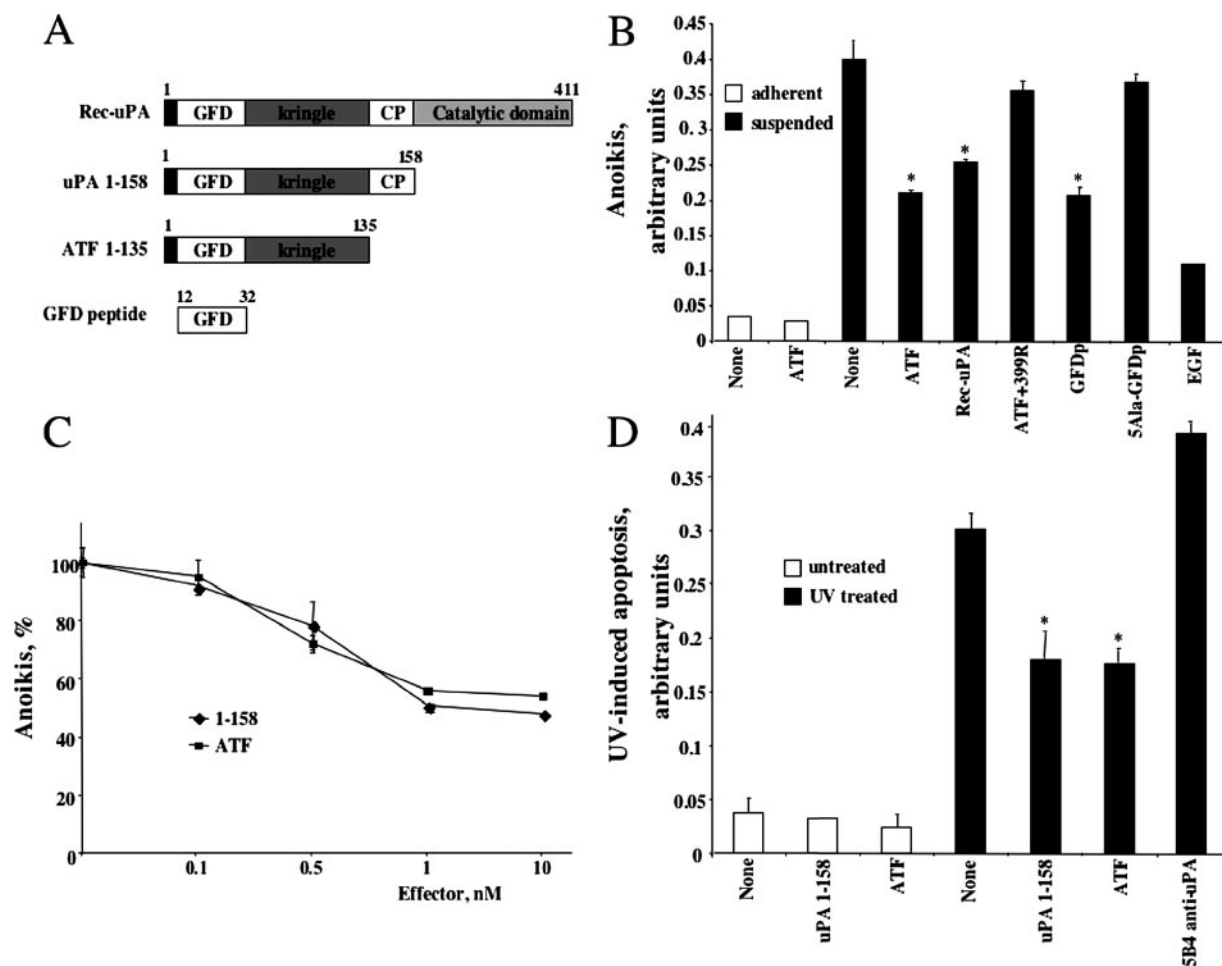


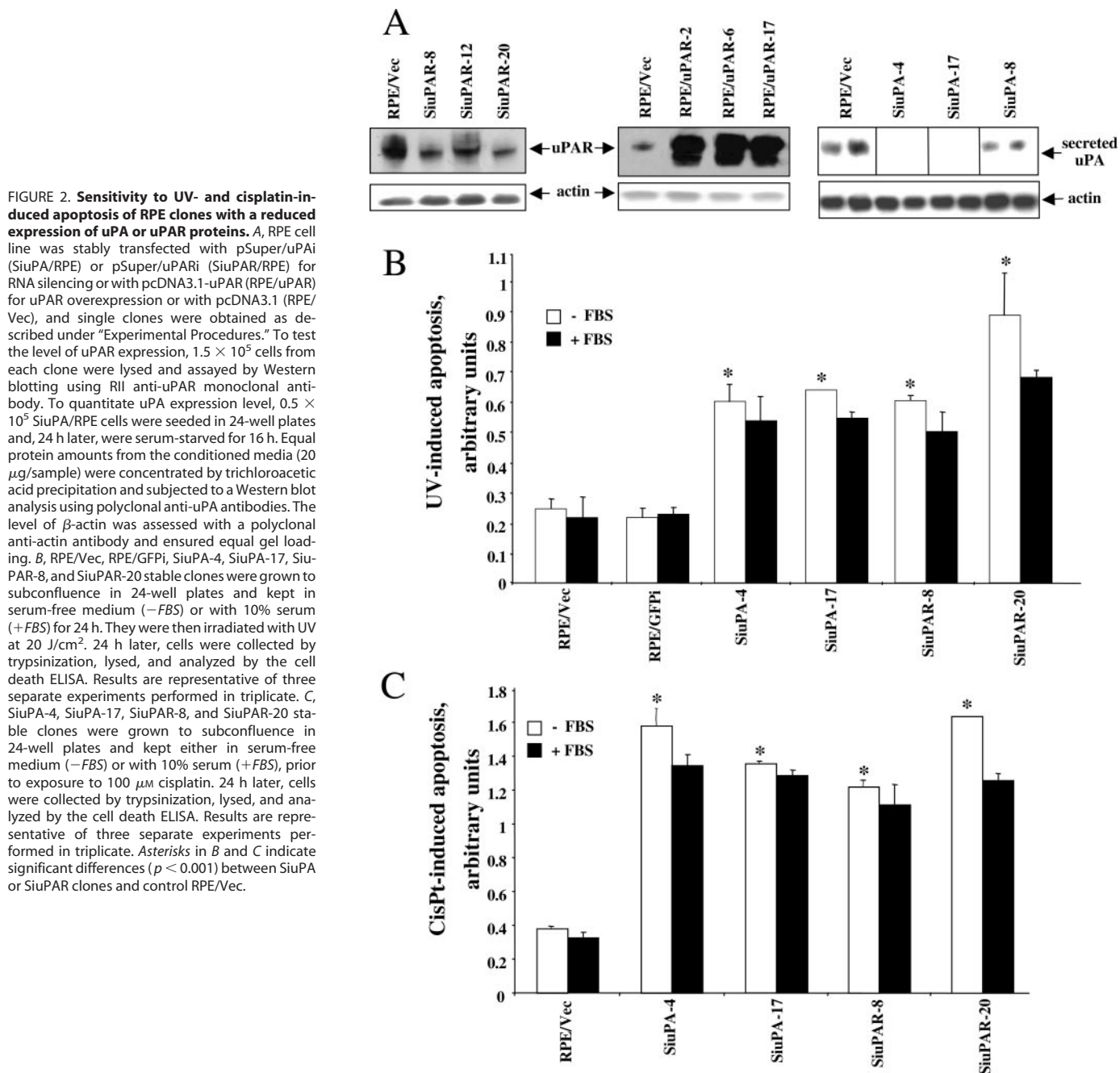
FIGURE 1. Protection from anoikis and UV-induced apoptosis by the amino-terminal region of human uPA. *A*, schematic representation of the human pro-urokinase structure showing the GFD (residues 1–49), the kringle domain (residues 50–131), the connecting peptide region (CP, residues 135–158), and the catalytic domain (residues 159–411). Recombinant pro-urokinase (residues 1–411) is purified from *Escherichia coli*. The uPA 1–158 and ATF (residues 1–135) variants were obtained as secreted products in the *P. pastoris* expression system. The GFD-derived peptide corresponds to residues 12–32 (GFDp). *B*, RPE cells were grown to subconfluence in 24-well plates and serum-starved for 24 h. Cells were then exposed to each of the following effectors: 10 nM bacterial recombinant pro-urokinase, 10 nM GFDp, 10 nM control peptide (5Ala-GFDp), 10 nM ATF, 10 ng/ml EGF for 8 h. When indicated, they were pretreated with 10 μ g/ml 399R anti-uPAR antibody for 30 min; cells were then detached by mild trypsinization. 5×10^4 cells/well were seeded onto poly-HEMA-coated or noncoated 24-well dishes. After 8 h, adherent or suspended cells were collected and analyzed for the extent of apoptosis by the cell death ELISA. Values represent the mean \pm S.D. absorbance at 405 nm of a representative experiment undertaken in triplicate. *C*, RPE cells were exposed to the indicated concentrations of uPA 1–158 or ATF as described in *B*. 5×10^4 RPE cells/sample were seeded into 24-well plates and 16 h later were serum-starved for 24 h; then cells were preincubated with 10 nM 1–158, 10 nM ATF, or 20 μ g/ml 5B4 anti-uPA antibody for 30 min, irradiated with UV at 20 J/cm², or untreated and cultured for 24 h under serum-free conditions. Then all cells were collected, lysed, and analyzed for the extent of cell death by ELISA. The results are the average of three determinations from three separate experiments with S.D. indicated by error bars. Asterisks in *B* and *D* indicate significant differences ($p < 0.003$) between treated samples and control (None).

length pro-uPA is fully retained by ATF, showing that proteolytic activity is dispensable to the anti-apoptotic effect of uPA.

uPA-mediated Protection from Anoikis Depends on uPAR Binding—Because ATF retains the receptor binding ability of uPA, we tested whether uPAR engagement is needed to achieve protection from anoikis. To answer this question we employed either a peptide corresponding to part of the GFD domain, shown to be the minimal requirement for receptor binding (GFDp, residues 12–32) (36), or a specific anti-uPAR polyclonal antibody (399R) that prevents uPAR binding (Fig. 1A) (17). As shown in Fig. 1B, unlike the effect of the control peptide (5Ala-GFDp, in which the residues critical to uPAR binding have been replaced with Ala residues), GFDp retains the protective ability of ATF. Furthermore, preincubation of RPE cells with 399R antibody completely abolished the survival action of ATF, indicating an obligate requirement for uPAR binding. Finally, a dose-response analysis indicates that the IC₅₀ of ATF and uPA 1–158 anti-apoptotic effects (~ 0.5 nM) is compatible with the high affinity of the uPA/uPAR interaction (Fig. 1C).

The Amino-terminal Region of uPA Protects RPE Cells Also from UV-induced Apoptosis—In order to investigate if uPA plays a more general role as a survival factor, we examined the effect of ATF on RPE cell survival following UV irradiation. Cell viability was reduced to 50% upon UV irradiation, as measured after 24 h by trypan blue exclusion (data not shown). This effect is due to increased apoptosis (Fig. 1D). However, if UV-treated RPE cells were pre-exposed to uPA 1–158 or ATF, cell death was reduced by nearly 40% (Fig. 1D). As mentioned earlier, RPE cells secrete uPA in the culture medium, suggesting the occurrence of an autocrine anti-apoptotic loop based on uPAR engagement. To test this possibility, RPE cells were acid-treated and exposed to 5B4 monoclonal antibody that inhibits the uPA/uPAR interaction (37). Following this treatment, UV-induced apoptosis increases by 30%, indicating that uPA availability in the culture medium supports RPE survival (Fig. 1D).

RPE Cell Clones with Reduced Expression of uPA or uPAR Exhibit an Increased Susceptibility to Apoptosis—The results shown in Fig. 1 indicate that the uPA/uPAR association has a positive effect on cell survival



following different apoptotic insults. Therefore, we hypothesized that a stable reduction of uPA and/or uPAR expression may enhance cellular susceptibility to apoptosis. To test this possibility, the pSUPER vector was engineered to express short hairpin RNAs targeting either human uPA or uPAR. A pSUPER encoding a short hairpin RNA targeting the green fluorescent protein (GFP) was employed as a negative control. All constructs were introduced into RPE cells, and the resulting single clones (30 SiuPA, 24 SiuPAR), as well as the pool of the GFPi transfectants, were analyzed by Western blotting of cell lysates with anti-uPAR antibodies or conditioned media with anti-uPA antibodies, followed by quantitative densitometry. This analysis revealed a significant decrease in uPAR (30–50% of control in the SiuPAR clones) and uPA protein levels (2–10% of control in the SiuPA clones) in the six clones that were employed for further analyses (Fig. 2*A*, left and right panels). SiuPA-4, SiuPA-17, SiuPAR-8, and SiuPAR-20 were tested for susceptibility to

UV irradiation without further uPA addition. In all cases, we could detect a 2–3-fold increase of cell sensitivity to UV-induced cell death with respect to control transfected cells (RPE/Vec or RPE/GFPi) (Fig. 2*B*). A 3–4-fold increase of sensitivity to cell death was also observed following treatment of SiuPA-4, SiuPA-17, SiuPAR-8, and SiuPAR-20 clones with the drug cisplatin (Fig. 2*C*). Interestingly, the reduced survival of the SiuPA and SiuPAR clones, exposed to UV or cisplatin, is observed also in the presence of serum (Fig. 2, *B* and *C*). As predicted, SiuPA and SiuPAR clones also exhibited an increased susceptibility to anoikis, as indicated by the level of cleaved PARP following forced detachment from the tissue culture dish (Fig. 3*A*). Finally, to establish if an increased level of surface receptors up-regulates cell resistance to anoikis, RPE stable cell clones with increased uPAR expression levels were obtained (Fig. 2*A*, middle panel). By quantitative receptor binding assays, the level of available uPAR on the cell surface was consistent with

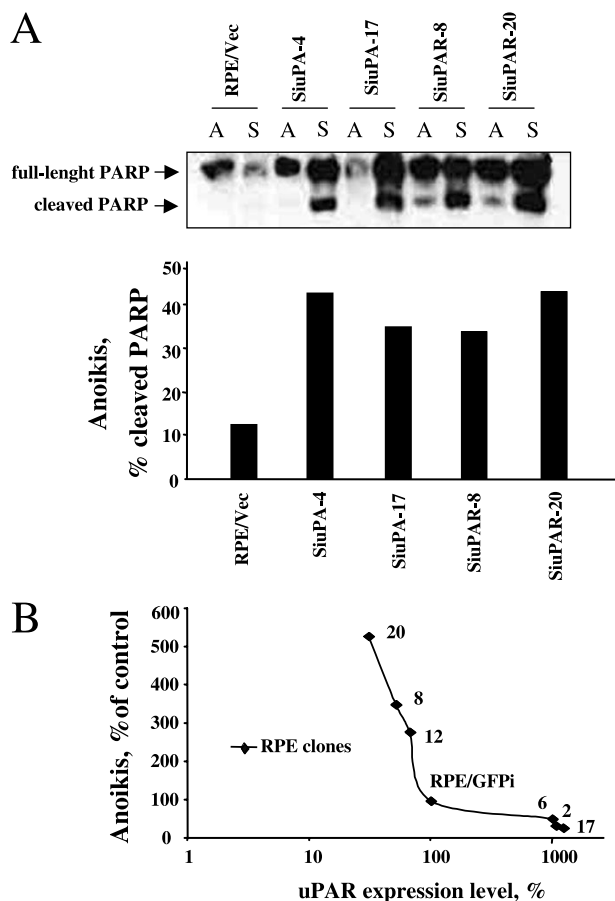


FIGURE 3. Relationship between the extent of uPAR expression and cell sensitivity to anoikis. *A*, equal protein amounts (50 μ g/sample) extracted from RPE/Vec, SiuPA-4, SiuPA-17, SiuPAR-8, or SiuPAR-20 clones were plated onto poly-HEMA-coated (S) or uncoated dishes (A), as described in the legend to Fig. 1B. They were then subjected to Western blot analysis using a polyclonal PARP antibody recognizing both full-length and cleaved PARP. The extent of cleaved PARP is calculated relative to the total PARP and is reported in the histogram. *B*, SiuPAR-20, SiuPAR-8, SiuPAR-12, and RPE/uPAR-2, RPE/uPAR-6, RPE/uPAR-17 clones were plated in poly-HEMA-coated dishes, and the extent of anoikis was determined by cell death ELISA as described under "Experimental Procedures." The level of uPAR expression was determined previously by Western blotting (Fig. 2A). The uPAR level of the RPE/GFPi is taken as 100%, and all values of the single clones were expressed relative to that.

the differences assessed by Western blotting (not shown). The selected RPE clones, namely uPAR-2, uPAR-6, and uPAR-17, produced 10–15-fold more uPAR than RPE/GFPi cells, and as expected, they exhibited a reduced sensitivity to anoikis (Fig. 3B). Interestingly, this analysis revealed a striking correlation between uPAR expression levels and susceptibility to anoikis, as shown in Fig. 3B.

To investigate whether uPAR plays a general role as a survival factor, we took advantage of the HEK-293 cell line, which does not express detectable urokinase receptors (38). These cells were compared with HEK-293 cells ectopically expressing uPAR, named HEK-293/uPAR (~350,000 uPAR/cell). Strikingly, uPAR-overexpressing HEK-293 cells, subjected to serum starvation, were more than two times more resistant to apoptosis than control (Fig. 4), suggesting that the uPAR-dependent positive effect on cell survival is not restricted to RPE cells but is a more general phenomenon.

Engagement of uPAR Activates the PI3K/Akt and MEK/ERK-dependent Pathways—Death by apoptosis is considered to be the output of a complex integration of signaling events, where the action of death-promoting molecules is constantly counterbalanced by the action of survival signaling pathways (39). The signaling mediators that have a more established role in survival signaling are the MEK/ERK- and the PI3K/

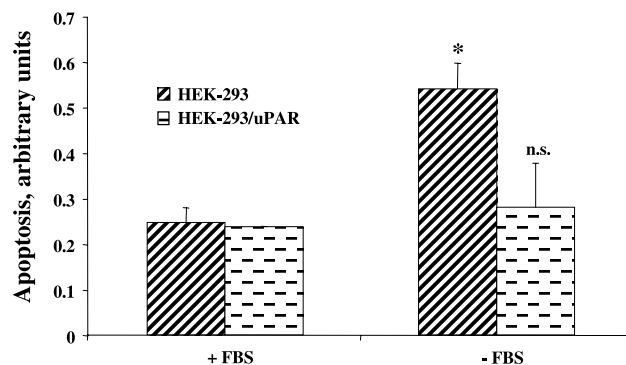


FIGURE 4. uPAR ectopic expression protects from apoptosis induced by serum starvation. HEK-293 and HEK-293/uPAR cell lines were grown to subconfluence in 6-well plates and cultured for 24 h in the presence (+FBS) or in absence of serum (–FBS). The adherent cells were then detached by mild trypsinization and collected together with floating cells; the extent of apoptosis was determined by the cell death ELISA. The results are the average of three determinations with S.D. indicated by error bars. *, $p < 0.005$ HEK-293 –FBS versus HEK-293 +FBS; n.s., HEK-293/uPAR –FBS nonsignificant versus HEK-293/uPAR +FBS.

Akt-dependent cascades (40, 41). To test the role of these pathways in the anti-apoptotic effect of uPA, the extent of phosphorylated Akt and ERK1/2 was assessed in RPE cells exposed to ATF or to uPA 1–158. As shown in Fig. 5A, both kinases were transiently phosphorylated following uPA 1–158 treatment, in a time-dependent fashion although with different kinetics. Furthermore, activation of both the ERK1/2 and Akt was strictly dependent on receptor binding, as preincubation of RPE cells with the uPAR blocking 399R antibody prevented ATF-dependent phosphorylation of both kinases (Fig. 5B). Interestingly, SiuPA-4 and SiuPA-17 as well as SiuPAR-20 and SiuPAR-8 RPE clones exhibited a lower basal level of phosphorylated Akt than control RPE/Vec (Fig. 5C). These data are in agreement with the observations by Adachi *et al.* (42) and Lakka *et al.* (43) on the inhibition of Akt and ERK phosphorylation following knockdown of uPAR expression in glioma cells. This finding suggests that the uPA/uPAR interaction may be responsible for keeping the basal level of active Akt and raises the possibility that in RPE cells there is a uPA/uPAR-dependent autocrine loop sustaining Akt phosphorylation.

Urokinase-dependent Stimulation of MEK and PI3K Leads to Bcl-xL Transcriptional Activation—The results presented above show that the uPA/uPAR system is directly involved in the protection of RPE and HEK-293 cell from apoptosis induced by different stimuli. The general effect observed on cell survival suggests that uPA might regulate one or more components of the core apoptotic machinery. Given that the anti-apoptotic factor Bcl-xL is a key protein in the establishment of susceptibility to cell death and is frequently regulated at the transcriptional and/or translational level, we asked if Bcl-xL protein levels were changed as a consequence of uPAR engagement. As shown in Fig. 6A, a 3–4-fold increase in the level of the 26-kDa Bcl-xL protein following treatment of RPE cells for 5 h with either ATF or uPA 1–158 was observed. In parallel experiments, the expression level of the pro-apoptotic member of the Bcl-2 family, Bax, was instead left unchanged by uPA 1–158 treatment (data not shown). The selective induction of Bcl-xL protein levels following incubation with uPA 1–158 was dependent on *de novo* protein synthesis, because it was prevented by cell pre-treatment with the protein synthesis inhibitor cycloheximide (Fig. 6B). The involvement of uPAR is indicated by the inhibitory effect of 399R polyclonal anti-uPAR antibody on the up-regulation of Bcl-xL (Fig. 6A). Moreover, to establish if uPA was affecting Bcl-xL expression at the transcriptional level, a real time PCR analysis of total RNA extracted from control and uPA 1–158-treated RPE cells was performed. As

FIGURE 5. uPA- and uPAR-dependent activation of Akt and ERK1/2 kinases. *A*, subconfluent RPE cells were serum-starved for 24 h in 6-well plates and exposed to 10 nM uPA 1–158 under serum-free conditions or to 10% FBS for the indicated times. The level of phosphorylated kinases was then assessed by Western blotting with anti-phospho-Akt or anti-phospho-ERK1/2 antibodies. Quantitation of total Akt with anti-Akt antibodies ensures equal protein loadings. *B*, subconfluent RPE cells were exposed to 10 nM ATF or 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*) for 3 min and then analyzed for the content of P-ERK1/2. To analyze Akt phosphorylation, cells were exposed to ATF for 45 min. When indicated, they were pretreated with 10 μ g/ml 399R polyclonal anti-uPAR antibody or control IgG for 30 min. *C*, RPE/Vec, SiuPA-4, SiuPA-17, SiuPAR-20, and SiuPAR-8 clones (1.5×10^5 cells/well) were grown for 16 h in 6-well plates and serum-starved for 24 h. The basal level of P-Akt, P-ERK1/2, and Akt tot was assessed as described in the legend to *A*.

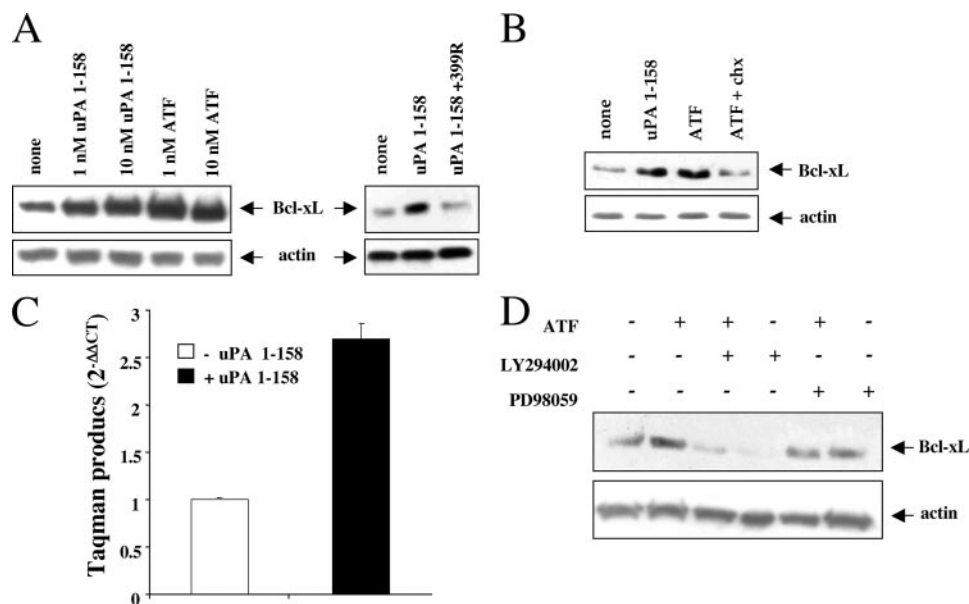
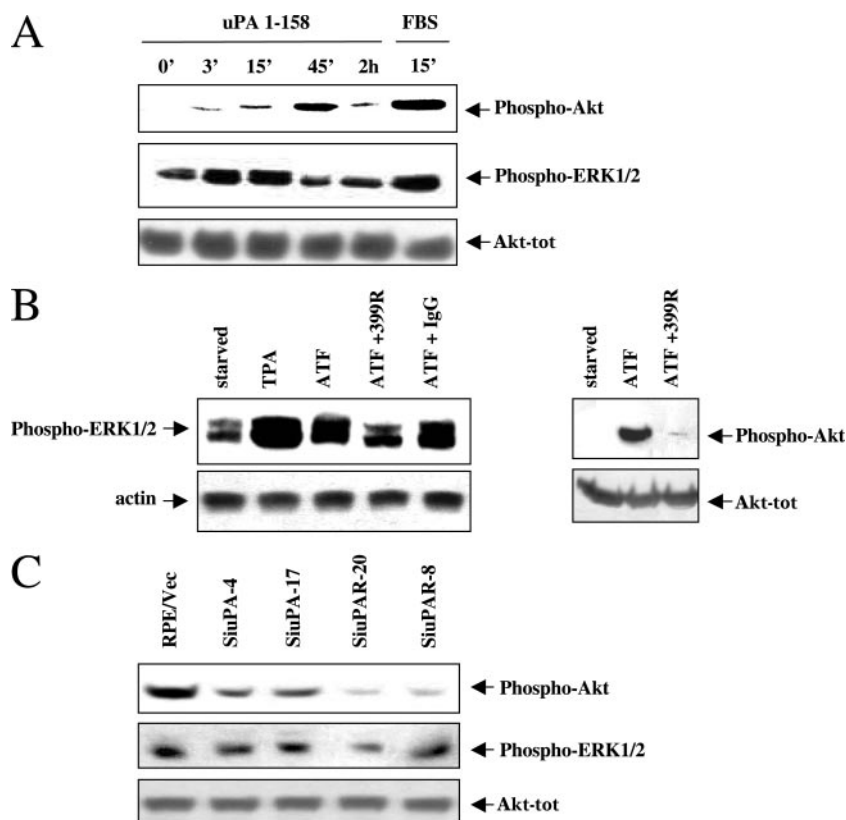


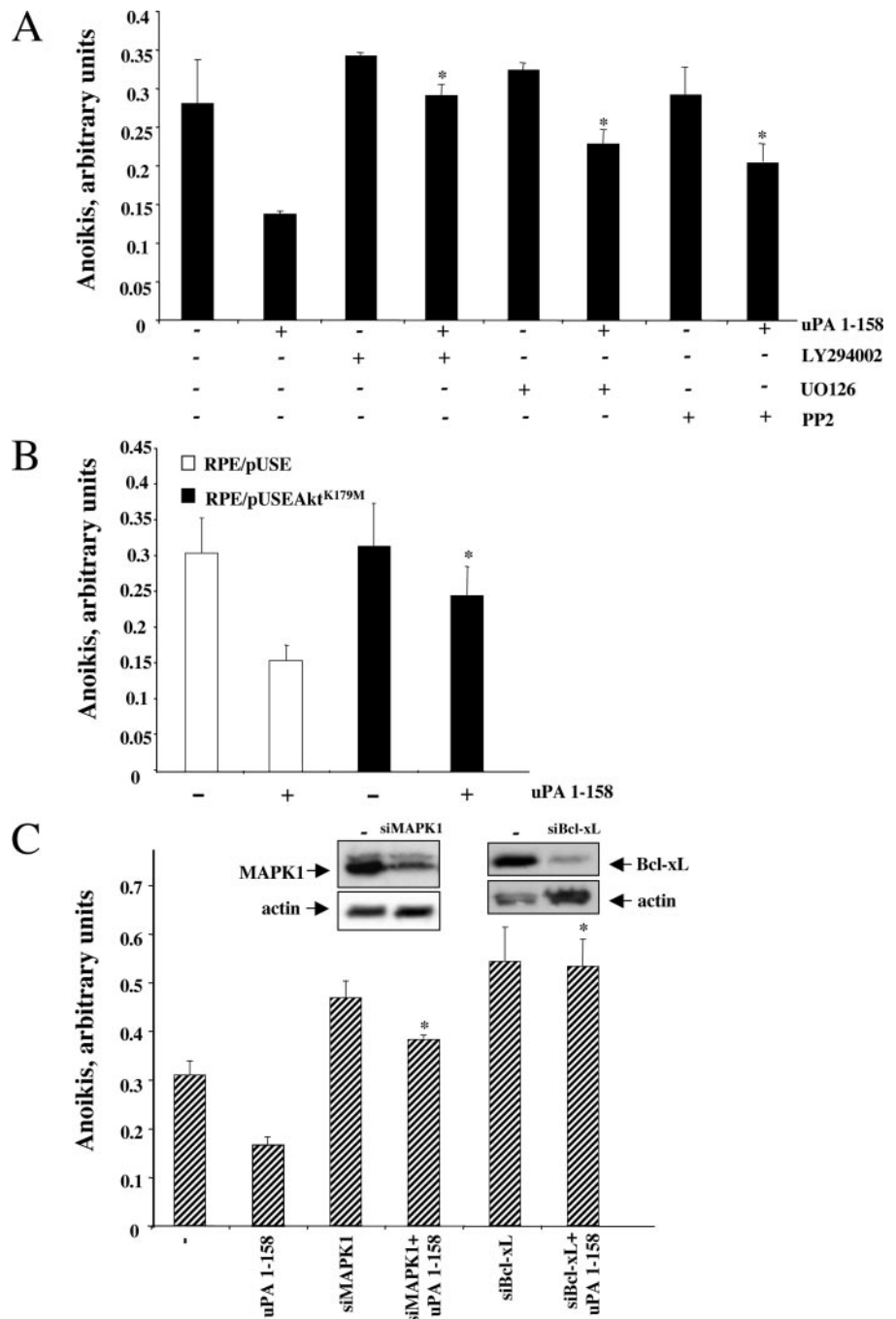
FIGURE 6. uPA-dependent transcriptional induction of Bcl-xL in RPE cells exposed to uPA 1–158 or ATF. *A*, control RPE (1.5×10^5 /sample) were serum-starved for 24 h, exposed for 5 h to the indicated concentrations of uPA 1–158 or ATF in serum-free medium, lysed, and analyzed for Bcl-xL expression by Western blotting analysis using a polyclonal anti-Bcl-x antibody. When indicated, they were pretreated with 10 μ g/ml 399R polyclonal anti-uPAR antibody for 30 min. *B*, RPE cells were treated as described in *A* and assayed for Bcl-xL protein level by Western blotting. When indicated, cells were pretreated with 10 nM uPA 1-158 or with 10 μ M cycloheximide (*chx*) for 1 h prior to the exposure to 10 nM ATF for 5 h. *C*, cDNAs obtained from RPE total RNA following treatment with uPA 1–158 for 5 h or diluents were analyzed by TaqMan real time PCR. The amounts of Bcl-xL mRNA were assessed relative to an internal control (glyceraldehyde-3-phosphate dehydrogenase mRNA). *D*, RPE were treated as described in *A*. When indicated, cells were preincubated with 20 μ M LY294002 or 50 μ M PD98059 for 30 min and then treated with or without 10 nM ATF for 5 h. The cells were then lysed and analyzed for the level of Bcl-xL protein by Western blotting. In all cases, membranes were reprobed with a polyclonal anti-actin antibody as a loading control. The results shown are representative of three independent experiments performed in triplicate with S.D. indicated by error bars.

shown in Fig. 6C, cell exposure to uPA 1–158 induced Bcl-xL mRNA levels by 2.65 ± 0.15 -fold, as compared with untreated cells.

To investigate whether the PI3K- and MEK/ERK-dependent pathways could mediate the up-regulation of Bcl-xL protein levels by uPA, we analyzed RPE cells exposed to uPA 1–158 in the presence of the

chemical inhibitors LY294002 or PD98059. As shown in Fig. 6D, unlike PD98059, LY294002 reduced Bcl-xL basal level. However, each inhibitor was sufficient to prevent the induction of Bcl-xL by uPA 1–158. Taken together, these data show that both pathways are required for uPA-dependent full induction of Bcl-xL expression.

FIGURE 7. The protective ability of uPA is mediated by Bcl-xL in a MAPK- and PI3K-dependent manner. *A*, 5×10^4 parental RPE cells were cultured in 24-well plate for 16 h and serum-starved for 24 h; cells were then pretreated with $20 \mu\text{M}$ LY294002, $10 \mu\text{M}$ UO126, or $10 \mu\text{M}$ PP2 for 30 min and then incubated with or without 10 nM uPA 1–158 for 8 h in poly-HEMA-coated dishes, as described in the legend to Fig. 1B. Then all cells were collected and subjected to the cell death ELISA. Data are expressed as mean \pm S.D. and represent one of at least three separate experiments undertaken in triplicate. *, $p < 0.005$ uPA 1–158 + LY294002 versus uPA 1–158, uPA 1–158 + UO126 versus uPA 1–158, and uPA 1–158 + PP2 versus uPA 1–158. *B*, 1×10^5 RPE cells were seeded in 6-well plates and transiently transfected with $1 \mu\text{g}$ of a plasmid encoding dominant negative Akt (pUSE/Akt^{K179M}). 16 h after transfection, cells were serum-starved for 24 h, exposed to 10 nM uPA 1–158 for 8 h, and assayed for the extent of anoikis. Values represent the average of two different experiments performed in triplicate. *, $p < 0.03$ uPA 1–158 RPE/pUSE/Akt^{K179M} versus uPA 1–158 RPE/pUSE. *C*, 3.5×10^4 RPE cells were transfected with 37.5 ng (5 nM) of siRNA targeted against MAPK1 or Bcl-xL using the RNAi transfection kit. The levels of MAPK and Bcl-xL of the transfectants were assessed 24 h after the transfection with control siRNA (–) or with siMAPK1 or with siBcl-xL by Western blotting with specific antibodies, as described under “Experimental Procedures” (insets). To test sensitivity to anoikis, 16 h after transfection, cells were serum-starved for 24 h and plated onto poly-HEMA-coated dishes for 8 h, as described in Fig. 1B. The results are the average of two separate experiments undertaken in triplicate with S.D. indicated by error bars. *, $p < 0.009$ siRNA MAPK1 + uPA 1–158 versus uPA 1–158, siRNA Bcl-xL + uPA 1–158 versus uPA 1–158.



PI3K/Akt and MEK/ERK Pathways Mediate the uPA-dependent Survival Signal—The finding that uPA/uPAR interaction may up-regulate the levels of the anti-apoptotic factor Bcl-xL through Akt and ERK activation suggests that these mediators may underlie its anti-apoptotic activity. To investigate whether these two kinases are required for the anti-apoptotic effect of uPA 1–158, RPE cells were pre-exposed to either LY294002 or UO126 and then plated onto poly-HEMA-coated dishes. Fig. 7A shows that uPA 1–158-dependent protection is greatly reduced by LY294002 and by UO126, although to a different extent. The Src inhibitor PP2 was also an effective inhibitor of uPA protection. Moreover, the role of Akt in the pathway elicited by uPA 1–158 was investigated by the use of a dominant negative Akt variant encoded by pUSE/Akt^{K179M} plasmid. RPE cells were transiently transfected with pUSE/Akt^{K179M} or with pUSE plasmids and analyzed after 48 h. As expected,

cells bearing pUSE/Akt^{K179M} exhibited a significantly reduced ability to respond to uPA 1–158 than controls (Fig. 7B). Finally, the relevance of MAPK and Bcl-xL in the uPA-dependent protection from anoikis was investigated by introducing specific siRNAs into RPE cells by transient transfection. As indicated in Fig. 7C (insets), the levels of MAPK1 and Bcl-xL proteins were markedly reduced by the specific siRNAs (siMAPK1 and Bcl-xL). The transient transfectants were then analyzed for the extent of anoikis in the presence or in the absence of uPA 1–158. As shown in Fig. 7C, siMAPK1 reduced and siBcl-xL abolished the protective effect of uPA 1–158. In parallel control samples, the non-silencing siRNA was ineffective. These data, taken together, show that uPA activates both the MEK/ERK- and the PI3K/Akt-dependent pathways, ultimately leading to the up-regulation of Bcl-xL. These events are all required to protect RPE cells from anoikis.

DISCUSSION

This study provides a causal link between uPAR signaling and protection from programmed cell death. Here we show that ligand engagement of uPAR counteracts the pro-apoptotic effect triggered by UV light, cisplatin, and forced detachment from the culture dish. Furthermore, we demonstrate that the expression level of uPAR positively correlates with resistance to anoikis in the RPE and embryonic kidney epithelial (HEK-293) cell lines. Finally, our data show that the uPA/uPAR interaction results in a marked up-regulation of the anti-apoptotic factor Bcl-xL, which is required to the uPA-dependent anti-apoptotic activity.

Our study is based on the use of purified recombinant uPA variants, an approach that enabled us to define the domains of the molecule active in the stimulation of cell survival. Given the complex structure-function relationship of the uPA protein, the use of variants and peptides was crucial to define whether the proteolytic activity and/or the binding to uPAR was relevant to its anti-apoptotic effect. A physiological significance to our findings is provided by the fact that several of these noncatalytic fragments have been found in human urine (44). On the contrary, no data are presented on the effects of proteolytically active uPA, as catalytic activity in the presence of traces plasminogen may mask the anti-apoptotic effect of the amino-terminal region. According to this possibility, it has been reported that the plasmin generated from surface-associated plasminogen induces cell retraction, fibronectin fragmentation, ultimately resulting in cell detachment and morphological/biochemical changes characteristic of apoptosis (45, 46). Furthermore, a negative effect on cell survival has been observed in a model system of retinal diseases caused by uPA-dependent ECM degradation (47). All these findings point to a pro-apoptotic role of active uPA in cellular physiology. The results presented here give instead a different picture, showing a clear survival activity associated with the amino-terminal domain of uPA and resulting from receptor binding and signaling. How can we reconcile these apparently contrasting findings? A reasonable hypothesis is that proteolytically active uPA has a built-in anti-apoptotic activity associated with its receptor binding domain that constantly counterbalances the pro-apoptotic effect of its serine protease activity. *In vivo*, the most abundant form of uPA present in plasma is indeed the inactive pro-enzyme, which is fully able to associate with uPAR and elicit intracellular signaling (48). According to our results, pro-uPA is also endowed with anti-apoptotic ability (Fig. 1B). Interestingly, RPE clones with a reduced expression of uPA exhibit an increased sensitivity to UV- and cisplatin-induced apoptosis even in the presence of serum (Fig. 2, B and C), supporting an important role in cell survival for the uPA/uPAR interaction. Therefore, it is reasonable to hypothesize that *in vivo* uPA mostly behaves as a typical motogen factor with intrinsic survival activity. Other chemotactic factors have in fact been shown to promote cell survival; hepatocyte growth/scatter factor, which causes the breakdown of epithelial intercellular adhesions, is also able to trigger resistance to anoikis (33). Also, macrophage-stimulating factors can protect epithelial cells from anoikis by cooperating with integrins and activating its transmembrane receptor tyrosine kinase, named RON in humans or SKT in mice (49). A likely explanation is that the cyclic attachment and detachment from substrate occurring during cell migration may promote apoptosis, if not counterbalanced by a protective effect.

How does the binding of uPA to a glycosylphosphatidylinositol-anchored extracellular receptor promote cell survival? The data presented here support a model in which uPA/uPAR binding delivers an intracellular signal that activates both the MEK/ERK and PI3K/Akt pathways, ultimately leading to the up-regulation of the anti-apoptotic factor

Bcl-xL. Activation of the MEK/ERK pathway by uPA has been reported previously (30, 50) and is probably because of an integrin-dependent activation of the Src/focal adhesion kinase (FAK) pathway (51). The involvement of integrin-type receptors is in agreement with the well documented functional and physical interaction between uPAR and integrins, in particular $\alpha v\beta 5$, $\alpha 5\beta 1$, and CD11b/CD18 (52). In our experiments, anti- $\alpha v\beta 5$ blocking antibodies reduced uPA-dependent protection from anoikis by 20%, suggesting a role for $\alpha v\beta 5$ in this response. On the other hand, activation of FAK by uPA has been reported, and protection from apoptosis in other systems has also been shown to be generated by the activation of Src/FAK through PI3K and MAPK (53, 54). In the RPE cell line, the involvement of the Src-FAK pathway is supported by the observation that a chemical inhibitor of the Src kinase (PP2) prevents uPA protection from anoikis (Fig. 7A). Based on these observations, a reasonable possibility is that following uPA/uPAR binding, a signal is transduced intracellularly by a cooperation between uPAR and integrins that activate PI3K and MEK through Src and FAK phosphorylation. Given the degree of connection existing between the MEK/ERK and PI3K/Akt pathways, it is difficult to assess the relative contribution of each pathway to the induction of cell survival by uPA. However, consistent with the report of Chandrasekar *et al.* (55), we noticed that silencing of uPA or uPAR dramatically affected the basal level of Akt phosphorylation, although it leaves the phosphorylation status of ERK1/2 unaltered. This observation suggests the existence of a uPA/uPAR-dependent autocrine loop strongly affecting the level of active Akt rather than that of ERK1/2. Furthermore, because the effect seems to be more pronounced in uPAR knocked down clones than in those silenced for uPA, is reasonable to speculate that in our experimental system uPAR might function as a co-receptor in the activation of the PI3K/Akt pathway, similarly to what has been suggested for the activation of the EGF receptor in COS-7 cells (56).

Whatever pathway plays the major role in the transduction of the survival signal triggered by ligand-engaged uPAR, the data presented here clearly show the requirement for an enhanced expression of the universal anti-apoptotic factor Bcl-xL. This finding explains why uPA has a survival effect against apoptotic stimuli as distant as anoikis and DNA damage. Accordingly, overexpression of the *Bcl-xL* gene confers resistance to a broad range of pro-apoptotic stimuli, such as the intrinsic apoptotic effect of oncogene activation, hypoxia, and matrix detachment (3, 57, 58). Furthermore, a striking relationship has been found between an increased Bcl-xL expression level and resistance to a wide panel of standard chemotherapeutic agents, indicating that Bcl-xL overexpression is selected during anticancer treatment and is preferred over other anti-apoptotic members of the same family (59). Based on the results presented in this paper, we suggest that at least part of the increased Bcl-xL expression during cancer formation could be the result of uPA/uPAR action; this possibility is supported by the elevated uPA and uPAR expression levels found in multiple types of cancer (60, 61). In agreement with these observations, targeting the uPAR with inhibitory peptides leads to a reduction of glioma tumor size in mice through inhibition of cell proliferation and increased tumor cell apoptosis (62). In the emerging picture, the uPA/uPAR system has the ability to support the malignant phenotype through several mechanisms: first, by virtue of its matrix-degrading ability that favors tumor dissemination; second, by stimulating cell motility; third, by eliciting cell proliferation; and fourth, by protecting cells from apoptosis, thus enhancing tumor survival.

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