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bcl-2 Induction of Urokinase Plasminogen Activator Receptor Expression in Human Cancer Cells through Sp1 Activation

INVOLVEMENT OF ERK1/ERK2 ACTIVITY*

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We have previously demonstrated that Bcl-2 overexpression in human breast carcinoma and melanoma cells synergizes with hypoxia to increase angiogenesis through up-regulation of vascular endothelial growth factor. In this work we demonstrated, for the first time, that Bcl-2 overexpression in cancer cells exposed to hypoxia modulates urokinase plasminogen activator receptor (uPAR) expression through Sp1 transcription factor and that the extracellular signal-regulated kinase (ERK) pathway plays a role in Sp1 transcriptional activity. In particular, an increase in uPAR protein and mRNA expression was found in melanoma *bcl-2* transfectants grown under hypoxia when compared with control cells, and a decrease of uPAR protein expression was induced by treatment of cells with specific *bcl-2* antisense oligonucleotides. Up-regulation of uPAR expression was accompanied by increased Sp1 protein expression, stability, serine phosphorylation, and DNA binding activity. Treatment of cells with mitramycin A, an inhibitor of Sp1 activity, confirmed the role of Sp1 transcriptional activity in uPAR induction by Bcl-2. The contribution of the ERK pathway in Sp1-increased transcriptional activity was demonstrated by the use of chemical inhibition. In fact, ERK kinase activation was induced in Bcl-2-overexpressing cells exposed to hypoxia, and the ERK kinase inhibitor UO126 was able to down-regulate Sp1 phosphorylation and DNA binding activity. Using a human breast carcinoma line, we obtained data supporting our findings with melanoma cells and identified a link between the induction of Sp1 and uPAR expression as a common *bcl-2*-controlled phenomenon in human tumors. In conclusion, our results strongly indicate that up-regulation of uPAR expression by Bcl-2 in hypoxia is modulated by Sp1 DNA binding activity through the ERK signaling pathway.

Angiogenesis is a fundamental process required for tumor growth, invasion, and metastasis and is strongly induced by hypoxia. In fact, several cell types including tumor cells, macrophages, and endothelial cells respond to hypoxia by producing angiogenic factors, fibrinolytic factors, and adhesion molecules involved in pathologic angiogenesis (1–5). Four sequential steps can be distinguished during angiogenesis: the degradation of the basement membrane and interstitial matrix, endothelial cell migration, endothelial cell proliferation, and the formation of tubular structures with a lumen and a new basement membrane (6). Three of these steps critically depend on proteolytic activity generated by the matrix metalloproteinases and the plasminogen activator/plasmin system. In particular, the role of urokinase plasminogen activator receptor (uPAR)[1] in tumor cell invasion and migration and in the formation of new microvascular structures has been largely demonstrated (7–9).

Angiogenesis is also controlled by alterations in oncogene and tumor suppressor gene expression (10–14). In this context, we previously demonstrated that the *bcl-2* oncogene increases *in vitro* and *in vivo* angiogenesis in two different tumor histotypes (15, 16). In particular, we found an increase in the level of vascular endothelial growth factor (VEGF) when breast carcinoma (15) and melanoma (16) cells overexpressing Bcl-2 were exposed to hypoxic conditions. We also demonstrated that Bcl-2 overexpression in human melanoma cells enhances hypoxia-induced VEGF mRNA stability and promoter activation (16) and that treatment of melanoma cells with a *bcl-2*/*bcl-xL* antisense oligonucleotide induces antiangiogenic activity (17).

The involvement of *bcl-2* in angiogenesis of prostate carcinoma (18) and microvascular endothelial cells has also been described (19). Thus, the leading property of *bcl-2* to inhibit apoptosis is associated with its ability to induce angiogenesis.

The aim of this study was to evaluate the role of Bcl-2 overexpression in the regulation of uPAR expression in human tumors. Since a minimal promoter region required for the basal transcription of the human *uPAR* gene has been demonstrated to contain GC-rich proximal sequences that are specifically bound by the transcription factor Sp1 (20, 21), we also investigated whether the regulation of uPAR by Bcl-2 overexpression is due to the effect of Bcl-2 on Sp1 expression, phosphorylation, and DNA binding activity. To address the molecular mechanism that mediates the effect of Bcl-2, the role of extracellular signal-regulated kinase (ERK) signaling in Sp1 transcriptional activity was investigated. Our results demonstrate that Bcl-2

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[1] The abbreviations used are: uPAR, urokinase plasminogen activator receptor; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor; ADR, adriamycin; MTR, mitramycin A; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay.

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overexpression in hypoxia increases Sp1 expression and activity through ERK signaling with the result of enhanced uPAR transcription and expression.

MATERIALS AND METHODS

Cell Lines and Cell Cultures—M14 human melanoma cells, MCF7 ADR human breast carcinoma cells resistant to adriamycin (ADR), and Bcl-2-overexpressing clones, previously obtained after transfection (16, 22), were maintained in RPMI medium (Invitrogen) containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics.

Hypoxic Treatment—Parental cells and *bcl-2* transfectants were seeded and grown for 24 h in complete medium. Then cells were incubated for 24 h in serum-free medium under normoxic or hypoxic conditions as reported previously (15). Cells were harvested, counted, and used for protein preparation and total RNA extraction.

Antisense Treatment—These studies were performed with antisense 2009, a 20-mer phosphorothioate oligonucleotide directed against the coding region (codons 141–147) of the *bcl-2* messenger RNA. The antisense 2009 and the scrambled control sequence were previously described (23). Oligonucleotides were delivered into cells in the form of complexes with the transfection reagent Lipofectin (Invitrogen). Lipofectin at 100 μg/ml was allowed to complex with oligonucleotides, and this solution was further diluted to the desired concentration in serum and antibiotic-free medium prior to addition to the cells. M14 parental cells and *bcl-2* transfectants were incubated for 24 h in the presence of 300 nM oligonucleotides and Lipofectin and then exposed to hypoxia for 24 h in serum-free medium. ELISA and Western blot analyses were performed at the end of treatment.

ELISA Analysis—To determine the amount of uPAR in the cell lysates and in tumor xenografts, an ELISA kit was used. The sensitivity of the uPAR assays was 0.1 ng/ml (American Diagnostica inc., Greenwich, CT). To evaluate *in vitro* uPAR expression, cells were exposed to normoxia or hypoxia for 24 h in the absence or presence of 100 or 200 nM mitramycin A (MTR; Sigma) or were treated with *bcl-2* antisense oligonucleotide as reported above. Then cells were harvested, washed twice in PBS, resuspended, and counted. Cells were then centrifuged, and the pellet was lysed in lysis buffer (partial cell lysate). The combined trypsin/EDTA treatment leaves a complex material, referred to as surface-adherent material (24), on the culture dish, which primarily represents the cell focal contacts and is enriched with uPAR. Thus, the partial cell lysate was replaced in the culture dish for 1 h at 4 °C to lyse the surface-adherent material-associated uPAR (total cell lysate) as well. The total cell lysate was stirred for 12 h at 4 °C to allow an optimal solubilization of the cell extract and centrifuged at $10,000 \times g$ for 60 min at 4 °C to separate cell debris. The material was frozen and maintained at −80 °C. For *in vivo* analysis of uPAR expression, tumor xenografts (22) (100–200 mg) was homogenized in 500 μl of lysis buffer. Homogenates were centrifuged (20 min at $16,000 \times g$), and the supernatant was used for uPAR protein analysis.

Immunoprecipitation Assay—Nuclear extracts were prepared as described previously (16). Extracts from $\sim 3 \times 10^6$ cells (100 μl containing 200 μg of nuclear proteins) were diluted to 1 ml in whole-cell extraction buffer (25 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and inhibitor proteases and phosphatases) and incubated with 3 μl of polyclonal rabbit antibody to Sp1 (Pep2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C with gentle shaking. After the addition of 50 μl of protein A-agarose beads (Santa Cruz Biotechnology), the suspension was incubated for another 2 h at 4 °C. The beads were pelleted by centrifugation, washed three times with 1.5 ml of cold extraction buffer, and resuspended in 50 μl of 2× SDS sample buffer. After the suspension was heated to 95 °C for 10 min, 20-μl samples were resolved on denaturing SDS-polyacrylamide gels and transferred to membranes. Anti-human anti-phosphoserine and anti-phosphothreonine antibodies (Chemicon International, Temacula, CA) were used at dilutions of 1:500 and 1:100, respectively.

Western Blot Analysis—For analysis of Sp1 protein expression, equal amounts of nuclear extracts (35 μg of protein) were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Monoclonal antibody against Sp1 (clone 1C6; BD Pharmigen Technical, Franklin Lakes, NJ) was used at a dilution of 1:200. For the Sp1 phosphorylation study, nuclear extracts obtained after 24 h of hypoxic treatment were treated for 60 min at 37 °C with 10 units of calf intestinal phosphatase (New England Biolabs) before Western blot analysis. For the Sp1 stability study, cycloheximide (CHX; Calbiochem) at a dose of 10 μg/ml was added after 24 h of hypoxic treatment, and the cells were further incubated under hypoxia for varying times ranging from 30 min to 4 h.

To evaluate uPAR and Bcl-2 expression and ERK1/ERK2 expression and phosphorylation, whole-cell protein extracts were electrophoresed on an 11% SDS-polyacrylamide gel. Anti-human uPAR mouse monoclonal antibody (American Diagnostica Inc.) was used at a 1:400 dilution. Anti-human Bcl-2 mouse monoclonal antibody (clone 124; Dako s.p.a., Milan, Italy) was used at a 1:200 dilution. To detect phospho-ERKs, the phospho-p44/42 mitogen-activated protein kinase (Thr^{202}/Tyr^{204}) antibody (Cell Signaling Technology Inc.) was used at a 1:1000 dilution; to probe total ERK1 and ERK2, the p44/42 mitogen-activated protein kinase polyclonal antibody (Cell Signaling Technology Inc.) was used at a 1:1000 dilution. Immunoreactive bands were visualized using horseradish peroxidase-coupled goat anti-rabbit immunoglobulin and the ECL detection system (Amersham Biosciences). To check the amount of proteins transferred to the nitrocellulose membranes, β-actin or heat shock protein (Hsp70/72) was used as control and detected by an anti-β-actin polyclonal antibody (Santa Cruz Biotechnology) or anti-Hsp70/72 monoclonal antibody (Calbiochem) at a 1:1000 dilution. Densitometric analysis was performed after Western blot analysis.

Western blot analysis of ERK1/ERK2 and Sp1 expression and phosphorylation was performed in the absence or presence of ERK inhibitor UO126 (10 μM; Calbiochem).

Northern Blot Analysis—Total RNA was prepared, and Northern blot analysis was performed as described previously (15). A 585-bp fragment of the plasmid specific for human uPAR kindly provided by Dr. F. Blasi (25), a 717-bp fragment of human Sp1 kindly provided by Dr. R. C. Simmen (26), and a probe for glyceraldehyde-3-phosphate dehydrogenase (27) were used.

Promoter Activity—For transient transfection, 3×10^5 cells were seeded into 60-mm dishes, and 24 h later each dish was transfected with 6 μg of pGL3-mouse uPAR promoter vector kindly provided by Dr. A. Maity (28). Cells were cotransfected in triplicates with an internal control PEQ-176 plasmid (1.5 μg) using a calcium-phosphate method (Promega Italia, Milan, Italy). Twenty-four h later, half of the dishes were subjected to hypoxia, and the other half were kept under normoxic conditions. Samples were collected 24 h after the induction of hypoxia and analyzed for luciferase and β-galactosidase activity. Relative luciferase expression was determined as a ratio of β-galactosidase activity. The mean of five independent experiments was calculated for each condition.

Electrophoretic Mobility Shift Assay (EMSA)—Cells were exposed for 24 h to hypoxia in the presence or absence of the Sp1 inhibitor MTR (100 or 200 nM) or ERK inhibitor UO126 (10 μM). Then nuclear proteins were prepared as described for immunoprecipitation, and EMSA was performed as previously described (29). Oligonucleotides were purchased from Invitrogen. The following double-stranded oligomer-containing Sp1 consensus sequence was used as labeled probes (10^4 cpm) or cold competitor (100 ng): 5′-ATTCGATCGGGGCGGGGCGAGC-3′ (30). To analyze the specificity of DNA-binding complexes, supershift with Sp1 (Pep2; Santa Cruz Biotechnology) and Sp3 (D-20; Santa Cruz Biotechnology) antibodies was performed by preincubating nuclear extracts with the antibody (2 μg) for 30 min prior to the addition of labeled DNA probe.

Statistical Analysis—Multiple comparisons were performed by the Student-Newman-Keuls test after demonstration of significant differences among medians by nonparametric variance analysis according to Kruskal-Wallis.

RESULTS

Bcl-2 Overexpression Increases uPAR Protein Expression under Hypoxic Conditions—We previously demonstrated the ability of Bcl-2 to synergize with hypoxia to increase angiogenesis in melanoma and breast carcinoma cells (15, 16). Since uPAR plays an important role in angiogenesis (7–9), we evaluated whether uPAR was also involved in the Bcl-2-induced angiogenesis. For this purpose, two Bcl-2-overexpressing clones, previously obtained after transfection of the M14 parental melanoma line (16), were used.

We first determined the levels of cell-associated uPAR protein in the M14 cell line, the MN8 control clone, and two *bcl-2* transfectants (MB5 and MB6) grown under normoxic (Fig. 1*A*, *white columns*) and hypoxic (*black columns*) conditions for 24 h. As shown in Fig. 1*A*, the difference in uPAR amounts measured in the control lines and *bcl-2*-transfected clones were not sta-

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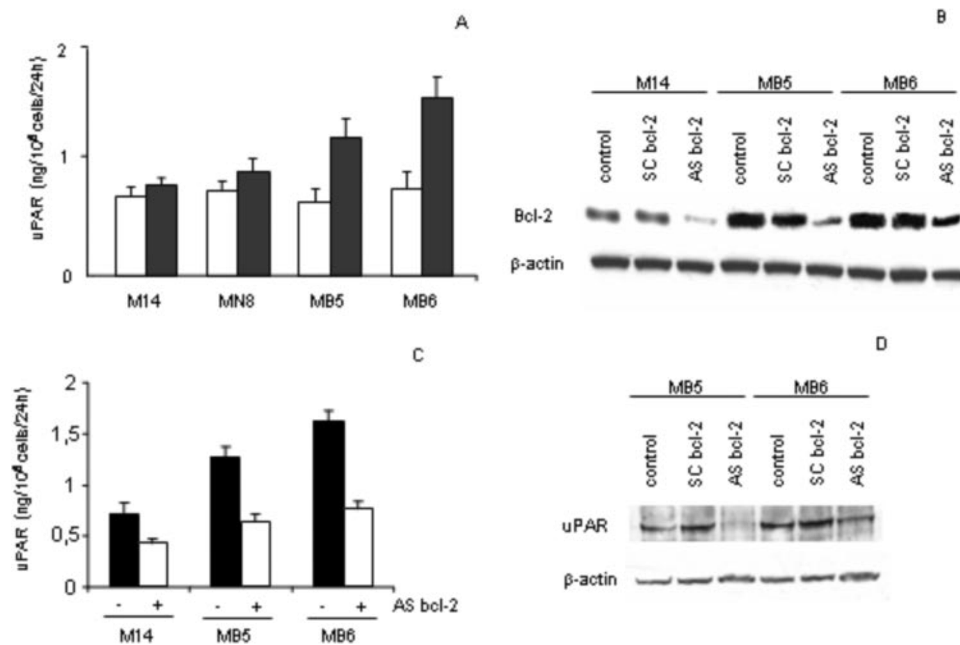

FIG. 1. **Bcl-2 overexpression increases uPAR protein expression, and *bcl-2* antisense oligonucleotide down-regulates Bcl-2 and uPAR protein expression in hypoxic conditions.** *A*, expression of uPAR evaluated by ELISA in cell lysates of the M14 parental line, the control clone (MN8), and two *bcl-2* transfectants (MB5 and MB6) grown in normoxia (*white columns*) and hypoxia (*black columns*) for 24 h. Each point represents the mean ± S.D. of six experiments performed in duplicate. *B*, *C*, and *D*, Bcl-2 and uPAR protein expression after treatment of parental cells (M14) and *bcl-2*-overexpressing clones (MB5 and MB6) for 24 h with 300 nM antisense oligonucleotide 2009 (*AS Bcl-2*) or scrambled sequence (*SC Bcl-2*). At the end of oligonucleotide treatment, cells were exposed to hypoxia for 24 h. Western blot analysis (*B* and *D*) and ELISA (*C*) were performed at the end of oligonucleotide/hypoxia treatment. For Western blotting, β-actin was used as a control for equal protein loading, and one representative experiment of three is shown.

tistically significant when cells were grown under normoxic conditions. In hypoxia, the M14 parental line and the MN8 control clone did not show significant differences, neither when compared with normoxic conditions nor between each other. On the contrary, the two *bcl-2* transfectants grown in hypoxia showed significantly higher levels of uPAR protein than in normoxia (~2-fold increase).

To confirm the role of *bcl-2* in the regulation of uPAR expression, Bcl-2 protein expression was down-regulated both in the M14 parental cells and the MB5 and MB6 *bcl-2* transfectants using 2009 antisense oligonucleotide (23) before cells were exposed to hypoxia for 24 h. Western blot analysis of Bcl-2 expression (Fig. 1*B*) showed that *bcl-2* antisense oligonucleotide treatment decreased Bcl-2 protein levels by about 45 and 60% in parental cells and in the two transfectants, respectively. Performing ELISA assays, also a decrease of the level of uPAR protein in parental cells by about 40% was observed. The reduction in the two *bcl-2* transfectants was about 60–70% (Fig. 1*C*). Down-regulation of uPAR expression in *bcl-2* transfectants by *bcl-2* antisense oligonucleotide treatment was also confirmed by Western blot. As revealed in Fig. 1*D*, antisense treatment induced about 60% inhibition of uPAR expression in both clones. The scrambled sequence oligonucleotide did not affect Bcl-2 or uPAR expression. These results confirm a link between Bcl-2 and uPAR expression.

Bcl-2 Overexpression Increases uPAR mRNA Expression and Promoter Activity under Hypoxic Conditions—Because uPAR expression can be modulated by transcriptional regulation (28), we were interested to know the mechanism through which Bcl-2 synergizes with hypoxia to induce uPAR expression. To this end, we determined whether transactivation of the *uPAR* promoter differently occurred in control cells and the *bcl-2* transfectants (Fig. 2*A*). Transient transfections were performed using the *uPAR* promoter coupled to the luciferase-reporter gene (28), and control and Bcl-2-overexpressing cells were exposed to normoxia or hypoxia for 24 h. As shown in Fig. 2*A*, under normoxic conditions, uPAR promoter activity was low and similar in all cells tested, regardless of the level of Bcl-2 protein. Exposure to hypoxia induced a slight increase in promoter activity in control cells, whereas an increase of about 4-fold was observed in Bcl-2-overexpressing cells. In the MN8 control clone, the uPAR promoter response was comparable with that of M14 both in normoxia and hypoxia (data not shown).

Since the steady-state level of mRNA also represents a control point for uPAR expression (28), we examined the effect of Bcl-2 on uPAR at the transcriptional level. Northern blot analysis of uPAR mRNA was performed after exposure of M14 parental line, MN8 control clone, and two *bcl-2* transfectants (MB5 and MB6) to hypoxic conditions for 24 h (Fig. 2*B*). An increase of about 2-fold of uPAR mRNA expression was observed in both *bcl-2* transfectants, compared with the M14 parental line and MN8 control clone exposed to hypoxia for 24 h.

Sp1 Protein Stability and Phosphorylation Is Increased in bcl-2 Transfectants Exposed to Hypoxia—A minimal promoter region, containing GC-rich proximal sequences that are specifically bound by the transcription factor Sp1, has been identified for the basal transcriptional activity of the human *uPAR* gene (20, 21). The regulation of Sp1-dependent transcription can be affected by changes in the overall amount of Sp1 or in transactivation activity due to biochemical modifications such as phosphorylation (31–36). To address this issue, we analyzed Sp1 protein expression, stability, and phosphorylation in M14 parental cells, the MN8 control clone, and the MB5 and MB6 *bcl-2* transfectants grown in normoxia or exposed to hypoxia for 24 h.

To evaluate the effect of Bcl-2 on Sp1 mRNA expression, Northern blot analysis was performed 24 h after exposure to hypoxic conditions (Fig. 3*A*). Similar levels of Sp1 were observed between the control lines and the *bcl-2* transfectants grown in hypoxia.

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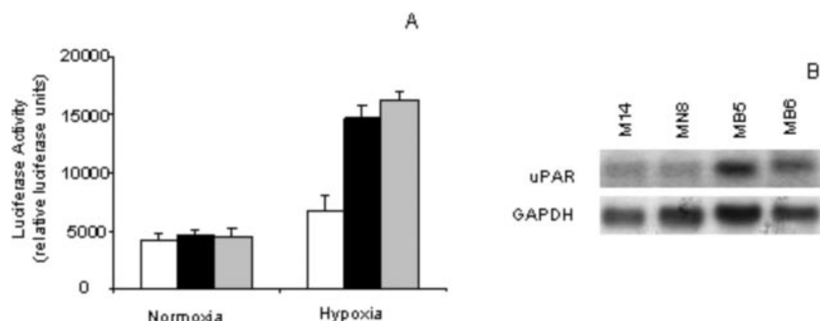

FIG. 2. **Bcl-2 overexpression increases uPAR transcriptional activity and mRNA expression in hypoxic conditions.** *A*, uPAR transcriptional activity of M14 cells (*white columns*) and two *bcl-2* transfectants (MB5 (*black columns*) and MB6 (*gray columns*)). Cells were transfected with a reporter plasmid (uPAR promoter) and exposed to normoxia or hypoxia for 24 h before assaying for luciferase activity. Luciferase values were normalized for transfection efficiency (luciferase/β-galactosidase ratios). Results are means of five independent experiments. *B*, Northern blot analysis of uPAR gene expression in the M14 parental line, the MN8 control clone, and two *bcl-2* transfectants (MB5 and MB6) exposed for 24 h to hypoxia. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is an internal control for mRNA quality and loading. One representative experiment of two is shown.

The expression of Sp1 was also analyzed at the protein level. A representative Western blot analysis of Sp1 protein in nuclear extracts is shown in Fig. 3*B*. As expected, Sp1 protein migrates as two bands with molecular masses of 95 and 105 kDa. The two species are the result of differential post-translational modification of the Sp1 polypeptide, corresponding to the unphosphorylated (lower band) and phosphorylated (upper band) protein (37). Whereas no differences in Sp1 expression between control and Bcl-2-overexpressing cells were observed under normoxic conditions (data not shown), after 24 h of hypoxia, an increase in both bands was observed in the *bcl-2* transfectants compared with parental cells. In particular, densitometric analysis revealed an increase of about 2- and 3-fold in the lower and the phosphorylated higher molecular weight forms of Sp1, respectively.

To assess whether Bcl-2 overexpression stabilizes the Sp1 protein, we monitored the levels of Sp1 protein after blocking *de novo* protein synthesis by CHX treatment. To this end, the M14 parental line and a representative *bcl-2* transfectant (MB5) were exposed to hypoxia for 24 h and then treated, under hypoxia, with 10 μg/ml CHX for varying times ranging from 30 min to 4 h (Fig. 3*C*). Western blot analysis showed a rapid decay of Sp1 protein in M14 parental cells within 30 min and Sp1 was hardly detectable 2 h after the start of CHX treatment. In contrast, Sp1 protein levels in Bcl-2-overexpressing cells remained stable during the first 2 h of CHX treatment and then gradually but slowly decreased about 40% within 4 h. The results obtained with the MB6 clone were comparable with those with MB5 clone (data not shown). These results clearly indicate that increased expression of Sp1 protein in *bcl-2* transfectants is due to increased Sp1 protein stability.

Since post-transcriptional modification of Sp1 correlates with enhanced transcription of "Sp1 site-dependent" genes, the ability of Bcl-2 to modulate the amount of phosphorylated Sp1 was also assessed in hypoxia by immunoprecipitation experiments. For this purpose, nuclear protein extracts of M14 parental line and a representative *bcl-2* transfectant (MB6) grown in normoxia and in hypoxia for 24 h were immunoprecipitated by a specific anti-Sp1 antibody and subsequently subjected to Western blot analysis using anti-phosphoserine and anti-phosphothreonine antibodies. As reported in Fig. 3*D*, the antibody directed against phosphoserine-containing proteins, reproducibly detected a strongly enhanced signal in the MB6 Bcl-2-overexpressing cells exposed to hypoxia compared with M14 parental cells kept under identical conditions. On the contrary, no difference was found between M14 parental and Bcl-2-overexpressing cells grown in normoxia. The antibody that recognizes phosphothreonine-containing proteins did not display enhanced signal strength after Bcl-2 overexpression relative to M14 parental cells both in normoxia and hypoxia. The addition of calf intestinal phosphatase to the cell lysates before Western blot analysis, demonstrated a reduction in the expression of the upper band both in parental and *bcl-2* transfectants (Fig. 3*E*). These results indicate that Bcl-2 increases Sp1 protein expression and phosphorylation, but not Sp1 mRNA level.

Sp1 Transcriptional Activity Is Increased in bcl-2 Transfectants Exposed to Hypoxia—Since Sp1 abundance and phosphorylation have been implicated in changes of Sp1 transcriptional activity (31–33), we determined whether the differences in Sp1 protein expression and phosphorylation detected after Bcl-2 overexpression were accompanied by an increase in Sp1 DNA binding activity. EMSA was performed after exposure of the different cell lines to hypoxia for 24 h. As reported in Fig. 4*A*, the two Bcl-2-overexpressing clones (MB5 in *lane 2*; MB6 in *lane 3*) showed a significant increase of the Sp1 DNA binding activity, compared with the parental M14 cells (*lane 1*). To analyze the specificity of the DNA-binding complexes, cold competition was performed (*lane 4*) using a representative *bcl-2* transfectant (MB5). A 100-fold molar excess of unlabeled Sp1 consensus oligonucleotide reduced Sp1 complexes. The addition of antibody directed against Sp1 induced a supershift and a significant reduction of Sp1-dependent binding activity (*lane 5*), which was not observed when an Sp3 antibody was used (*lane 6*). In all of the experiments, the results obtained with the MN8 control clone and MB6 *bcl-2* transfectant were comparable with those with M14 cells and MB5 clone, respectively (data not shown).

To evaluate whether the increase of uPAR protein expression by Bcl-2 is mediated by the activation of Sp1, cells were treated with MTR, an inhibitor of Sp1 interaction with its consensus sequences, that specifically inhibits the expression of several genes (38–40). As reported in Fig. 4*A*, treatment of one representative *bcl-2* transfectant (MB5) with 100 (*lane 7*) and 200 nM (*lane 8*) MTR inhibited, in a dose-dependent manner, the enhanced Sp1 DNA binding activity induced by Bcl-2. In particular, 100 nM MTR decreased the level of Sp1 activity induced by Bcl-2 to that in the M14 parental cells. The results obtained with the MB6 clone were comparable with those with the MB5 clone (data not shown).

To evaluate the expression of uPAR after inhibition of Sp1 transcriptional activity with MTR, *bcl-2* transfectants were exposed to hypoxia for 24 h in the presence of 100 or 200 nM MTR, and uPAR protein levels were measured by ELISA (Fig. 4*B*). MTR reduced uPAR protein expression in Bcl-2-overexpressing cells in a dose-dependent manner. About 50% of inhi-

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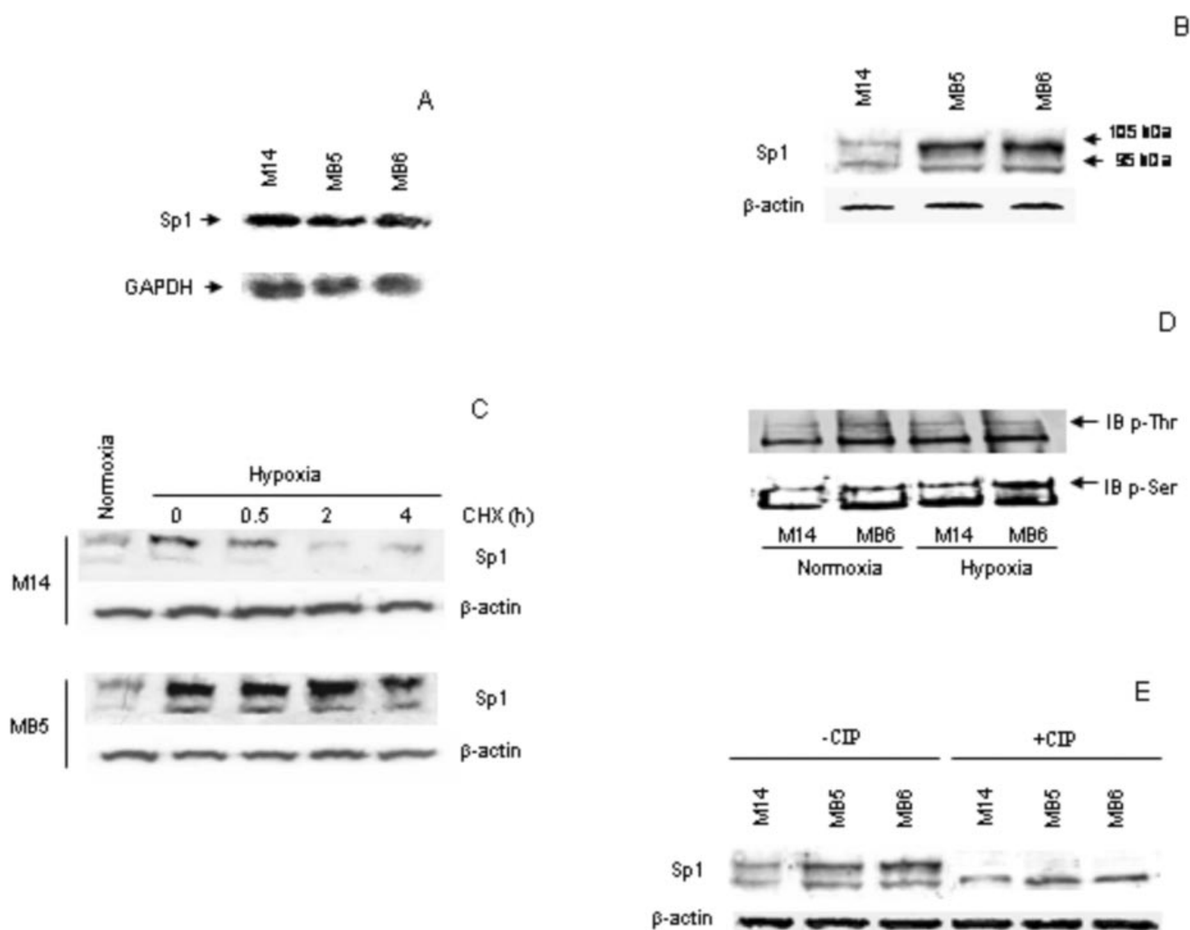

FIG. 3. **Bcl-2 overexpression in hypoxia increases Sp1 protein expression, stability, and phosphorylation but not mRNA expression.** *A*, Northern blot analysis of Sp1 gene expression in the M14 parental line and two *bcl-2* transfectants (MB5 and MB6) exposed for 24 h to hypoxia. GAPDH is an internal control for mRNA quality and loading. One representative experiment of three is shown. *B*, Western blot analysis of Sp1 protein in M14 cells and two *bcl-2* transfectants (MB5 and MB6) grown in hypoxic conditions for 24 h. β-actin expression served as controls for uniformity of protein gel loading and blotting. As expected, two species at 95 and 105 kDa resulted in all of the samples (*arrows*). One representative experiment of two is shown. *C*, M14 control cells and MB5 Bcl-2-overexpressing clone were exposed to hypoxia for 24 h and then treated with 10 μg/ml CHX for different times under hypoxic conditions. Sp1 protein levels were assessed by Western blotting with an Sp1-directed antibody. The protein level of Sp1 after 24 h of exposure to normoxia is also reported. β-actin was used as a control for equal protein loading. One representative experiment of three is shown. *D*, nuclear extracts of cells grown in normoxia or hypoxia for 24 h were immunoprecipitated with antibody to Sp1 and immunoblotted (*IB*) with anti-phosphoserine (*p-Ser*) or anti-phosphothreonine (*p-Thr*) antibodies. *E*, Western blot analysis of Sp1 protein in M14 cells and two *bcl-2* transfectants (MB5 and MB6) grown in hypoxic conditions for 24 h. Nuclear extracts were untreated or treated with 10 units of calf intestinal phosphatase (*CIP*) for 60 min at 37 °C. β-Actin expression served as controls for uniformity of protein gel loading and blotting. One representative experiment of two is shown.

bition of uPAR expression was calculated in both clones after treatment with 200 nM MTR.

Effect of Bcl-2 Overexpression on ERK Signaling Pathway—Since Sp1 is phosphorylated by a number of cellular kinases, including protein kinase A, different members of the protein kinase C family, and ERK2 (31, 41), we investigated whether the increase in Sp1 phosphorylation and DNA binding activity found in *bcl-2* transfectants exposed to hypoxia was due to activation of the ERK signaling pathway. Using an anti-phospho-ERK antibody that recognizes the active forms of ERK1 and ERK2, an increase in both ERK1 and ERK2 phosphorylation was observed in *bcl-2* transfectants exposed to hypoxia when compared with the parental line exposed at the same experimental conditions (Fig. 5*A*). Using polyclonal antibody raised against total ERK1 and ERK2, no modification in the level of ERK kinases expression was detected (Fig. 5*A*), indicating that the increase in ERK phosphorylation was not due to an increase in total ERK proteins. To evaluate the contribution of the ERK pathway in Sp1 phosphorylation and transcriptional activity, we performed Western blot and EMSA assays in the absence or presence of ERK inhibitor UO126 (10 μM). Abrogation of ERK activation by the ERK inhibitor UO126 (Fig. 5*A*) was paralleled by reduction of Sp1 phosphorylation (Fig. 5*B*) and DNA binding activity (Fig. 5*C*), clearly suggesting that Sp1 phosphorylation and DNA binding activity were ERK-dependent.

Effect of Bcl-2 Overexpression on uPAR Expression and Sp1 Activity in Breast Cancer Cells—To examine whether Bcl-2 overexpression results in the induction of uPAR expression and Sp1 activity also in another tumor cell type other than in M14 melanoma cells, a human breast carcinoma model was used. To this end, MCF7 ADR human breast carcinoma cells and two Bcl-2-overexpressing clones (MAB25 and MAB30), previously obtained after transfection (22), were exposed to hypoxia for 24 h, and the various analyses were performed as described above. We previously demonstrated that Bcl-2 overexpression in this cell line enhances its metastatic potential and synergizes with hypoxia to increase VEGF expression and *in vivo* angiogenesis (15, 22).

Western blot analysis of Sp1 protein showed an increased

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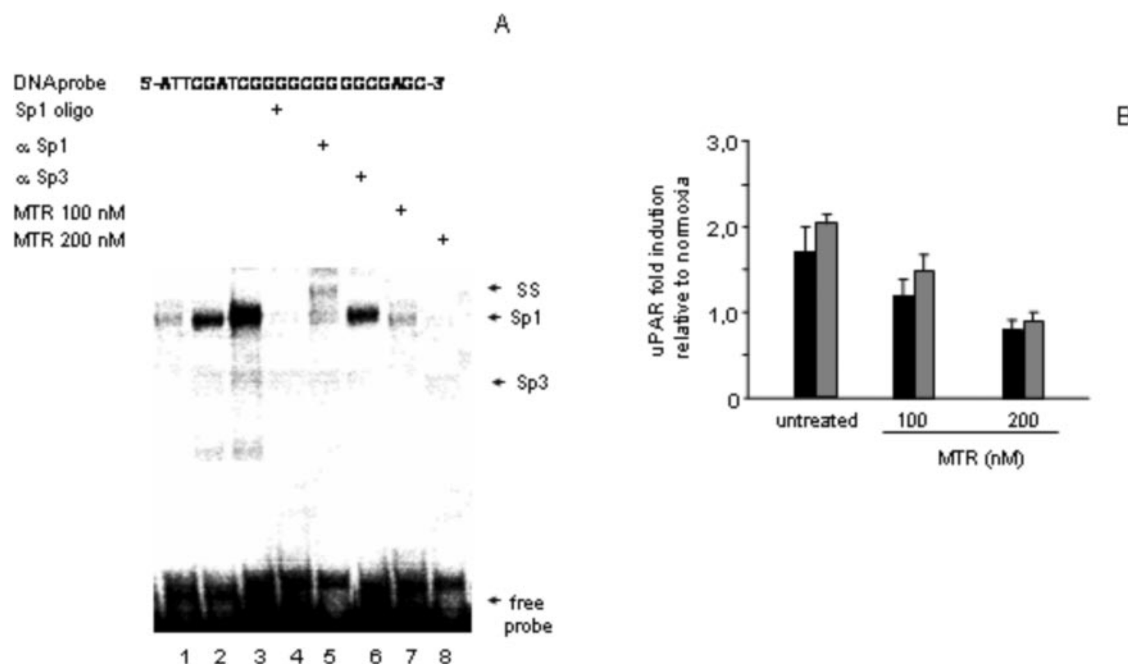

FIG. 4. **Bcl-2 overexpression in hypoxia increases Sp1 DNA-binding activity and MTR blocks Sp1 DNA-binding activity and uPAR expression.** *A*, EMSA was performed using ^{32}P-labeled oligonucleotides as a probe in the presence of nuclear extract from M14 (*lane 1*) and two *bcl-2* transfectants (MB5 (*lanes 2* and *4–8*) and MB6 (*lane 3*)) grown in hypoxic conditions for 24 h. 100 ng of double-stranded Sp1 oligonucleotide competitor (*Sp1 oligo*; *lane 4*) was added to the reaction mixture. Supershift assay (*SS*) of Sp1 DNA-binding complexes in M14 cells was performed by the addition of anti-Sp1 (*lane 5*) and anti-Sp3 (*lane 6*) antibodies to the reaction mixture. MTR (100 nM (*lane 7*) or 200 nM (*lane 8*)) was added to inhibit Sp1 DNA binding activity. *B*, expression of uPAR evaluated by ELISA in cell lysates of the two *bcl-2* transfectants (MB5 (*black columns*) and MB6 (*gray columns*)) grown in hypoxia for 24 h in the presence or absence of 100 and 200 nM MTR. Each point represents the mean ± S.D. of six experiments performed in duplicate.

expression of the transcription factor of about 3-fold in two *bcl-2* transfectants (MAB30 and MAB25) compared with MCF7 ADR control cells (Fig. 6*A*).

As shown both by Western blot analysis (Fig. 6*B*) and ELISA (Fig. 6*C*), the expression of uPAR protein in MAB25 and MAB30 *bcl-2* transfectants was about 2–3-fold higher than in MCF7 ADR parental cells.

Since low pressure of oxygen is a hallmark of many solid tumors, we also investigated whether Bcl-2 overexpression can modulate uPAR levels also in tumor xenografts obtained after injection of the different cell lines in nude mice (37). Interestingly, we found an increased level of uPAR protein expression in tumors derived from the two Bcl-2-overexpressing clones as compared with tumors from the MCF7 ADR parental cells (Fig. 6*C*).

Moreover, transactivation of the uPAR promoter was dependent on the levels of Bcl-2 expression, since a 24-h exposure to hypoxia induced an increase of about 3-fold in promoter activity in Bcl-2-overexpressing cells compared with control cells (Fig. 6*D*).

In all of the experiments, the results obtained with the MAN9 control clone were comparable with those with MCF7 ADR cells (data not shown).

These data corroborate and extend our finding with M14 melanoma cells, attesting for a more general phenomenon that links overexpression of Bcl-2 and Sp1-induced uPAR expression in human tumors.

DISCUSSION

In this paper, we demonstrate, for the first time, that Bcl-2 overexpression in human cancer cells synergizes with hypoxia to increase the expression level of uPAR through up-regulation of Sp1 transcriptional activity and that *uPAR* promoter activation is mediated via the ERK/Sp1 signaling pathway.

The uPAR is a cell surface receptor that, by its ligand uPA, leads to a very efficient plasmin-mediated degradation of matrix components. Thus, it is one of the molecules promoting invasion and metastasis and predicting a poor clinical prognosis of diverse cancer types (7, 9, 42–44). Binding of Sp1 transcription factor to the human *uPA* and *uPAR* promoters has been demonstrated and correlated with the invasive potential of tumor cells (37, 47). Sp1 is a ubiquitously expressed transcription factor that recognizes GC-rich sequence present in regulatory sequences of numerous housekeeping genes and genes involved in growth regulation and cancer (31). Sp1 undergoes post-translational modifications such as phosphorylation and glycosylation (31). Whereas glycosylation may enable nuclear localization and DNA binding and may determine the stability of Sp1 in the cell (46), phosphorylation may assist in stabilizing the Sp1-DNA complex (31), and there is sufficient evidence that Sp1 phosphorylation serves to regulate transcriptional initiation.

Using Bcl-2-overexpressing clones of the M14 melanoma cell line, we observed that under hypoxic conditions, uPAR expression increased at the protein and mRNA level compared with parental cells grown under identical conditions. A decreased expression of uPAR protein was observed after down-regulation of Bcl-2 protein using *bcl-2*-specific antisense oligonucleotides. In addition, we found enhanced binding of nuclear proteins to the ^{32}P-labeled Sp1 oligonucleotides as well as increased uPAR promoter activity in *bcl-2* transfectants exposed to hypoxia. Moreover, overexpression of Bcl-2 leads to increased Sp1 steady-state levels, probably as a result of increased protein stability. In fact, protein stability studies after CHX treatment indicated a significant increase in Sp1 protein stability after Bcl-2 overexpression. Since both phosphorylation (45) and glycosylation (46) of Sp1 have been demonstrated

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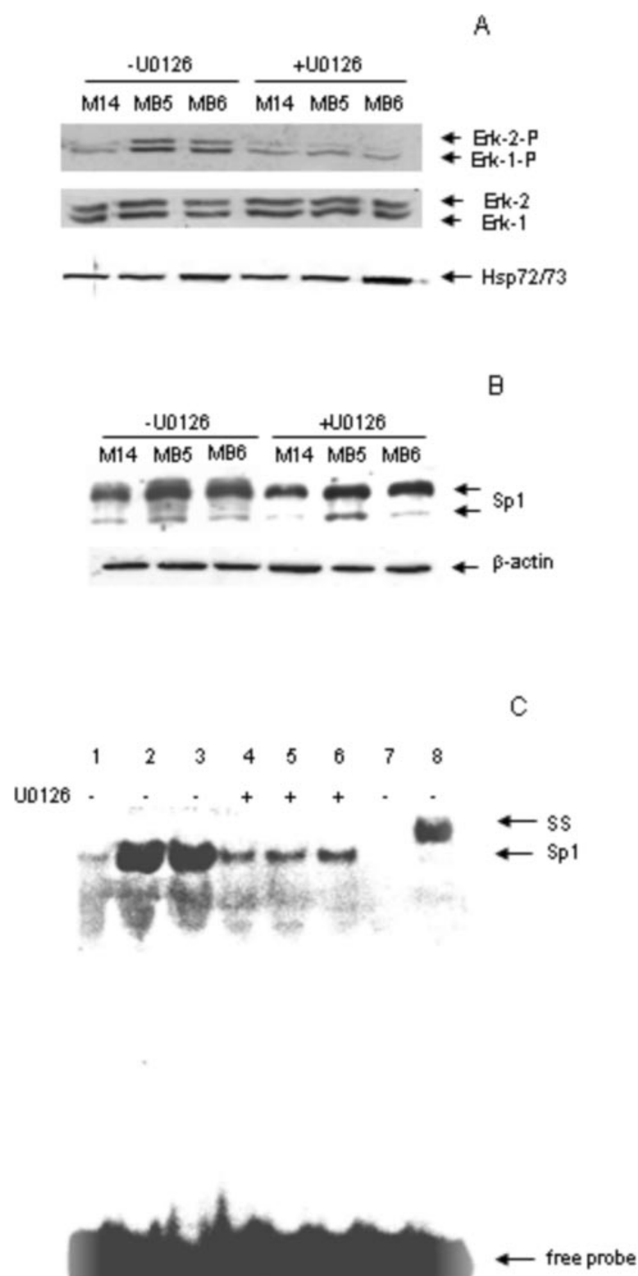

FIG. 5. **Bcl-2 overexpression in hypoxia increases ERK1/ERK2 activity and the ERK inhibitor UO126 reduces Sp1 phosphorylation and DNA-binding activity.** M14 and two *bcl-2* transfectants were exposed to hypoxia for 24 h in the presence or absence of UO126 (10 μM). Total protein extracts were used to perform Western blot analysis of ERK1/ERK2 phosphorylation and expression (*A*), whereas nuclear extracts were employed to evaluate Sp1 expression (*B*) and DNA binding activity (*C*). EMSA of nuclear extract from M14 (*lanes 1* and *4*) and two *bcl-2* transfectants (MB5 (*lanes 2* and *5*) and MB6 (*lanes 3* and *6–8*)) grown in hypoxic conditions for 24 h in the absence (*lanes 1–3*, *7*, and *8*) or presence (*lanes 4–6*) of UO126 ERK inhibitor (10 μM) is shown. 100 ng of double-stranded Sp1 oligonucleotide competitor (*lane 7*) was added to the reaction mixture. Supershift assay (*SS*) of Sp1 DNA-binding complexes in MB6 cells was performed by the addition of anti-Sp1 antibody (*lane 8*) to the reaction mixture. One representative experiment of two is shown.

to be associated with proteasome degradation, these mechanisms can be responsible for the increased Sp1 stability found in our experimental model.

We also demonstrated that Bcl-2 overexpression induced uPAR and Sp1 expression in the MCF7 ADR human breast carcinoma cell line, confirming our findings with M14 melanoma cells. This strongly suggests a link between *bcl-2* and uPAR provided by the Sp1 transcription factor as a more general phenomenon in human cancer.

In the present study, we also investigated the status of Sp1 phosphorylation after hypoxic exposure of parental and Bcl-2-overexpressing melanoma cells. Experiments of Sp1 immunoprecipitation performed to evaluate the presence of serine- and threonine-phosphorylated proteins revealed increased intracellular amounts of serine-phosphorylated Sp1 in response to Bcl-2 overexpression. In agreement with other reports, demonstrating that Sp1 phosphorylation of serine residues correlates with enhanced transcription of “Sp1 site-dependent” genes (31, 34), we hypothesized that Bcl-2 is involved in the increase of Sp1 transcriptional activity through serine phosphorylation of the transcription factor. MTR, a pharmacological inhibitor of Sp1, prevented the induction of Sp1 DNA binding activity and uPAR expression by Bcl-2, thus confirming the role of Sp1 in Bcl-2-induced uPAR expression.

The induction of uPAR expression by Sp1 observed in our experimental model is indicative of another potential mechanism by which Sp1 promotes angiogenesis. In fact, a number of genes that can mediate angiogenesis have been shown to be modulated by Sp1, including VEGF (34), thymidine phosphorylase (48), and metalloprotease 2 (49).

Experiments were also performed to evaluate the signal transduction pathway that mediates uPAR induction by Bcl-2 and to determine what effect this pathway has on Sp1. The results reported here evidenced that Bcl-2 overexpression in hypoxia increases phosphorylation of ERK. To pursue the role of this kinase in regulating Sp1 binding activity, we modulated ERK activity. We found that modulation of ERK activity by UO126 inhibitor is correlated with reduction of ERK phosphorylation and with Sp1 DNA binding activity and transcriptional activity. Taken together, these results indicate that Sp1 is a phosphoprotein that becomes hyperphosphorylated after Bcl-2 activation of the ERK kinase cascade in hypoxia. Sp1 hyperphosphorylation stimulates an increase in Sp1 binding and contributes to Sp1-mediated transactivation and increased promoter activity. These data are in agreement with those demonstrating Sp1 phosphorylation by ERK with consequent stimulation of DNA binding (41, 49) and with those demonstrating that uPAR production in glioblastoma cells is regulated by ERK-dependent signaling (49). Although several papers have demonstrated a mechanism for cellular survival that involves ERK activation (51, 52), our results indicate that Bcl-2 directly or indirectly activates the signal transduction cascade irrespective of an apoptotic stimulus. In fact, no apoptotic cells were observed after hypoxic treatment (16).

Together with our previous results, demonstrating the ability of Bcl-2 overexpression to increase VEGF expression and hypoxia-inducible factor 1 activity (16), the present study suggests that activation of ERK signaling by Bcl-2 may increase angiogenesis through activation of different genes that play a critical role in the angiogenic switch. In fact, the involvement of ERK signaling in the expression of several molecules related to angiogenesis, such as metalloprotease 2 (34), VEGF (49), and hypoxia-inducible factor 1 (53) has been demonstrated.

Although seven potential ERK phosphorylation sites in Sp1 have been predicted by computer analysis (49), other kinase activities such as casein kinase 2 and protein kinase A, have been demonstrated to affect Sp1 phosphorylation, binding, and presumably transactivation (33, 54). Thus, we cannot exclude the possibility that kinases other than ERK are involved in Sp1 phosphorylation.

The nature of the transduction pathway from Bcl-2 to the activation of kinases is still unknown and remains to be elucidated. Reactive oxygen species may play a role in such a phe-

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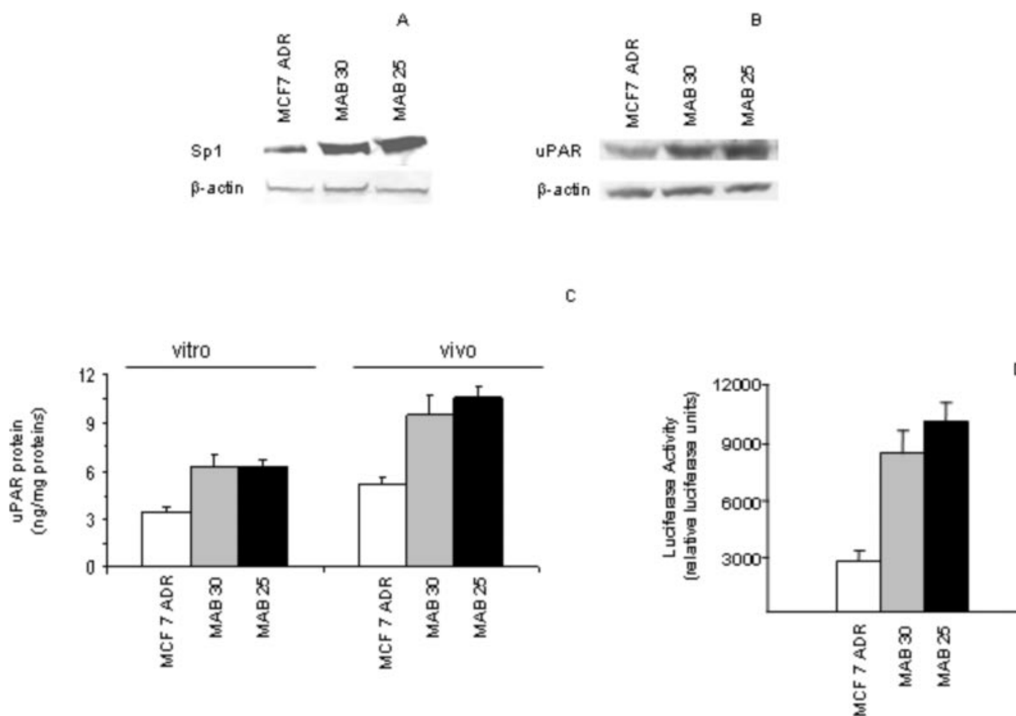

FIG. 6. **Bcl-2 overexpression in MCF7 ADR line increases uPAR and Sp1 protein expression and uPAR promoter activity.** MCF7 ADR cells and two *bcl-2* transfectants (MAB25 and MAB30) were exposed for 24 h to hypoxia, and the different analyses were performed. *A*, Western blot analysis of Sp1 expression in nuclear extracts. β-Actin expression served as controls for uniformity of protein gel loading and blotting. *B*, Western blot analysis of uPAR expression in total cell lysates. β-Actin expression served as controls for uniformity of protein gel loading and blotting. One representative experiment of three is shown. *C*, uPAR expression evaluated by ELISA in *in vitro* tumor cells and *in vivo* xenografts. *D*, uPAR promoter activity. Cells were transfected with a reporter plasmid (uPAR promoter), and after exposure to hypoxia, they were assayed for luciferase activity. Luciferase values are normalized for transfection efficiency (luciferase/β-galactosidase ratios). Results are means of five independent experiments.

nomenon. In fact, although the increase of reactive oxygen species production during hypoxia is still much debated, ERK activation upon hypoxia (52) and reactive oxygen species (55, 56) has been demonstrated, and Bcl-2 has been found to modulate reactive oxygen species production (57, 58). Thus, Bcl-2 could induce ERK activation through modulation of reactive oxygen species eventually induced by hypoxia. Bcl-2 could also activate ERK signaling through modulation of the expression of protein kinase C, Ca^{2+}/calmodulin, or other molecules involved in ERK activation. It is also possible that Bcl-2 interacts with another factor or factors to regulate ERK activation and that these factors are induced by hypoxia. Another mechanism can consider an unknown molecule induced by hypoxia and able to interact with Bcl-2.

To the best of our knowledge, this is the first demonstration of a direct relationship between Bcl-2 and uPAR expression through Sp1 activity. Our results are also in good agreement with those of others describing a correlation between Sp1 activity and uPAR expression exclusively in resected tumors but not in normal control tissues (30, 40). In particular, a coordinated up-regulation of uPAR expression and Sp1 DNA-binding activity was found in a study including patients with breast carcinoma and benign lesions as control (30). Tumor-specific Sp1 binding to the *uPAR* promoter was also demonstrated in a large series of resected colorectal cancers as well as in gastric carcinomas that represent different tumor entities (40). In addition, urokinase receptor gene expression was found to be regulated by Src partly via increased Sp1 binding (21), and a more recent paper has demonstrated uPA regulation by Sp1 (47).

Increased Sp1 phosphorylation and activity has also been proposed to result from oxidative stress-, tumor necrosis factor- and hepatocyte growth factor-induced VEGF transcription (34, 35, 59). Thus, it is likely that this transcription factor also plays a role in Bcl-2-induced VEGF expression that we previously demonstrated (15, 16).

We suggest that the increase of Sp1-mediated uPAR expression induced by Bcl-2 under hypoxic conditions facilitates binding of the uPA ligand to its receptor, which ultimately enhances the plasminogen activation required for metastasis and angiogenesis (54–62). The finding that Bcl-2 not only up-regulates uPAR but also VEGF (15–17) and matrix metalloproteinases (21) is of particular significance and suggests a series of well coordinated events that control a set of genes required for the angiogenic phenotype. Moreover, a direct correlation between VEGF and uPAR expression may also exist. Since treatment of capillary endothelial cells with VEGF causes up-regulation of uPAR (63), it is possible that the increased VEGF expression observed in our model systems (15, 16) directly stimulates uPAR expression.

In a previous study, we demonstrated that Bcl-2 overexpression in M14 cells increases angiogenesis through hypoxia-inducible factor 1-mediated VEGF transcriptional activity (16). Taken together, our results indicate that Bcl-2-induced angiogenesis involves distinct signaling pathways and molecules playing a role in the angiogenic switch in many tumor systems.

In view of the fact that VEGF and uPAR levels correlate with tumor progression in melanoma and other tumor histotypes, prevention of the angiogenic switch using Bcl-2 inhibitors, such as antisense oligonucleotides or synthetic ligands, or blocking antibodies that directly target uPAR or VEGF may represent a therapeutic option for patients with cancer at high risk of progression.

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bcl-2 Induction of Urokinase Plasminogen Activator Receptor Expression in Human Cancer Cells through Sp1 Activation: INVOLVEMENT OF ERK1/ERK2 ACTIVITY

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