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CITED4 Inhibits Hypoxia-Activated Transcription in Cancer Cells, and Its Cytoplasmic Location in Breast Cancer Is Associated with Elevated Expression of Tumor Cell Hypoxia-Inducible Factor 1α

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ABSTRACT

The interaction of hypoxia-inducible factor 1α and the CH1 domain of the transcriptional coactivator p300/CBP is necessary for the expression of hypoxia responsive genes and tumor angiogenesis. The transcription factor CITED2 binds p300/CBP at the CH1 domain and functions as a negative regulator of hypoxia signaling by competing with hypoxia-inducible factor 1α. CITED4, a recently identified member of the CITED family, binds p300/CBP via the CH1 domain and functions as a coactivator for transcription factor AP-2. Here, we show that CITED4 blocks the binding of hypoxia-inducible factor 1α to p300 *in vitro* and inhibits hypoxia-inducible factor-1α transactivation and hypoxia-mediated reporter gene activation. These studies suggest that CITED4 might function as an inhibitor of hypoxia-inducible factor 1α. To explore the function of CITED4 in breast cancer, we determined its expression in normal, *in situ* and invasive breast cancers. We also correlated its expression in 286 invasive breast tumors with clinicopathological, hypoxia markers and survival. In contrast to the nuclear localization of CITED4 in normal breast tissue, breast tumors were characterized by cytoplasmic and nuclear localization. Nuclear CITED4 expression was significantly inversely associated with tumor hypoxia-inducible factor 1α ($P < 0.05$), tumor size ($P = 0.03$), tumor grade ($P = 0.0001$), and Chalkley vessel count ($P = 0.04$). CITED4 showed no significant correlation with patient age ($P = 0.45$), estrogen receptor ($P = 0.11$), or epidermal growth factor receptor ($P = 0.48$). These results show that breast cancer development is characterized by either nuclear loss or cytoplasmic translocation of CITED4, with consequent loss of hypoxia-inducible factor-1α transcriptional antagonist activity. This may be an important mechanism by which tumors enhance hypoxia-inducible factor expression and result in an aggressive phenotype.

INTRODUCTION

Angiogenesis is essential for tumor growth and metastasis. The activation of the hypoxia-induced transcription factor 1α is often a crucial step for the expression of angiogenic factors controlling tumor neovascularization. To activate transcription, hypoxia-inducible factor 1α must recruit the transcriptional adapter/histone acetyltransferase proteins, p300 and CBP to the target gene promoters. This occurs by direct physical interactions between the transactivation domain of hypoxia-inducible factor 1α and the first cysteine-histidine (CH1) domain of p300/CBP. This domain of p300/CBP also binds members of the CITED (CBP/p300 interacting transactivator with ED-rich tail) family of transcription factors via their conserved COOH-terminal transactivation domain. We have showed previously that CITED2 (also known as p35srj or Mrg1), a hypoxia- and growth factor-induced transcription factor, will disrupt the interaction between hypoxia-inducible factor 1α and p300 and inhibit transactivation by hypoxia-inducible factor 1α and gene activation by hypoxia (1, 2). The solution structure of the hypoxia-inducible factor 1α-p300 and the CITED2-p300 complexes provides a molecular mechanism for this competition. CITED2 binds with 33-fold greater affinity to CH1 in comparison to hypoxia-inducible factor 1α. Both CITED2 and hypoxia-inducible factor 1α share a LPXL (Leu-Pro-X-Leu) motif that interacts with an overlapping binding site on p300-CH1 (3, 4). Genetic evidence indicates that loss of CITED2 is associated with increased activation of hypoxia-inducible factor-1 target genes, supporting this model (5). CITED2 also functions as a coactivator for the transcription factor AP-2, linking it to p300/CBP (6, 7). These interactions are thought to underlie the neural and cardiac developmental malformations associated with loss of CITED2 (5, 6, 8). CITED2 also functions to control cell proliferation and loss of CITED2 results in premature senescence of mouse embryonic fibroblasts. This results from a deficiency of Bmi1 and Mel18, which function to repress the cell proliferation inhibitors INK4a/ARF (9).

We have identified recently a new member of the CITED family, called CITED4, and showed that it directly binds the p300-CH1 domain and functions as a coactivator for transcription factor AP-2 (10). CITED4 is expressed ubiquitously in both human and mouse tissues. Cited4 expression is weak in nulliparous mouse breast tissues but is activated strongly by mid-gestation, with the protein showing a nucleocytoplasmic distribution. This high level of expression continues on through gestation and weaning. Consistent with this, Cited4 expression was stimulated by prolactin-induced differentiation of mouse mammary epithelial Scp2 cells in culture (11).

The CITED4 transactivation domain is highly conserved with that of CITED2 and also contains the LPXL motif. This suggests that CITED4, like CITED2, may function as an inhibitor of hypoxia-inducible factor 1α transactivation. In this report, we test this hypothesis and show that CITED4 is able to inhibit hypoxia-inducible factor 1α by disrupting the interaction with p300. Because we have demonstrated previously expression of CITED4 in human breast cancer cell lines (10), and we have shown hypoxia to be an important microenvironmental influence in breast cancer (12), we also examined the expression of CITED4 and hypoxia-inducible factor 1α in breast carcinomas. Our findings show that tumors are able to down-regulate expression of CITED4 or translocate CITED4 to the cytoplasm, which is associated with elevated hypoxia-inducible factor 1α and increased tumor size, grade, and angiogenesis.

MATERIALS AND METHODS

Generation of Monoclonal Antibody to CITED4

Monoclonal antibody HT13 was raised by immunizing mice with a purified glutathione *S*-transferase (GST)-CITED4 fusion protein as described previously (13). Positive hybridoma supernatants were identified by ELISA, confirmed by Western blotting of a maltose binding protein-CITED4 fusion

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protein, and immunoprecipitation of *in vitro* translated CITED4 (data not shown). GST-CITED4 and maltose binding protein-CITED4 were generated as described previously (10). The HT13 hybridoma was cloned, and high-titer cell-culture supernatants were retested by Western blotting. Isotyping of HT13 using a commercial kit (Roche Diagnostics, Sussex, United Kingdom) gave positive bands for IgG1, IgG2a, and κ chains.

CITED4 Regulation of Hypoxia-Inducible Factor 1α

Plasmids. Plasmids expressing CITED4 full-length or truncated CITED4 lacking the p300/CBP interacting domain (CITED4Δ), untagged or HA-tagged, have been described previously (10). GAL4-hypoxia-inducible factor 1α and hypoxia-inducible factor 1α expressing plasmids used for generating ^{35}S-labeled peptides have been described previously (1, 14). The GAL4-luc reporter and pCMV-lacZ plasmids were gifts from Ron Evans (Salk Institute, San Diego, CA). The 3xHRE-TK-luc reporter (15) was a gift from Steve McKnight (University of Texas, Dallas, TX).

GST Fusion–Binding and Peptide Competition. GST fusion proteins were generated as described previously (16). ^{35}S-labeled peptides were synthesized using a TNT kit (Promega) following the manufacturer's instructions. *In vitro* binding reactions were done as described previously (1). Peptides used for competition experiments were DEEALTSLELELGLHRVRELPELFLGQSEFDCF (residues 138–170 of human CITED4) and YAGPGMDSGLRPRGA (residues 32–46 of mouse CITED4). GST-p300-CH1 or GST (1 μg of eluted protein) was preincubated with the indicated peptide (20 μg) for 5 hours in 300 μl Hyb-75 + 1% milk buffer. All of the reaction mixtures contained equal amounts of the DMSO vehicle used to dissolve the peptides. ^{35}S-labeled, *in vitro* translated proteins were then added, and binding reactions were carried out overnight, after which the relevant proteins were precipitated with glutathione-Sepharose beads and analyzed as described above.

Tumors and Patients

Breast carcinomas (n = 286) and normal tissue from reduction mammoplasties (n = 6) were collected from patients undergoing surgery at the Oxford Radcliffe Hospitals (Oxford, United Kingdom). Tumors were treated by simple mastectomy (n = 71) or lumpectomy (n = 215) with axillary node sampling. All of the patients with tumors had axillary node status confirmed histologically. Histological types included 205 ductal carcinomas not otherwise specified, 20 lobular carcinomas, and 21 other types (data unavailable for 40 tumors). Grading of carcinomas was done according to the modified Bloom and Richardson method. The clinicopathological characteristics are given in Table 1. In patients <50 years, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil i.v. for six courses was administered if tumors were node-positive or estrogen receptor-negative and/or ≥3 cm. Patients ≥50 years with estrogen receptor-negative, node-positive tumors also received cyclophosphamide, methotrexate, and 5-fluorouracil. Patients with estrogen receptor-positive tumors received tamoxifen for 5 years; median follow-up was 7.3 years (range 0.4–11.0) and patients showed 97 relapses and 74 deaths.

Table 1 *Clinicopathological data from the series of patients stratified by CITED4 nuclear immunopositivity*

	CITED4 nuclear-negative (N = 124)	CITED4 nuclear-positive (N = 162)	P
Age (years)			
<50	38	43	0.45
≥50	86	119	
Nodal status			
negative	68	88	0.96
positive	56	74	
Tumor size			
≤2 cm	57	96	0.03*
>2 cm	67	66	
ER			
negative	47	47	0.11
positive	77	115	
EGFR			
negative	51	60	0.48
positive	70	98	
Grade			
I	16	31	0.0001
II	27	54	
III	49	29	
CVC			
<7	12	22	0.04*
≥7	19	12	
HIF-1α tumor			
negative	81	123	0.05*
positive	29	24	
VEGF-A			
negative	58	83	0.50
positive	57	69	

NOTE: Where n < 286 data unavailable.

Abbreviations: ER, estrogen receptor; EGFR, epidermal growth factor receptor; CVC, Chalkley vessel count; HIF, hypoxia-inducible factor.

*, significant.

Assessment of CITED4

To determine the pattern and variation in expression of CITED4, full-tissue sections in 6 normal, 8 *in situ*, and 86 invasive tumors were examined. To perform an analysis of the level of tumor CITED4 expression with clinicopathological variables and molecular markers of hypoxia, a tissue array of 286, including the above-mentioned invasive carcinomas, was constructed using a Beecher Instruments (Silver Spring, MD) arrayer.

Four-micron sections from formalin-fixed paraffin-embedded tissues from both series were cut onto silane-coated slides. These were dewaxed through xylene and graded alcohols, before placing in PBS for 5 minutes. Primary antibody (HT13; for characterization see Results) was applied as neat supernatant for 30 minutes without any antigen-retrieval techniques, followed by EnVision (Dako, United Kingdom) detection system.

In a preliminary analysis, some tumors were noted to be negative for CITED4, whereas others showed immunopositivity in the nucleus and/or cytoplasm of neoplastic cells. Because p300 and CBP are nuclear proteins and hypoxia-inducible factor-p300 interactions occur in the nucleus, it is likely that CITED4 physical inhibition of hypoxia-inducible factor 1α at the CH1 domain also takes place in the nucleus. Therefore, we stratified tumors into functionally negative for CITED4 (*i.e.*, no or <10% nuclear staining) and functionally positive for CITED4 (tumors that showed ≥10% nuclear staining).

Assessment of Hypoxia-Inducible Factor 1α Expression, Vascular Endothelial Growth Factor (VEGF), and Microvessel Quantitation

The hypoxia-inducible factor 1α protein was detected using the monoclonal antibodies ESEE 122 (IgG1; dilution 1:40; ref. 12) and the Envision-HRP kit (DAKO, Glostrup, Denmark). The intensity of nuclear staining was compared with that seen in parallel stained-control sections, as reported previously, with scoring based on the intensity and extent of nuclear and cytoplasmic reactivity (12). Cases with absent or weak cytoplasmic expression of hypoxia-inducible factor were considered as negative. Cases with nuclear expression or strong cytoplasmic expression were considered as positive.

To assess whether localization of CITED4 was related to function of hypoxia-inducible factor 1α, this transcription factor together with the hypoxia-inducible factor-1α target gene VEGF was analyzed. VEGF expression was semiquantitatively scored on the cytoplasmic tumor intensity and proportion of cell expressing VEGF, using VG1 (17, 18). Chalkley vascular counts were quantitatively determined by assessing the three most vascular areas at ×250 magnification, as reported previously (19). Two observers assessed the stainings using a conference microscope.

Statistical Analysis

Spearman rank correlation coefficients were used for studying the association between continuous variables. Tests of hypotheses on the location parameter (median) were done using rank statistics (Mann-Whitney, Kruskall-Wallis, and adjusted Kruskall-Wallis for ordered groups). The chi-square test was used to test for independence of categorical variables including categorized continuous variables. The log-rank test was used to test for differences in survival, and Cox proportional hazards model was used for multivariate models of survival. All of the statistics were done using the Stata package release 7.0 (Stata Corporation, College Station, Texas).

RESULTS

Specificity of HT13 Monoclonal Antibody to CITED4. To test the specificity of the HT13 for CITED4 peptide, U2-OS cells, which

do not express CITED4 (data not shown; Fig. 1), were transfected with a plasmid expressing NH_2-terminal HA-tagged CITED1, CITED2, and CITED4 human genes. The whole cell extracts prepared from the transfected U2-OS cells were separated on SDS-PAGE and immunoblotted with the anti-HA antibody or HT13 (Fig. 1 *A*, top and middle panels, respectively). The anti-HA antibody detected HA-tagged CITED1, CITED2, and CITED4 peptides (Fig. 1*A*, top panel), indicating that the three CITED peptides were expressed in the transfected cells. When HT13 was used, only HA-CITED4 peptide was detected, indicating that HT13 does not cross-react with the other closely related CITED peptides and is highly specific for CITED4 peptide.

We have shown previously that ECV304 (a bladder cell line with endothelial characteristics) expresses high levels of CITED4 transcripts, whereas CITED4 transcripts were not detected in the breast cancer cell line MDA-MB-231. To validate additionally the specificity of detection of CITED4 protein by HT13, we performed Western blotting using whole cell extracts prepared from ECV304 and MDA-MB-231 cells (Fig. 1*B*). A specific band with a molecular weight (M_r ~24,500) similar to the human CITED4 was detected in ECV304 but not in MDA-MB-231 protein extracts. This again indicates that HT13 detects CITED4 specifically.

We then examined the specificity of HT13 for CITED4 detection by immunostaining. U2-OS cells were transfected with plasmids expressing NH_2-terminal HA-tagged CITED1, CITED2, or CITED4 peptides. The cells were immunostained simultaneously with HT13 and with a rat anti-HA-tag antibody (Fig. 1*C*). The HA-antibody was able to detect all of the HA-CITED proteins (Fig. 1*C*, panels b–d, green), whereas HT13 specifically detected HA-CITED4 peptide (Fig. 1*C*, panel b, red). Both signals detected in cells transfected with the plasmid-expressing HA-CITED4 colocalized, indicating that the HA-CITED4 peptide was detected simultaneously by the anti-HA and HT13 antibodies. The HA-tagged CITED4 peptide was detected mainly (by both antibodies) in the cytoplasm of transfected U2-OS cells. But in some cells, HA-CITED4 also localizes in the nucleus (Fig. 1*C*, panel b).

The Effect of CITED4 on Hypoxia-Activated Transcription. We used GAL4-hypoxia-inducible factor 1α, a fusion of hypoxia-inducible factor 1α residues 723–826 to the GAL4 DNA-binding domain to test whether CITED4 affects hypoxia-inducible factor 1α transactivation. These hypoxia-inducible factor 1α residues bind p300-CH1 and constitute a transactivation domain. Cotransfection of GAL4-hypoxia-inducible factor 1α with a GAL4-luciferase reporter plasmid into cells activated the luciferase gene only after stimulation

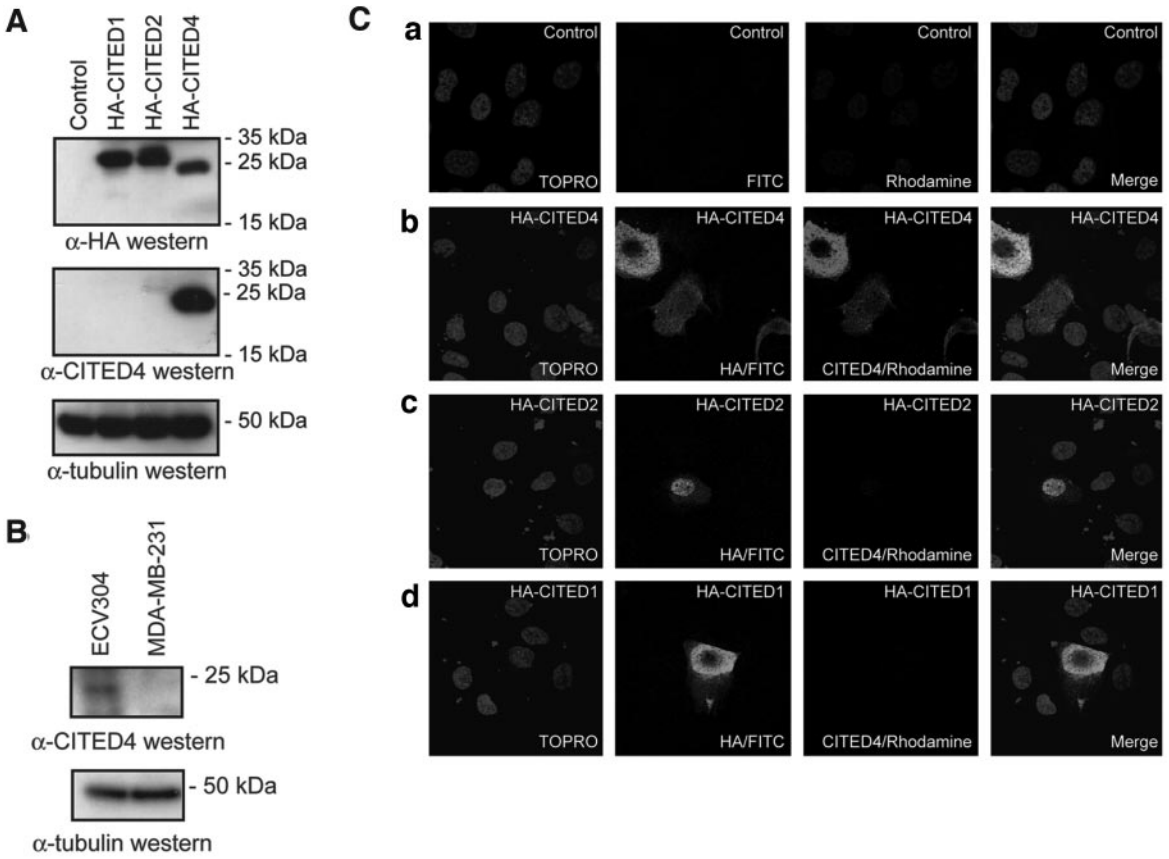

Fig. 1. Characterization of anti-CITED4 monoclonal antibody HT13. *A*, Western blot performed using 50 μg of whole cell extracts prepared from U2-OS cells transfected with plasmids expressing HA-CITED1, HA-CITED2, or HA-CITED4 or untransfected cells (*Control*). Whole cell extracts were prepared as described previously (10). The extracts were fractionated on a 12% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and probed with a rat anti-HA tag antibody (Roche) used at 1:200 dilution (*top panel*) or with HT13 anti-CITED4 antibody used at 1:5 dilution (*middle panel*). The whole cell extracts were also probed using anti-β-tubulin antibody (T-5293, Sigma, Chemical Co., Poole, Dorset, United Kingdom) at dilution 1:1,000 to confirm equal loading. *B* (*top panel*), Western blot of 50-μg ECV304 and MDA231 whole cell extracts fractionated on a 12% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and probed using HT13 antibody supernatant diluted 1:5. *Bottom panel*, Western blot of the same membrane using anti-β-tubulin antibody (Sigma T-5293) at dilution 1:1,000 to confirm equal loading. *C*, immunofluorescence: U2-OS cells were transfected with plasmids expressing HA-CITED1 (*panels d*), HA-CITED2 (*panels c*), HA-CITED4 (*panels b*), or untransfected (*panels a, Control*). Forty-eight hours after transfection cells were coimmunostained. Rat anti-HA tag (Roche) was used at 1:200 dilution followed by secondary antibody [rabbit antirat IgG coupled to FITC (Sigma)] at 1:400 dilution. Neat culture supernatant containing the anti-CITED4 monoclonal HT13 was used in combination with rabbit antimouse antibody coupled to rhodamine (Chemicon, Harrow, United Kingdom) at 1:200 dilutions. Nuclei were counterstained with TOPRO (Molecular Probes Ltd., Eugene, OR). Cells were mounted in Vectamount (Vector Laboratories, Peterborough, United Kingdom) and were visualized using a Bio-Rad MRC 1024 confocal microscope. Data from TOPRO (*blue*), FITC (*green*), and rhodamine (*red*) channels were sequentially accumulated. The merged image is shown in the panels on the extreme right.

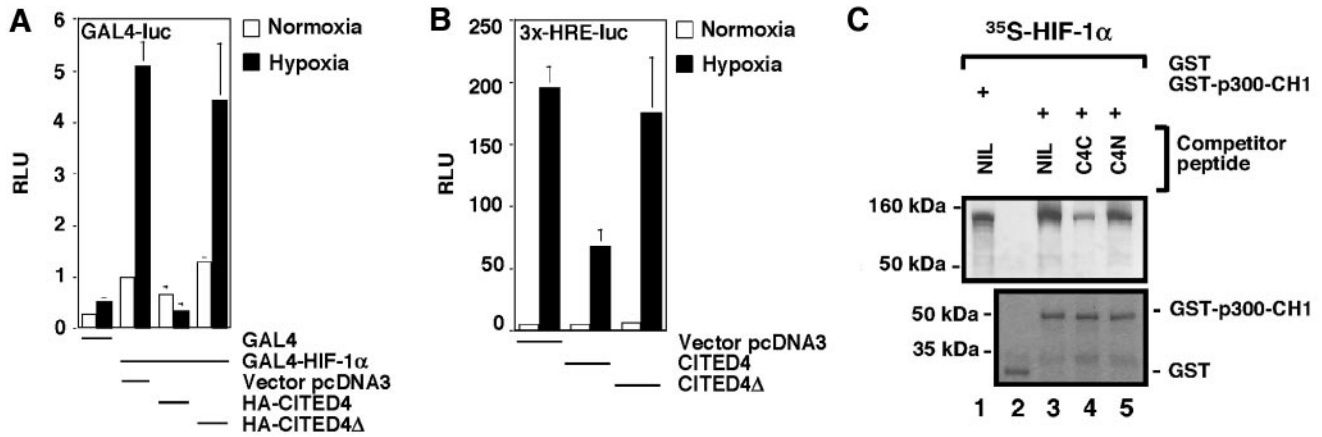

Fig. 2. CITED4 inhibits hypoxia-activated transcription. *A*, CITED4 inhibits hypoxia-inducible factor 1α transactivation. Hep3B cells were transiently cotransfected with GAL4-hypoxia-inducible factor 1α or GAL4 DNA-binding domain alone (40 ng), 3xGAL4-luc reporter (100 ng), CMV-lacZ (100 ng), and the indicated HA-CITED4 expressing plasmids (40 ng). HA-CITED4Δ plasmid expresses HA-fused to residues 1–137 of CITED4. The vector plasmid served as an additional control. The effect of HA-CITED4 proteins on GAL4-hypoxia-inducible factor 1α transactivation was tested on cells maintained in normoxia (21% O2) or stimulated with hypoxia (1% O2) for 16 hours. Results are presented as relative luciferase units, corrected for lacZ activity, and show the mean of three independent experiments. *B*, CITED4 blocks the transcriptional activation of a hypoxia response element. Hep3B cells were cotransfected with 3xHRE-luc reporter (40 ng), CMV-lacZ (100 ng), and the indicated CITED4 or CITED4Δ expressing plasmids, or pcDNA3 vector control (40 ng). The effect of CITED4 proteins was tested on cells maintained in normoxia or stimulated with hypoxia for 16 hours. Results are presented as for *A*. *C*, CITED4 blocks hypoxia-inducible factor 1α interaction with p300-CH1. *Top panels:* Autoradiograms of SDS-PAGE gels. *Lane 1*, 20% of the input of hypoxia-inducible factor 1α generated as an *in vitro* translated ^{35}S-labeled peptide. The relative amount of the factor bound to bacterially expressed GST (*Lane 2*) or to GST-p300CH1 (p300 residues 300–528, *Lanes 3–5*) immobilized on glutathione-Sepharose beads is shown. Binding was tested in the absence (*Lane 3*) or presence of 20 μg of a synthetic peptide corresponding to the p300-CH1 binding domain of CITED4 (*Lane 4*, *C4C*) or in the presence of 20 μg of a control peptide from the NH_2 terminus of CITED4 (*Lane 5*, *C4N*). *Bottom panels*, Coomassie-stained gel showing relative amounts of GST and GST-p300CH1 proteins. *Bars*, ±SD. (*RLU*, relative luciferase units; *HRE*, hypoxia response element; *HIF*, hypoxia-inducible factor)

by hypoxia (Fig. 2*A*). Cotransfection of a CITED4-expression plasmid (HA-CITED4) led to a complete loss of induced GAL4-hypoxia-inducible factor 1α transcriptional activity. A CITED4 mutant lacking the p300-CH1 binding domain (HA-CITED4Δ) failed to interfere with GAL4-hypoxia-inducible factor 1α transcriptional activity. In analogous experiments, CITED4 also inhibited the transactivation function of GAL4-EPAS1/hypoxia-inducible factor 2α (residues 19–870; data not shown).

We next tested whether CITED4 inhibits the activation of natural hypoxia-inducible factor-1 response elements activated by hypoxia (Fig. 2*B*). A reporter plasmid containing the luciferase gene under the control of three hypoxia-inducible factor 1 consensus DNA-binding elements (3xHRE-luc) was used to assess the effect of CITED4 expression on endogenous hypoxia-inducible factor 1 activity. As shown in Fig. 2*B*, transcription from the transfected reporter gene is activated when cells are stimulated by hypoxia, and this is markedly inhibited by CITED4. The inhibition conferred by CITED4 is dependent on the presence of its COOH-terminal domain, because the expression of CITED4Δ is unable to suppress hypoxia-stimulated transcription.

The Effect of CITED4 on Hypoxia-Inducible Factor 1α Interaction with p300-CH1. To determine the biochemical mechanism by which CITED4 inhibited transactivation by hypoxia-inducible factor 1α, we generated ^{35}S-labeled hypoxia-inducible factor 1α by coupled *in vitro* transcription-translation. This peptide was tested for interaction with bacterially expressed purified GST-p300-CH1 fusion protein (containing GST fused to p300 residues 300–528; Fig. 2*C*, Lane 3). This experiment showed that, *in vitro*, hypoxia-inducible factor 1α interacts with the CH1-domain of p300. To determine whether CITED4 would affect its binding, we performed a similar *in vitro* binding experiment in the presence of a peptide corresponding to residues 138–170 of CITED4, which is homologous to the p300-CH1 binding domain mapped previously in CITED2 (1). Addition of the CITED4 peptide in the reaction mix led to a hypoxia-inducible factor 1α decreased binding to the p300-CH1 domain (Fig. 2*C*, Lane 4). A control peptide derived from the NH_2 terminus of CITED4 had no effect (Fig. 2*C*, Lane 5).

The Pattern of Expression of CITED4 in Normal and Neoplastic Breast Tissues. Whole tissue sections of all of the normal and *in situ* carcinomas together with 86 invasive tumors were stained satisfactorily. Expression of CITED4 was observed in normal breast tissues. This was present in epithelial elements, mostly in the ductal cell but also in myoepithelial cell layers (Fig. 3*A* and *B*). Occasional cases showed strong myoepithelial cell without ductal cell staining. Expression was also identified in other elements, including inter- and intralobular stroma and occasional endothelial cells. Expression was mostly nuclear but also cytoplasmic (Fig. 3*C*).

CITED4 was highly expressed in breast neoplasia (Fig. 3, *D–G*). Expression was observed in both *in situ* and invasive disease (Fig. 3, *D–G*). Expression was present mostly in the cytoplasm of malignant epithelium. In *in situ* disease, staining was heterogeneous and weak (Fig. 3*E*), whereas in invasive carcinomas, it was homogenous and generally stronger (Fig. 3, *F–G*). In 30 cases, tumor-associated microvessels were also positive for CITED4. Stromal staining was frequently present, and occasional cases were also positive in arteriolar smooth muscle and pericytes (Fig. 3*G*, inset). In tissue microarrays, 124 cases showed no or <10% nuclear staining and 162 ≥10% nuclear staining. Forty-nine cases showed no cytoplasmic expression, 106 weak, 91 moderate, and 40 strong cytoplasmic staining.

Relationship among Tumor CITED4 Protein Expression and Clinicopathological, Microvessel Density, Hypoxic Markers, and Survival. To investigate additionally the change in location from nuclear to cytoplasmic compartment, we examined the relationship among potentially functional (conserving nuclear expression) and nonfunctional (no nuclear expression) CITED4 and clinicopathological variables and survival. This stratification split the tumors such that 124 were considered to lose CITED4 function and 162 were considered to have retained it. We observed significant inverse associations between retention of nuclear CITED4 and tumor hypoxia-inducible factor 1α expression ($P < 0.05$), tumor size ($P = 0.03$), tumor grade ($P = 0.0001$), and Chalkley vessel count ($P = 0.04$; Table 1). CITED4 showed no significant correlation with patient age ($P = 0.45$), estrogen receptor ($P = 0.11$), or epidermal growth factor receptor ($P = 0.48$; Table 1). In a survival analysis, patients with

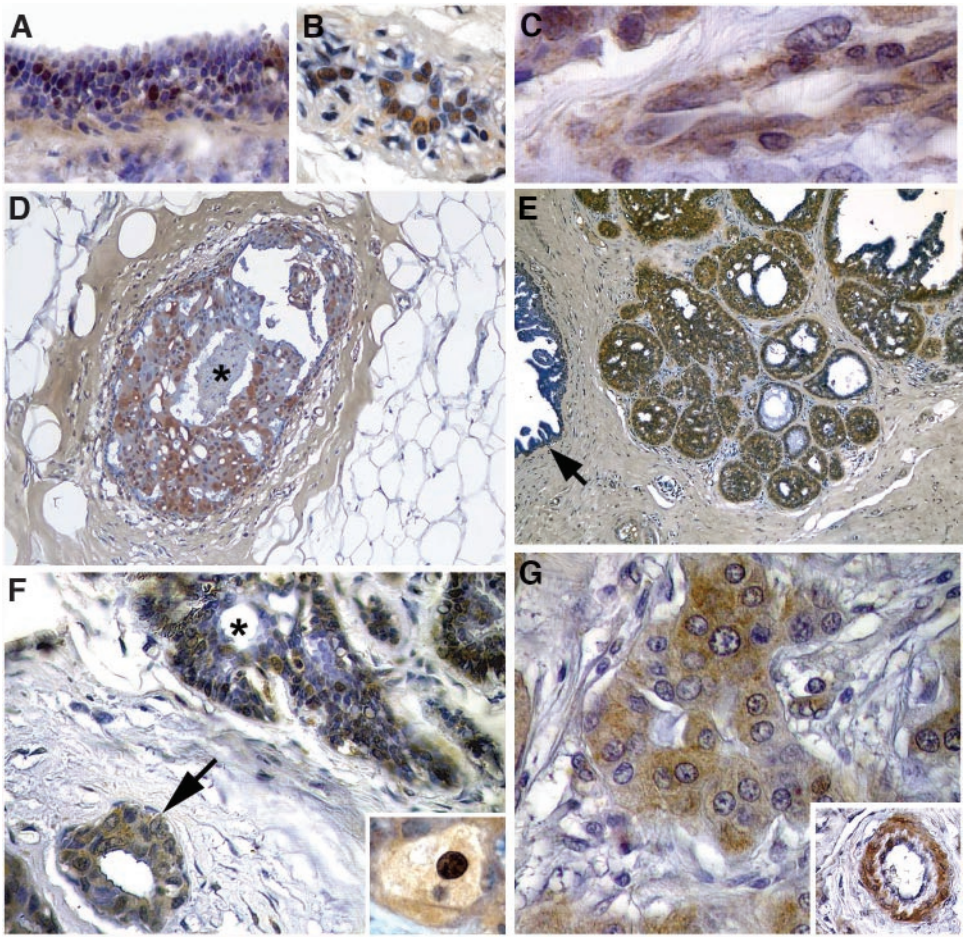

Fig. 3. Immunohistochemistry for CITED4 strong nuclear staining of CITED4 in the nuclei of luminal cells of a duct (*A*) and in an acinus (*B*) of a terminal duct lobular unit. CITED4 staining in the nucleus and cytoplasm of the endothelium of a small capillary (*C*). Heterogeneous nuclear and cytoplasmic CITED4 immunoreactivity within one duct of an intermediate nuclear grade ductal carcinoma *in situ*; ∗, absence of up-regulation around the area of necrosis (*D*). Homogeneous strong staining of CITED4 within individual ducts involved by low-grade ductal carcinoma *in situ* but with low expression of CITED4 in ducts in an adjacent terminal duct lobular unit (*arrow; E*). Strong CITED4 positivity in the cytoplasmic compartment of malignant glands (*black arrow*) in contrast to entrapped normal elements, where CITED4 is predominantly nuclear (∗); *inset*, occasional tumors showed strong nuclear expression (*F*). Strong cytoplasmic and weak nuclear CITED4 staining of an invasive ductal carcinoma; *inset*, CITED4 expression in pericytes surrounding a small vessel (*G*).

CITED4 function positive tumors had an improved relapse-free and overall survival of borderline significance ($P = 0.06$; Fig. 4).

DISCUSSION

CITED2 can act as a hypoxia-inducible factor 1α antagonist through a functionally critical sequence motif (LPXL), and we tested whether CITED4, which also contains the identical motif, could act in a similar manner. Our results show that cotransfection of CITED4 efficiently inhibited hypoxia-inducible factor 1α transcriptional activity, and that this effect requires the p300-CH1 binding domain of CITED4. We show additionally that CITED4 strongly inhibits the interaction of p300-CH1 with hypoxia-inducible factor 1α *in vitro*, indicating that the transcriptional inhibitory effects of CITED4 are because of direct competition for available p300/CBP binding sites.

Because CITED4 is expressed in murine mammary tissues, in human breast cancer cell lines, and hypoxia is an important microenvironmental influence in breast cancer biology (10–12), we also examined the expression of CITED4, hypoxia-inducible factor 1α, and the hypoxia-inducible factor 1α target gene VEGF in breast cancer. In normal breast tissues, CITED4 was expressed in several tissue elements with a predominant nuclear distribution, consistent with a role in the normal physiological changes in the breast that occur during the menstrual cycle. In support of this is differential expression of different CITED proteins during the murine milk cycle and changes in their expression between nulliparous and pregnant breast tissues (10, 11). Nevertheless, unlike breast tissues in the mouse, we identified expression of CITED4 in nonepithelial tissue elements, suggesting the role of CITED4 in human tissues is more complex.

In contrast to normal breast tissue, CITED4 in tumors showed several patterns of cellular location. These included retention of nuclear expression, cytoplasmic expression, and no expression. Using a rabbit polyclonal antibody, we have shown previously that CITED4 is predominantly in the nucleus in ECV304 cells (10). However, CITED1 expression is exclusively cytoplasmic in keeping with the ability of these proteins to be expressed in different cellular compartments (20). Thus, it is possible that during neoplastic transformation, tumors either suppress CITED4 and/or locate it in the cytoplasmic compartment, physically distancing CITED4 from binding p300/CBP and, thus, allowing enhanced hypoxia-inducible factor 1α function with increased effectiveness of any given level of hypoxia-inducible factor 1α. A change in nuclear cytoplasmic location of many proteins has been noted in breast cancer and other tumor types and may represent a change in regulation of these proteins (21). In additional support of this notion is the observation of a less aggressive tumor phenotype in tumors retaining nuclear expression with improved survival in the univariate analysis. Nevertheless, this was not independent in a multivariate analysis, which is likely to be because of the strong associations with tumor grade, size, and Chalkley vessel count.

There is increasing evidence of multiple pathways that may synergize with hypoxia to regulate hypoxia-inducible factor 1α in cancer, including PTEN mutation, Ras activation, and growth factor signaling pathways (22). We describe a novel pathway, the cytoplasmic localization of a transcriptional antagonist by which hypoxia-inducible factor may also be regulated. The intracellular processing of CITED4 in tumor cells may occur through several mechanisms, including increased cytoplasmic binding or decreased nuclear import/export.

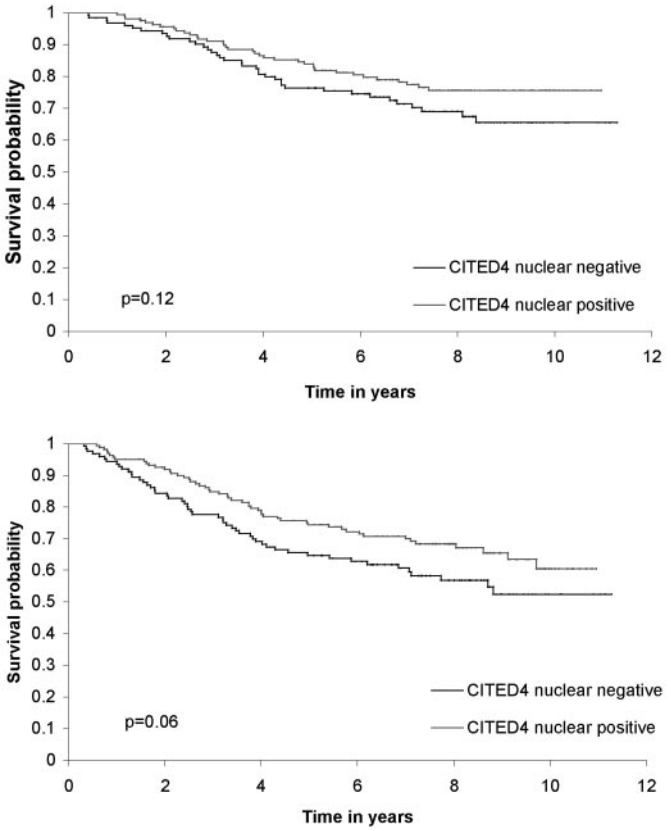

Fig. 4. Kaplan and Meier relapse-free (*top graph*) and overall survival (*bottom graph*) stratified by CITED4 function.

Although we have been unable to show additional nuclear CITED4 accumulation in Hep3b or T47D cells (which mainly localizes in the cytoplasm) with leptomycin B (data not shown), suggesting cytoplasmic binding as the likely mechanism, reports of nuclear export insensitive to leptomycin B have been described previously (23). The cytoplasmic export has been reported for several other nuclear proteins including p53, mdm2, and p21 (24, 25) that would also similarly affect function. In keeping with this, CITED1 is reported to show exclusive nuclear expression in human melanoma (20).

It is unlikely that CITED4 is directly regulating hypoxia-inducible factor 1α expression, but clearly the pathways converge and are selected in tumor biology. Although not addressed in this study, it is possible that displacement of hypoxia-inducible factor 1α from p300/CBP allows it to be more efficiently targeted for degradation by the von Hippel-Lindau-ubiquitin ligase complex. Additionally, the association of high nuclear hypoxia-inducible factor 1α and the cytoplasmic antagonist CITED4 would be associated with more aggressive phenotypes, as reported here for CITED4 and by others for hypoxia-inducible factor 1α (26).

In addition to being a transcriptional inhibitor, CITED4 is a strong coactivator for AP2, which is able to up-regulate VEGF (27). However, in agreement with other studies (28), we observed no relationship between tumor cell CITED4 function and VEGF expression, which may be because of cell-type specific AP2 coactivation, or, more likely, that human tumors use several pathways to regulate VEGF, such as Sp-1, AP-1, CREB-1, and RTEF-1. Nevertheless, the exact role of AP2 in breast cancer is also unclear, because AP2 overexpression is associated with high expression of HER2 and estrogen receptor in breast cancer cell lines (29) but low AP2 levels with disease progression (30). In the latter study, AP2 localized to the cytoplasm in half of the carcinomas (30), again indicating the aberrant localization of transcriptional regulators in cancer.

In summary, we have shown that CITED4 strongly inhibits the interaction of p300-CH1 with hypoxia-inducible factor 1α and is an efficient inhibitor of transactivation by hypoxia-inducible factor 1α. We have observed that in tumors, there is an alteration in the expression pattern of CITED4 from nucleus to cytoplasmic. This loss may allow p300/CBP to interact with hypoxia-inducible factor 1α and oncogenes to enhance their transcriptional activity leading to an aggressive tumor phenotype. Because CBP/p300 has been reported to function as a transcriptional coactivator of BRCA1 (31), it would be of interest to assess the expression of CITED4 in breast cancers derived from BRCA1-affected individuals. Study into enhancing the effect of CITED4 to interfere with hypoxia-inducible factor function is warranted, particularly because it does not have a nuclear localization signal. It may be possible to generate small molecules that bind particular elements CITED4-hypoxia-inducible factor-1α-p300 complex and differentially affect components of the hypoxia response (4).

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REFERENCES

1. Bhattacharya S, Michels CL, Leung MK, et al. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. Genes Dev 1999;13:64–75.
2. Leung MK, Jones T, Michels CL, Livingston DM, Bhattacharya S. Molecular cloning and chromosomal localization of the human CITED2 gene encoding p35srj/Mrg1. Genomics 1999;61:307–13.
3. Freedman SJ, Sun ZY, Kung AL, et al. Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. Nat Struct Biol 2003;10:504–12.
4. Bhattacharya S, Ratcliffe PJ. ExCITED about HIF. Nat Struct Biol 2003;10:501–3.
5. Yin Z, Haynie J, Yang X, et al. The essential role of Cited2, a negative regulator for HIF-1alpha, in heart development and neurulation. Proc Natl Acad Sci USA 2002;99:10488–93.
6. Bamforth SD, Braganca J, Eloranta JJ, et al. Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfap2 coactivator. Nat Genet 2001;29:469–74.
7. Braganca J, Eloranta JJ, Bamforth SD, et al. Physical and functional interactions among AP-2 transcription factors, p300/CREB-binding protein, and CITED2. J Biol Chem 2003;278:16021–9.
8. Barbera JP, Rodriguez TA, Greene ND, et al. Folic acid prevents exencephaly in Cited2 deficient mice. Hum Mol Genet 2002;11:283–93.
9. Kranc KR, Bamforth SD, Braganca J, et al. Transcriptional coactivator Cited2 induces Bmi1 and Mel18 and controls fibroblast proliferation via Ink4a/ARF. Mol Cell Biol 2003;23:7658–66.
10. Braganca J, Swingler T, Marques FI, et al. Human CREB-binding protein/p300-interacting transactivator with ED-rich tail (CITED) 4, a new member of the CITED family, functions as a co-activator for transcription factor AP-2. J Biol Chem 2002;277:8559–65.
11. Yahata T, Takedatsu H, Dunwoodie SL, et al. Cloning of mouse cited4, a member of the CITED family p300/CBP-binding transcriptional coactivators: induced expression in mammary epithelial cells. Genomics 2002;80:601–13.
12. Talks KL, Turley H, Gatter KC, et al. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 2000;157:411–21.
13. Eckner R, Ludlow JW, Lill NL, et al. Association of p300 and CBP with simian virus 40 large T antigen. Mol Cell Biol 1996;16:3454–64.
14. Bhattacharya S, Eckner R, Grossman S, et al. Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. Nature (Lond) 1996;383:344–7.
15. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 1997;11:72–82.
16. Ausubel F, Brent R, Kingston R, et al. Short protocols in molecular biology. 3rd ed. Hoboken, NJ: John Wiley and Sons, Inc.;1995.
17. Turley H, Scott PA, Watts VM, et al. Expression of VEGF in routinely fixed material using a new monoclonal antibody VG1. J Pathol 1998;186:313–8.
18. White JD, Hewett PW, Kosuge D, et al. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res 2002;62:1669–75.
19. Fox SB, Leek RD, Weekes MP, et al. Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. J Pathol 1995;177:275–83.

20. Li H, Ahmed NU, Fenner MH, et al. Regulation of expression of MSG1 melanocyte-specific nuclear protein in human melanocytes and melanoma cells. Exp Cell Res 1998;242:478–86.
21. Blagosklonny MV. Are p27 and p21 cytoplasmic oncoproteins? Cell Cycle 2002;1: 391–3.
22. Harris AL. Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38–47.
23. Wiechens N, Fagotto F. CRM1- and Ran-independent nuclear export of beta-catenin. Curr Biol 2001;11:18–27.
24. Lu W, Pochampally R, Chen L, et al. Nuclear exclusion of p53 in a subset of tumors requires MDM2 function. Oncogene 2000;19:232–40.
25. Winters ZE, Hunt NC, Bradburn MJ, et al. Subcellular localisation of cyclin B, Cdc2 and p21(WAF1/CIP1) in breast cancer. association with prognosis. Eur J Cancer 2001;37:2405–12.
26. Bos R, van der Groep P, Greijer AE, et al. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer (Phila) 2003:97:1573–81.
27. Brenneisen P, Blaudschun R, Gille J, et al. Essential role of an activator protein-2 (AP-2)/specificity protein 1 (Sp1) cluster in the UVB-mediated induction of the human vascular endothelial growth factor in HaCaT keratinocytes. Biochem J 2003; 369:341–9.
28. Jubb AM, Pham TQ, Hanby AM, et al. Expression of vascular endothelial growth factor, hypoxia inducible factor 1α and carbonic anhydrase IX in human tumours. J Clin Pathol 2004;57:504–12.
29. Hilger-Eversheim K, Moser M, Schorle H, Buettner R. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. Gene (Amst) 2000;260:1–12.
30. Pellikainen J, Kataja V, Ropponen K, et al. Reduced nuclear expression of transcription factor AP-2 associates with aggressive breast cancer. Clin Cancer Res 2002;8: 3487–95.
31. Pao GM, Janknecht R, Ruffner H, Hunter T, Verma IM. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. Proc Natl Acad Sci USA 2000; 97:1020–5.