CITED2 is expressed in human adrenocortical cells and regulated by basic fibroblast growth factor

Matthias Haase, Matthias Schott, Stefan R Bornstein[1], Ludwik K Malendowicz[2], Werner A Scherbaum and **Holger S Willenberg**

Department of Endocrinology, Diabetes and Rheumatology, University Hospital Duesseldorf, Moorenstr. 5, D-40225 Duesseldorf, Germany
[1]University of Technology Dresden, University Clinic III, Dresden, Germany
[2]School of Medicine, Department of Histology and Embryology, Poznan, Poland
(Requests for offprints should be addressed to H S Willenberg; Email: holger.willenberg@uni-duesseldorf.de)

Abstract

CITED2 gene deletion in mice leads to adrenal agenesis. Therefore, we analyzed CITED2, a CBP/p300 interacting transactivator with transforming activity, in the human adrenal gland. In this study, we examined CITED2 expression in human embryonic and adult adrenal glands as well as adrenocortical carcinomas. As ACTH and basic fibroblast growth factor (bFGF) are connected to the physiology and growth of adrenocortical cells we studied the regulation of CITED2 by these factors in the NCI-H295R adrenocortical carcinoma cell line. We found CITED2 expression in the adult adrenal cortex as well in adrenocortical carcinomas. At an early stage of human adrenal organogenesis CITED2 could be located to the definitive zone of the developing adrenal gland using immunohistochemistry. In NCI-H295R cells, stimulation by bFGF led to a dose-dependent increase in CITED2 promotor activity, mRNA and protein expression while ACTH had no significant effect. The stimulatory effect of bFGF could be reduced by blocking mitogen-activated protein kinase activity using the MAPkinase kinase (MEK1)-inhibitor PD98059. CITED2 is expressed in embryonic and adult human adrenal glands as well as in adrenocortical cancer. It is connected to the signaling cascades of bFGF and its expression is modulated by mitogen-activated protein kinases. This suggests a novel role for CITED2 in human adrenal growth and possibly in adrenal tumorigenesis.
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Introduction

CITED2 (CBP/p300 interacting transactivator with ED-rich tail 2) is a widely expressed transcriptional co-regulator of the cAMP response element binding protein (CREB) binding protein (CBP) and p300 (Bhattacharya *et al.* 1999). CITED2 expression leads to the modulation of different CBP or p300 dependent pathways including the co-activation of transcription factor AP2 by synergistic binding to CBP/p300 (Bamforth *et al.* 2001, Braganca *et al.* 2003) or the inhibiton of hypoxia inducible factor-1 by competitive binding to CBP/p300 (Bhattacharya *et al.* 1999, Freedman *et al.* 2003). CITED2 overexpression has been associated with oncogenic transformation and enhanced proliferation in different cell types (Sun *et al.* 1998, Kranc *et al.* 2003, Tien *et al.* 2004).

Mice lacking CITED2 exhibit a phenotype of cardiovascular malformations, neural crest defects, exencephaly and adrenal agenesis (Bamforth *et al.* 2001). Therefore, CITED2 is assumed to play a fundamental role in adrenal organogenesis, adrenal tissue growth and/or adrenal differentiation.

The human adrenal cortex arises from a combined adrenal and gonadal precursor at 4 weeks of gestation as a condensation of coelomic cells between the dorsal mesentery and the urogenital ridge (Mesiano & Jaffe 1997). From this site cells are believed to migrate in two waves to the cranial pole of the mesonephros forming the adrenal primordium. First, they present as a homogenous cell aggregation that gives rise to the fetal and definitive zones later. The fetal zone expresses 17-α-hydroxylase (CYP17; Hanley *et al.* 1993, Mesiano *et al.* 1993, Narasaka *et al.* 2001), a key enzyme of steroid synthesis, and produces mainly dehydroepiandrosterone-sulfate. After birth, the fetal zone degenerates within the first years of life. The definitive zone lacks expression of CYP17 (Hanley *et al.* 1993, Mesiano *et al.* 1993, Narasaka *et al.* 2001) and it is believed that the fetal zone as well as parts of the adult adrenal zones derive from precursors of the definitive zone (Mesiano & Jaffe 1997, Keegan & Hammer 2002, Muench *et al.* 2003). Development of the adrenal medulla is initiated by neural crest-derived chromaffin cells traveling to the primordial complex of adrenocortical cells (Keegan & Hammer 2002).

On one hand it has been speculated that adrenal agenesis observed in CITED2$^{-/-}$ mice could be an indirect result of defects in tissues interacting with adrenocortical cells, such as the adrenal medulla. On the other hand CITED2 could be essential for the function of adrenocortical cells themselves.

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However, there is no data about CITED2 in the human adrenal gland. Therefore, in this study we focussed on the role of CITED2 in the adrenal cortex. First, we examined the expression of CITED2 during early organogenesis, in adult adrenal glands as well as in adrenocortical carcinomas. Secondly, we analyzed if CITED2 is under control of factors that are relevant for adrenal function. Since corticotropin (ACTH) is a key regulator of adrenal physiology and basic fibroblast growth factor (bFGF) has been implicated in the control of adrenocortical growth (Mesiano *et al.* 1991, Basile & Holzwarth 1993, Ho & Vinson 1997, Feige *et al.* 1998, Boulle *et al.* 2000) we hypothesized that CITED2 may be a downstream effector of these factors. Therefore, we applied NCI-H295R cells to study the regulation of CITED2 by ACTH and bFGF in adrenocortical cells.

Materials and Methods

Tissues

Four embryonic adrenal glands from two embryos at 8 weeks of gestation were obtained from an earlier study that has been approved by the Ethical Committee of the Medical School of Poznan, Poland (Ehrhart-Bornstein *et al.* 1997). Histological analysis was applied to determine the age of gestation. Normal adult adrenal glands ($n=4$) were obtained from four patients who had undergone nephrectomy for renal cancer. Examination of the adrenal glands did not reveal infiltration by the renal tumor or metastases. The cancer species ($n=4$) were obtained from tumor resection from four patients with diagnosis of adrenocortical carcinoma. All tissues were fixed in formalin and subsequently embedded in paraffin.

Immunohistochemistry

The paraffin-embedded specimen of adrenal tissues were characterized immunohistochemically using antibodies to 17-α-hydroxylase (courtesy of Prof. Waterman, Vanderbilt University School of Medicine, Nashville, TN, USA) for adrenocortical cells and to chromogranin A (clone DAK-A3, DakoCytomation, Hamburg, Germany) for chromaffin cells as previously described (Ehrhart-Bornstein *et al.* 1997). For CITED2 staining, sections were deparaffinized in xylene, washed in ethanol 100% and hydrated in a descending ethanol row. Then sections were processed for 4 min by microwave irradiation in an antigen retrieval solution (DakoCytomation). Subsequently, endogenous peroxidase was quenched with 3% H_2O_2 and non-specific epitopes were blocked using serum-free blocking solution (supplied with DakoCytomation CSA Kit). The sections were incubated using monoclonal primary antibodies to CITED2 (Novus Biologicals, Littleton, CO, USA) in a dilution of 1:100 at room temperature for 1 h and signals were detected using the catalyzed signal amplification system (CSA, DakoCytomation), according to the manufactor's instructions. Negative control slides were incubated without primary antibody. After each step, except after the blocking procedure, slides were washed at least three times in 0·05 mol/l Tris–HCl (pH 7·6) containing 0·3 mol/l NaCl and 0·1% Tween 20 (TBST, DakoCytomation). Labeled protein was visualized using 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in Tris–HCl (pH 7·6) containing 0.016% H_2O_2 for 5–7 min. Nuclei were counterstained with hematoxylin (Merck) and slides were mounted in glycerol gelatine (Hollborn and Soehne, Leipzig, Germany).

Cell culture and in vitro *studies*

For stimulation experiments we employed the permanent adrenocortical carcinoma cell line NCI-H295R. The NCI-H295R cells were cultured in RPMI1640+L-glutamin (Invitrogen) supplemented with 2% fetal bovine serum, insulin (66 nM), hydrocortisone (10 nM), apo-transferrin (10 μg/ml), β-estradiol (10 nM), Na-Selenit (30 nM), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air, and 5% CO_2. Medium was changed every 3 days and cells were subcultured at confluency using Accutase (PAA Laboratories, Cölbe, Germany). Dependent on the experiments cells were plated in six-well culture plates for RNA-extraction, in 24-well culture plates for transfections or on poly-D-lysine-coated cover slips for immunofluorescence microscopy as described in the following sections.

Plasmids, transfections and luciferase assays

A firefly luciferase containing CITED2-promotor reporter plasmid was kindly provided by Bhattacharya and the sequence has been described already (Leung *et al.* 1999). For internal control we used pRL-TK plasmid (Promega) providing moderate expression of renilla luciferase.

NCI-H295R cells were cultured in 24-well plates at a density of 200 000 cells/well and transiently co-transfected for 24 h using 1·5 μl FuGENE6 transfection reagent (Roche Applied Science) with 0·25 μg of the CITED2 reporter construct and 0·25 μg of the renilla luciferase vector pRL-TK. Subsequently, cells were washed with PBS and incubated in cell culture medium for 48 h in the absence or presence of ACTH (100 nM, Synacthen, Ciba-Geigy, Wehr, Germany), forskolin (10 μM, Sigma-Aldrich) or human bFGF (0·1, 1 or 10 ng/ml, PromoCell, Heidelberg, Germany) either with or without PD98059 (20 μM, Promega). Firefly and renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega). For analysis, firefly activity was normalized to the activity of the renilla luciferase. The data is expressed as the mean±S.D. of three independent experiments. Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparison post test using Graph Pad Prism 4 (San Diego, CA, USA).

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Reverse transcriptase-PCR (RT-PCR)

RNAs of normal adult human adrenal cortices were obtained from BD Biosciences Clontech (Palo Alto, CA, USA) and total RNA from NCI-H295R cells was extracted using the RNeasy MiniKit (Qiagen) including a DNase I digestion step (New England Biolabs, Ipswich, MA, USA). Reverse transcription was done using Ready-To-Go t-primed first strand kit (Amersham Biosciences AB) according to the manufactor's instructions. PCR analysis was carried out using a PCR Master Kit (Roche Applied Science) and the following conditions: initial denaturation of 120 s at 94 °C followed by 30 three-step cycles of 30 s at 94 °C, 60 s at 58 °C, and 120 s at 72 °C. The whole procedure skipping reverse transcription reaction served as negative control.

Sequences of primers specific for CITED2 are given in Table 1. Amplification products were separated and visualized by agarose gel electrophoresis (3%) and ethidium bromide staining.

Semi-quantitative TaqMan PCR

NCI-H295R cells were cultured in six-well plates at a density of 600 000 cells/well and incubated in cell culture media for 8 h in the absence or presence of ACTH (100 nM), forskolin (10 μM), and human bFGF (0·1, 1 or 10 ng/ml). At the end of the incubation period the medium was removed and cells were washed with PBS before the RNA extraction procedure was started. Total RNA was extracted from NCI-H295R cells using the RNeasy MiniKit (Qiagen) including a DNase I digestion step (New England Biolabs) and reverse transcribed with the random-primed first strand cDNA Kit (Roche Applied Science) according to the manufactor's instructions. For negative control reactions reverse transcription was skipped. The CITED2 specific primers and probes were selected using the software PrimerExpress (PE Applied Biosystems, Foster City, CA, USA) and are given in Table 1. Sequences of primers and probes for the endogenous control 18S are also given in Table 1. Semiquantitative TaqMan PCR was carried out in 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 58 °C for one minute using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). All experiments were carried out in triplicates and average C_T units were obtained as the average of the results. Relative quantification of the CITED2 expression was done using the comparative C_T method in separate tubes. All data are expressed as the mean ± S.D. of four independent experiments. Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparison post test using Graph Pad Prism 4.

Table 1 Primer and probes for quantitative TaqMan analysis

Name	**Oligonucleotide**	**Sequence** (5′→3′)
18S	Forward primer	CGG CTA CCA CAT CCA AGG AA
18S	Reverse primer	GCT GGA ATT ACC GCG GCT
18S	Labeled probe	TGC TGG CAC GAG ACT TGC CCT C
CITED2	Forward primer	CCA CTA CAT GCC GGA TTT GC
CITED2	Reverse primer	TCT CGG AAG TGC TGG TTT GTC
CITED2	Labeled probe	CCC TGC TGC AGG CCA CCA GAT

Immunofluorescence microscopy

For immunofluorescence, NCI-H295R cells were seeded on poly-D-lysine (Sigma-Aldrich) coated glass cover slips and grown until they were about 70% confluent. Then cells were incubated in cell culture media for 24 h in the absence or presence of ACTH (100 nM) or human bFGF (10 ng/ml) either with or without PD98059 (20 μM). At the end of the incubation period, cells were fixed with methanol for 15 min at −20 °C, permeablized with 0·3% Triton X-100 (Sigma-Aldrich) for 5 min and unspecific sites were blocked for 1 h with 5% normal goat serum (DakoCytomation). Then cells were incubated with monoclonal anti-CITED2 antibodies (Novus Biologicals) in a dilution of 1:100 for 1 h at room temperature. The fluorescence dye conjugated secondary antibody (Alexa Fluor 488, goat anti-mouse IgG, Molecular Probes, Leiden, The Netherlands) was used in a dilution of 1:500 for 1 h at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 min. After each step, except after the blocking procedure, cells were washed at least three times in PBS.

Finally, cover slips were mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA) and preparations were examined with a Nikon Eclipse TE-300 fluorescent microscope.

Results

CITED2 is expressed during early adrenal organogenesis

Positive immunoreactivity with antibodies to CYP17 was found in the cytoplasm of cells within the inner zone of the embryonic adrenal gland at 8 weeks of gestation, marking the fetal zone (Fig. 1E). The outer zone did not show CYP17 staining and could accordingly be identified as the definitive zone.

CITED2 was expressed within the definitive zone of the adrenal gland (Fig. 1B). The expression was predominantly characterized by the labeling of small round and polygonal nuclei (Fig. 1D). In addition, we compared the expression patterns of CITED2 and chromogranin A in non-corresponding serial sections. Unlike CITED2, chromogranin A immunoreactivity was restricted to the cytoplasm of cells within the medial part of the definitive zone, representing chromaffin cells migrating from the para-aortic region to the adrenal (Fig. 1F).

CITED2 is expressed in adult adrenocortical cells

Immunohistochemistry demonstrated CITED2 protein expression in adrenocortical cells in the zonae glomerulosa

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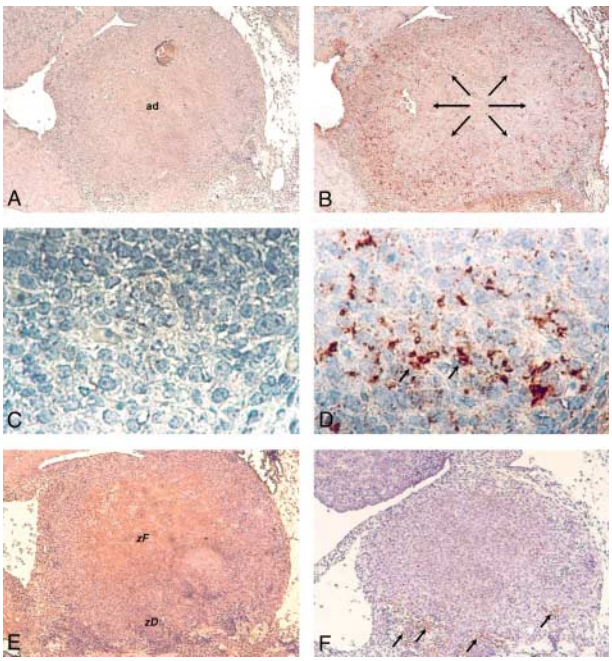

Figure 1 Representative sections of human embryonic adrenal glands at 8 weeks of gestation. Panel A: negative control without primary antibody (ad = adrenal, 100×). Panel B: immunostaining with antibodies to CITED2 revealed that CITED2 is located in the definitive zone (arrows, 100×). Panel C: high magnification of a negative control demonstrates nuclear morphology in the definitive zone (1000×). Panel D: high magnification of a representative section of the definitive zone. Predominantly, small nuclei were stained with an antibody to CITED2 (arrows, 1000×). Panel E: immunohistochemistry with an antibody to 17-α-hydroxylase identified the fetal zone (*zF*) while the definitive zone (*zD*) did not stain (100×). Panel F: chromogranin A immunoreactive cells in the outside margin of the adrenal gland (arrows, 100×).

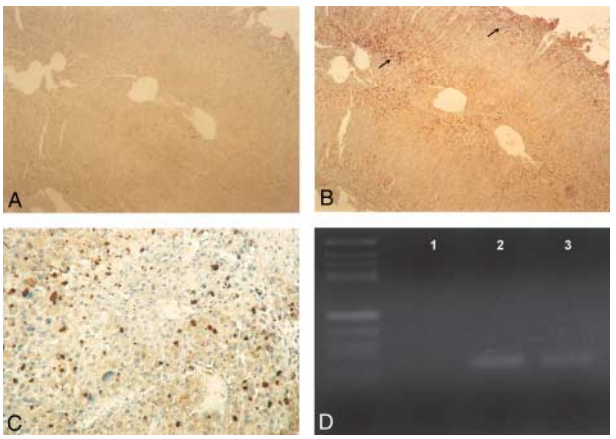

Figure 2 Representative sections of stained normal adult adrenal glands and adrenocortical carcinomas. Panel A: negative control for normal adult adrenal gland without primary antibody (40×). Panel B: normal adult adrenal gland stained for CITED2. Arrows indicate immunoreactivity in the zonae glomerulosa and reticularis at the corticomedullary junction (40×). Panel C: adrenocortical carcinoma stained for CITED2 showed strong nuclear expression of CITED2 (200×). Panel D: gel of RT-PCR analysis of mRNA from normal adult human adrenal cortex (lane 2) and from NCI-H295R cells (lane 3) the 69 bp fragments corresponded to the predicted amplification product of CITED2. As control for PCR we used RNA skipping the reverse transcription reaction (lane 1).

and reticularis at the cortico-medullary junction (Fig. 2B) whereas staining of cells in the zona fasciculata was somewhat weaker. In most cells, CITED2 immunoreactivity was located to the nuclei. Molecular studies using RT-PCR confirmed the expression of CITED2 on mRNA level in normal adult adrenocortical cells (Fig. 2D).

In addition, we could detect CITED2 expression in three out of four adrenocortical carcinomas using immunostaining techniques. CITED2 was distributed ubiquitously and expression was represented by a strong nuclear signal (Fig. 2C). Expression of CITED2 was also found in NCI-H295R cancer cells using RT-PCR (Fig. 2D).

CITED2 is regulated by bFGF

In NCI-H295R cells, promotor activity of CITED2 was increased by bFGF in a concentration-dependent manner when assayed after an incubation period of 48 h (Fig. 3A). Exposure of NCI-H295R cells to bFGF at concentrations of 10 ng/ml enhanced CITED2-promotor activity significantly. The stimulatory effect of bFGF could be fully blocked by co-incubation with the MEK1-inhibitor PD98059 at concenctrations of 20 μM. The results were confirmed using semiquantitative TaqMan PCR (Fig. 3B). Exposure of NCI-H295R cells to bFGF resulted in a marked and significant induction of CITED2-mRNA level in a concentration-dependent manner after 8 h of stimulation. The maximum level of induction after treatment with bFGF (10 ng/ml) varied between 15- and 38-fold above the unstimulated control.

Immunofluorescence technique demonstrated the induction of CITED2-protein by bFGF (Fig. 4). Exposure of NCI-H295R cells to bFGF (10 ng/ml) for 24 h resulted in an increased nuclear fluorescence signal. The co-inucbation of bFGF (10 ng/ml) with the MAPK-inhibitor PD98059 at concentrations of 20 μM attenuated CITED2 nuclear fluorescence signal.

CITED2 is regulated by forskolin

Incubation of NCI-H295R cells for 48 h in the presence of forskolin (10 μM) enhanced CITED2 promotor activity 1·8-fold in comparison to the unstimulated control (Fig. 3C). This observation was accompanied by a 3·5-fold increase in CITED2-mRNA level after exposure to forskolin (10 μM) for 8 h (Fig. 3C). In contrast to forskolin, ACTH (100 nM) did not exert a significant effect on CITED2-promotor activity, CITED2-mRNA or protein expression in NCI-H295R cells (Figs 3C and 4).

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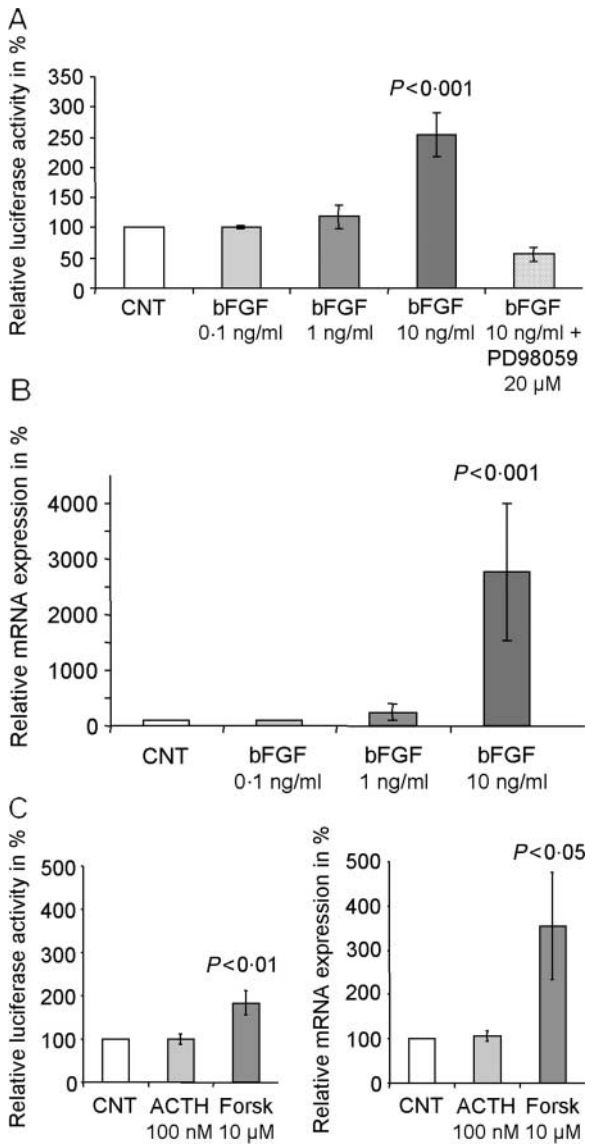

Figure 3 Regulation of CITED2-promotor activity and mRNA-level in NCI-H295R cells. Panel A: relative activity of the CITED2-promotor in NCI-H295R cells after exposure to bFGF in different concentrations either with or without PD98059 for 48 h. Panel B: relative levels of CITED2-mRNA in NCI-H295R cells after exposure to bFGF in different concentrations for 8 h. Panel C: effects of ACTH and forskolin (Forsk) on the CITED2-promotor activity after 48 h of stimulation (left panel) or on the CITED2-mRNA levels (right panel) after 8 h of stimulation in NCI-H295R cells. In all panels *P*-values indicate significant differences from the control (CNT).

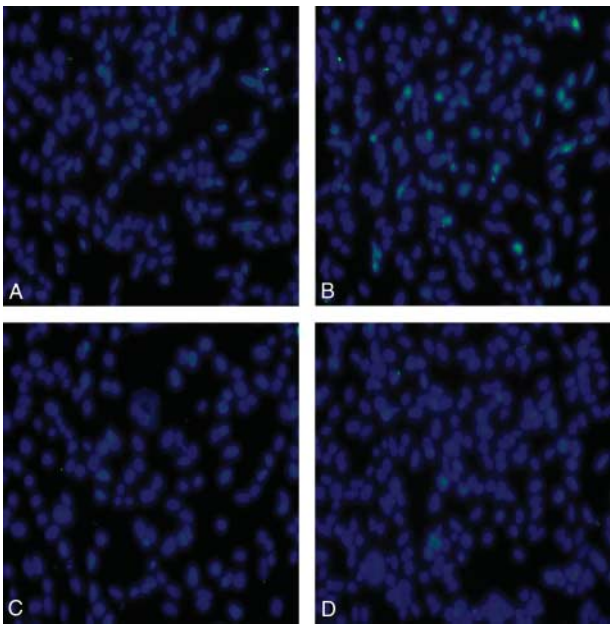

Figure 4 Immunofluorescence technique demonstrates CITED2 protein expression in NCI-H295R cells. Panel A: unstimulated control. Blue fluorescence dye DAPI marks nuclei. CITED2 protein is represented by a green fluorescence signal in the nuclei. Panel B: exposure of NCI-H295R cells for 24 h to bFGF (10 ng/ml) increased CITED2 nuclear expression in comparison with the control. Panel C: exposure of NCI-H295R cells to bFGF (10 ng/ml) in combination with PD98059 (20 µM) for 24 h attenuated CITED2 nuclear fluorescence signal in comparison with the stimulation with bFGF (10 ng/ml) alone. Panel D: exposure of NCI-H295R cells to ACTH (100 nM) for 24 h did not exert an effect on CITED2-protein expression in comparison with the control.

Discussion

CITED2 has been shown to be essential for the development of the adrenal gland of the mouse (Bamforth *et al.* 2001). In this study, we demonstrate that CITED2 also seems to play a role in the human adrenal gland.

The data provide the first evidence that CITED2 is expressed during early human adrenocortical organogenesis and that it is related to cells within the definitive zone. Though not studied by double-immunohistochemistry, the pictures obtained from the staining patterns in non-corresponding serial sections suggest that CITED2 expressing cells are cells other than chromogranin A positive cells. In concordance with these findings, the majority of normal adult adrenals also showed staining within the adrenal cortex: in the zona glomerulosa and in the zona reticularis at the corticomedullary junction. Interestingly, the definitive zone has been thought to represent a pool of proliferating and differentiating adrenocortical progenitor cells (Mesiano & Jaffe 1997, Muench *et al.* 2003, Ratcliffe *et al.* 2003).

Injection of CITED2-overexpressing cells in nude mice leads to tumor formation (Sun *et al.* 1998) and complementation with CITED2 enhanced proliferation in fibroblasts (Kranc *et al.* 2003) as well as in hepatocytes (Tien *et al.* 2004). Therefore, it seems possible that CITED2 also co-activates factors that are important for the regulation of proliferation and growth in adrenocortical cells. In this context, our data suggest that adrenal agenesis observed in CITED2$^{-/-}$ mice may develop as a result of primary dysfunction of adrenocortical precursor cells.

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In addition to our studies on the expression of CITED2 in adrenal tissues, we examined if CITED2 is controlled by factors important in the physiology of the adrenal gland. Many effects of ACTH are associated with the intracellular increase of cyclic AMP (cAMP). Here we demonstrate that forskolin, an activator of the adenylyl cyclase, increased both CITED2-promotor activity and mRNA levels.

In addition, we identified basic fibroblast growth factor as a potential candidate for CITED2 regulation since it is a potent mitogen for fetal and adult adrenocortical cells (Mesiano *et al.* 1991, Basile & Holzwarth 1993, Ho & Vinson 1997, Boulle *et al.* 2000) and since it is expressed in several adrenal tissues (Schweigerer *et al.* 1987, Westermann *et al.* 1990, Ho & Vinson 1995, 1997). Our *in vitro* experiments demonstrated that CITED2 promotor activity, mRNA- and protein levels were increased by bFGF in the NCI-H295R cell-line. The stimulatory effects of bFGF were dependent on the activity of MAPKs, a familiy of extracellular regulated kinases that control a multidude of cellular functions such as survival, proliferation and differentiation. Consistent with our findings MAPKs have been shown to constitute one of the intracellular signaling pathways that are activated by bFGF in adrenocortical cells (Lepique *et al.* 2000, Lotfi *et al.* 2000, Mattos & Lotfi 2005). Furthermore, the MAPKs are clearly linked to adrenal tumor formation. Therefore, CITED2 may also play a role in adrenal tumorigenesis. In this light, it is remarkable that CITED2 is also expressed in adrenocortical cancer.

In summary, our findings demonstrate that CITED2 is expressed in adrenocortical cells of the definitive zone of human embryonic adrenal glands, adult adrenal glands, and adrenal tumors. It is regulated by the cAMP pathway and by bFGF involving the MAPK-pathway. Therefore, similar to the murine model CITED2 is also relevant to human adrenal development and growth.

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