Novel mechanism of regulation of the DNA repair enzyme OGG1 in tuberin-deficient cells

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Tuberin (protein encoded by tuberous sclerosis complex 2, Tsc2) deficiency is associated with the decrease in the DNA repair enzyme 8-oxoG-DNA glycosylase (OGG1) in tumour kidney of tuberous sclerosis complex (TSC) patients. The purpose of this study was to elucidate the mechanisms by which tuberin regulates OGG1. The partial deficiency in tuberin expression that occurs in the renal proximal tubular cells and kidney cortex of the Eker rat is associated with decreased activator protein 4 (AP4) and OGG1 expression. A complete deficiency in tuberin is associated with loss of AP4 and OGG1 expression in kidney tumour from Eker rats and the accumulation of significant levels of 8-oxodeoxyguanosine. Knockdown of tuberin expression in human renal epithelial cells (HEK293) with small interfering RNA (siRNA) also resulted in a marked decrease in the expression of AP4 and OGG1. In contrast, overexpression of tuberin in HEK293 cells increased the expression of AP4 and OGG1 proteins. Downregulation of AP4 expression using siRNA resulted in a significant decrease in the protein expression of OGG1. Immunoprecipitation studies show that AP4 is associated with tuberin in cells. Gel shift analysis and chromatin immunoprecipitation identified the transcription factor AP4 as a positive regulator of the OGG1 promoter. AP4 DNA-binding activity is significantly reduced in Tsc2−/− as compared with Tsc2+/+ cells. Transcriptional activity of the OGG1 promoter is also decreased in tuberin-null cells compared with wild-type cells. These data indicate a novel role for tuberin in the regulation of OGG1 through the transcription factor AP4. This regulation may be important in the pathogenesis of kidney tumours in patients with TSC disease.

Introduction
Tuberous sclerosis complex (TSC) is a genetic disorder associated with renal cell carcinoma (1). Several studies have identified the TSC2 protein tuberin as an Akt substrate providing another plausible signaling pathway to place mammalian target of rapamycin (mTOR) downstream from Akt (2–5). Phosphorylation by Akt results in phosphorylation and inactivation of Tsc2 by several mechanisms, including degradation of the Tsc1–Tsc2 complex (6–11). Loss of heterozygosity at the Tsc2 locus has been detected in Tsc-associated renal cell carcinoma as well as in the sporadic forms of these tumours (12,13). In the Eker rat where the Tsc2 gene is mutated, the incidence of renal cell tumours in gene carriers approaches 100% by 1 year of age (14–16). The precise mechanism by which tuberin deficiency predisposes to renal carcinogenesis is yet not known.

Abbreviations: AP4, activator protein 4; ChIP, chromatin immunoprecipitation; dG, deoxyguanosine; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; 8-oxodG, 8-oxo-deoxyguanosine; OGG1, 8-oxoG-DNA glycosylase; siRNA, small interfering RNA; TSC, tuberous sclerosis complex.

Materials and methods

Cell cultures
Isolation and culture of primary proximal tubular cells. Primary proximal tubular cells were isolated and cultured following the method of Glynnie with minor modifications (40) from wild-type and Eker kidney rats. Renal cortical tissue was collected in cooled Hank’s balanced salt solution containing penicillin (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (0.125 μg/ml). After the capsule was removed, the cortex was cut into small pieces and the tissue fragments were suspended in 1 mg/ml (in Hank’s balanced salt solution) of type II collagenase (Worthington Biochemical, Lakewood, NJ) and incubated for 1 h at 37°C. The cells were centrifuged (200 g, 5 min, 4°C) and seeded into 75 cm2 tissue culture flasks that had been coated with collagen I. The cells were grown in serum-free medium (Dulbecco’s modified Eagle’s medium/F-12: glucose concentration, 17 mM) containing 15 mM N2-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer, 1-glutamine and pyridoxine hydrochloride. The medium was supplemented with epidermal growth factor (10 ng/ml), insulin (10 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (0.125 μg/ml). The cells were incubated at 37°C in humidified 5% CO2 in air.

Mouse embryonic fibroblasts. Mouse embryonic fibroblasts derived from Tsc2−/−, Tsc2−/+ and Tsc2+/+ embryos were generously provided by Dr D.J.Kwiatkowski (Harvard Medical School, Boston, MA). The cells were

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grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum. Cells were used between passages 2 and 6.

Human embryonic kidney epithelial cells. Human embryonic kidney epithelial cells (HEK293) were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO2.

Animals
Male wild-type and Eker rats (wild-type, Tsc-2+/+ and mutant Tsc-2−/−) were purchased from a breeding colony maintained in house at the University of Texas M. D. Anderson Cancer Center, Smithville, TX. The animals were allowed food and water ad libitum during the experiments. Animals were euthanized at 12 months for nephrectomy. Kidneys were quickly removed and dissected longitudinally, half preserved in 10% formalin in phosphate-buffered saline (PBS), pH 7.4 for immunostaining and the remaining tissues were snap frozen in liquid nitrogen for biochemical analysis. Tumour tissues from Eker rats were dissected for immunostaining and biochemical analysis. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio approved these animal studies.

Protein extraction and western blot analysis
Cell lysate and kidney tissue homogenates were prepared as described previously (37,38). Protein concentrations were determined with the Bradford assay (41). Cell lysate and kidney tissue homogenates were prepared as described previously (38). Tsc2−/−cell extracts with AP4 antibody using protein G-agarose according to manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Cells lysates were immunoblotted with tuberin and AP4 antibodies.

Electrophoretic mobility shift assay
Nuclear proteins were extracted from Tsc-2+/+, Tsc-2−/+ and Tsc-2−/−cells as described previously (38). The protein concentration of the nuclear extracts was determined using Bradford method (41). Electrophoretic mobility shift assay (EMSA)-binding reactions were incubated in a 20 μl final volume for 20 min at room temperature containing 5 μg of the nuclear extract, 20–30 fmol of the 32P-end-labelled double-stranded 21 bp oligonucleotide: 5'-AAAGC-GAGCAGCTGGCAGAGA-3' (covering the region of the hOGG1 promoter from −156 to −176 (control), and 1 μl of polyclonal antibody or protein G-agarose beads. The super shift assays were performed by pre-incubating nuclear extracts with 1 and 5 μg of AP4 antibody (Santa Cruz Biotechnology) into the reaction. In addition, labelled AP4 oligonucleotide at −163/−188 nt region with Mut-1 (aAGCTG), Mut-2 (CACGTTG) or Mut-3 (aAGCTG) was incubated with nuclear extracts of Tsc-2−/+cells. The reaction was carried out at room temperature for 30 min prior to adding the radiolabelled probe. Competition was performed in the presence of a 100-fold excess of the unlabelled oligonucleotides. The complexes were resolved using a 5% non-denaturing polyacrylamide gel. The gels were dried and exposed overnight at −70°C.

Chromatin immunoprecipitation assay
The assay was performed using a ChIP kit (Active Motif, Carlsbad, CA). Briefly, HEK293 cells grown in 100 mm dish were cross-linked using 1% formaldehyde (38). After washing twice with PBS, cells were resuspended in lysis buffer containing protease inhibitor cocktail. DNA was digested (5 min), and all fragments ranged from 100 to 500 bp by enzymatic method. The supernatant was then pre-cleared using protein G-agarose beads. After centrifugation, supernatant was incubated with either anti-AP4 antibody or control IgG for overnight. Incubation was further carried out with protein G-agarose beads for 2 h and beads were washed with buffers provided in the kit. The immunoprecipitated DNA was eluted from the beads with 1% sodium sulphate dodecyl and 1 M NaClO4 solution and reverse cross-linked using 5 M NaCl and RNase A at 65°C for overnight. Samples were then digested with proteinase K and DNA was purified using column method. Polymerase chain reaction (PCR) was performed on the purified DNA using hOGG1 promoter-specific primers forward (−209/−228) (5'-CCAGATGGAACCTTGAAGG-3') and reverse (−9/−29) (5'-CCGACCCAAGCACCCGGA-3').

Transcriptional activity of OGG1 promoter
The reporter plasmid consists of the OGG1 promoter with luciferase as reporter gene [courtesy of Dr Bioteux (43)] was used to determine the transcriptional activity of OGG1 promoter in mouse embryonic fibroblasts cells (38). Plasmids were transfected into HEK293 cells using the LipofectAMINE and Plus ReagentTM method (Invitrogen, Life Technologies, Carlsbad, CA). The cells were grown in six-well plates to 60–70% confluence. Prior to transfection, cells were washed twice with PBS and media was replaced with 1 ml of OPTI-MEM I (Invitrogen). Pre-complex of the DNA with Plus ReagentTM in Opti-MEM was mixed and incubated at room temperature for 15 min. LipofectAMINE was added to the complex of DNA and Plus ReagentTM and incubated for 15 min at room temperature. DNA and Plus ReagentTM/LipofectAMINE complexes were added to each well and incubated at 37°C with 5% CO2. After incubation for 4 h, 1 ml of fresh media with 20% serum was added to a final concentration of 10%. Cells were harvested after 48 h of transfection. Cells were washed twice with PBS and lysed in 0.2 ml of lysis buffer. Luciferase activity was determined using the Luciferase Reporter Assay System by 800 μl of OPTI-MEM I (Invitrogen, Carlsbad, CA). In parallel, 4 μl of oligo-fectametase (Invitrogen) were combined with 11 μl of OPTI-MEM I and incubated at room temperature for 10 min. SMART selected small interfering RNA (siRNA) duplexes of Tsc2 or AP4 with ‘HU’ overhangs and 5′ phosphate on the antisense strand. The siRNA specific for Tsc2 and AP4 were a mixture of two different DNA duplexes. Tsc2 siRNA kit from Santa Cruz Biotechnology. According to the manufacturer, these siRNA efficiently blocks tuberin and AP4 expression by 50–70%. The indicated duplex of 1.5 μg were diluted into 180 μl of OPTI-MEM I, added to the Oligofectamine/OPTI-MEM I mixture and incubated and then harvested at room temperature for 20 min (38). The siRNA complexes were then added to the cells. After incubation for 3–4 h in 5% CO2, 1 ml of fresh medium was added to a final serum concentration of 10%. Forty-eight hours after transfection, cells were harvested for western blot analysis. The control construct used in parallel experiments contains four pooled non-specific siRNA duplexes provided by with the kit (42).
a luminescent reading device according to the manufacturer's instructions (Promega, Madison, WI) and normalized to protein content.

**Mutations in the AP4-binding site of the OGG1 promoter**

Plasmids containing mutations at AP4-binding site in OGG1 promoter were constructed using PCR followed by cloning of PCR products into pGL3-basic vector (Promega, Madison, WI). Briefly, two forward primers containing a KpnI site at 5' and sequences −171 to +74 of OGG1 promoter with mutations were synthesized: AP4 no mutation, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3'. AP4 Mut 1, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3', AP4 Mut 2, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3' and AP4 Mut 3, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3'. (Nucleotide bases in bold are referring to the mutation sites). A reverse primer containing a HindIII site at 3' sequences corresponding to pGL3 basic vector was also synthesized: 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3'. The PCR products were digested with KpnI and HindIII and ligated into pGL3 basic vector using T4 ligase (Promega, Madison, WI). Briefly, two forward primers containing a KpnI site at 5' and sequences −171 to +74 of OGG1 promoter with mutations were synthesized: AP4 no mutation, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3'. AP4 Mut 1, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3', AP4 Mut 2, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3' and AP4 Mut 3, 5'−CATGCTGATCCGAGCAGCTGGCAAGAGCCCA-GTGGCAGAGAGCCCAGTGCCGGCCA-3'. (Nucleotide bases in bold are referring to the mutation sites). The mutations in the constructs were confirmed by DNA sequencing. The reporter plasmids with the OGG1 promoter driving firefly luciferase were used to determine the transcriptional activity of OGG1 promoter with and without mutations at AP4-binding site. Plasmids were transfected into mouse embryonic fibroblasts cells using the LipofectAMINE and Plus ReagentTM method (Invitrogen, Life Technologies) as described above.

**Statistics**

Data are presented as mean ± standard error. Statistical differences were determined using analysis of variance followed by Student–Dunnett's (Experiment versus Control) test using 1 trial analysis. P-values <0.01 were considered statistically significant.

**Results**

**Tuberin deficiency is associated with downregulation of AP4 and OGG1 expression in primary proximal tubular cells of the Eker rat**

To determine if deficiency in tuberin is associated with decreased AP4 and OGG1 expression, we performed western blot analysis on cell lysates of proximal tubular cells isolated from kidneys of Eker and wild-type rats. Decreased tuberin expression was associated with decreased AP4 and OGG1 expression in primary cells derived from normal Eker rats compared with wild-type cells (Figure 1A&B).

**Deficiency of tuberin is associated with the loss of AP4 and OGG1 expression in kidney tumour of Eker rat**

To determine if tuberin deficiency in kidney tumour of Eker rat is associated with downregulation of AP4 and OGG1 expression, kidney cortex homogenates from wild-type, normal and tumour of Eker rats were analysed by western blot. Tuberin expression in kidney tumours of Eker rats is significantly reduced compared with normal kidney tissue from wild-type rat and 6-fold decrease from wild-type cells is indicated by *P < 0.01.

**Loss of OGG1 is associated with significant accumulation of 8-oxodG**

The basal levels of 8-oxodG as measured by high-performance liquid chromatography were 2-fold higher in kidney cortex of Eker rat compared with wild-type rat (Figure 3). In addition, tumour kidney of Eker rat is associated with an 8-fold accumulation of 8-oxodG compared with normal kidney tissue from wild-type rat and 6-fold compared with kidney tissue of Eker rats (Figure 3A). Immunohistochemistry of 8-oxodG confirmed that significant accumulation of 8-oxodG in tumour kidney from Eker rat compared with normal kidney from Eker and wild-type rats (Figure 3B). The histological appearance of haematoxylin- and eosin-stained kidney sections of wild type showed normal tubular architecture of kidney. Tumours containing cells with clear cytoplasm and eccentric in large nuclei of clear cell carcinoma type were appeared in the kidney section of Eker rat (Figure 3C).

**Tuberin deficiency decreases AP4 binding to OGG1 promoter**

To further investigate the mechanism by which tuberin deficiency affects OGG1 expression, EMSAs were performed to determine if
To determine the role of the AP4-binding site on OGG1 promoter activity, cells were transfected with AP4 mutant and wild-type reporter plasmids (Figure 5A). Mutation of the first ‘C’ of the AP4-binding site (CAGCTG to AAGCTG, AP4-Mut 1) had no effect on the OGG1 promoter activity. Mutation of the second C (CAGCTG to CAGATG, AP4-Mut 2) significantly inhibited OGG1 promoter activity (Figure 6) as did mutation of both Cs of the AP4 motif from CAGCTG to AAGATG (AP4-Mut 3) (Figure 6). These results strongly suggest that the potential AP4-binding site at −163/168 is important for the OGG1 promoter activity.

Discussion

These data comprise the first report to provide that tuberin regulates OGG1 expression through the transcription factors AP4 in cultured cells and in rat kidney cortex. Several approaches were utilized to study the potential role of tuberin in the regulation of OGG1. First, we showed that decreased tuberin expression is associated with decreased AP4 and OGG1 expression in renal proximal tubular cells isolated from Eker rat as compared with wild-type cells. Second, decreased tuberin expression is associated also with decreased AP4 and OGG1 expression as well as significantly increased accumulation of 8-oxodG in kidney cortex of Eker compared with wild-type rat. In the kidney tumours of Eker rat, loss of tuberin is associated with loss of AP4 and OGG1 expression and an 8-fold increase in 8-oxodG compared with kidney tissue of wild-type rat. Third, we show that reduction of tuberin expression in human epithelial cells (293) using siRNA resulted in a marked decrease in the expression of OGG1 and AP4. Fourth, introduction of Tsc2 complementary DNA into tuberin-deficient cells restored OGG1 and AP4 expression. In addition, reduction of AP4 using siRNA results in significant decrease in OGG1 protein expression. Our data also show that AP4 is associated with tuberin in cells. We investigated the mechanism by which tuberin regulates OGG1 by gel shift analyses and ChIP and identified the transcription factor AP4 is a regulator of OGG1 promoter. Furthermore, we found that the DNA-binding activity of AP4 was significantly reduced in Tsc2−/− compared with Tsc2+/+ cells. In addition, the transcriptional activity of the OGG1 promoter was also decreased in Tsc2−/− cells. We find also that mutation(s) in AP4-binding region significantly inhibited OGG1 promoter activity suggesting that AP4 in an important enhancer of OGG1 promoter activity.

Tuberin expression in kidney from Eker rats is significantly reduced compared with expression in normal kidney tissues from wild-type littersmates (37). In the current study, we show that loss of tuberin was associated with abolished AP4 and OGG1 expression as well as accumulation of significant levels of 8-oxodG in the kidney tumours of Eker rats. Downregulation of tuberin using siRNA directed specific for the Tsc2 gene in 293 human renal epithelial cells was associated with decreased in AP4 and OGG1 expression. In addition, overexpression of tuberin by adenovirus (Ad-Tsc2) shows an increased in AP4 and OGG1 expression, indicating that tuberin is upstream of AP4 and OGG1. The immunoprecipitation analysis data of AP4 show that tuberin co-precipitates with AP4 indicating that AP4 is associated with tuberin in cells.

AP4 is an ubiquitously expressed transcription factor. It binds to the consensus sequence ‘CAGCTG’ (34). AP4 recognizes specific binding sites in the promoter as well as enhancer sequences of viral genes (34). The enhancer activity is abolished by mutations in these regions that also affect binding of AP4 in vitro (34). AP4 has recently been identified as a transcriptional repressor of HIV-1 (35). In the present study, we screened for enhancer-binding proteins to explore the molecular mechanisms that regulate OGG1 gene expression. We found that the OGG1 promoter contains a putative binding site for AP4 close to 3’ of the promoter region (−163/−168 nt).

Further investigation to test the role of tuberin in the regulation OGG1 by AP4 was performed by EMSA and ChIP analysis. Our data showed that AP4 binding was significantly lower in Tsc2−/− and Tsc2+/− cells compared with Tsc2+/+ cells. In addition, incubation of nuclear extracts with AP4-specific antibodies was competed the protein–DNA complex demonstrate that the Tsc2+/− cells contain a protein, AP4, that binds specifically to a region in the OGG1 promoter.
promoter. In addition, mutations of oligonucleotide (CAGαTG and aAGaTG) at −163/−168 nt region of AP4 show significant decrease in the protein–DNA complex. ChIP analysis further provided confirmation that Ap4 binds the OGG1 promoter at sites located at −163/−168 nt region. Collectively, the data indicate decreased binding of AP4 to the OGG1 promoter in tuberin-deficient cells.

**Fig. 3.** Significant increase in 8-oxodG levels in kidney tumour of Eker rat. (A) DNA was extracted from normal kidney of wild-type and Eker rat as well as from kidney tumour of Eker rat. Detection of dG and 8-oxodG was performed by High Pressure Liquid Chromatography-Electrochemical analysis. Authentic standards of 8-oxodG and dG were analysed simultaneously. Standard curves for dG and 8-oxodG were prepared and quantitated by linear regression analyses. Histograms represent means ± SE of three animals per group. Significant difference between wild-type, Eker rat and tumour of Eker rat is indicated by **P < 0.01. (B) These data confirmed by immunohistochemistry in kidney sections from wild-type and Eker rat as well as from kidney tumour of Eker rats. FITC for 8-oxodG (green color) was detected with excitation wavelengths at 450–490 nm. (C) Haematoxylin and eosin staining of kidney sections of wild type showed normal tubular architecture. Kidney sections of Eker rat show atypical tubular (AT) in dark color and tumors (T) containing cells with clear cytoplasm and eccentric in large nuclei of clear cell carcinoma type.
In addition, the mutations in consensus sequence of AP4 motif (CAGCTG to CAGATG) significantly inhibited OGG1 promoter activity strongly suggest that CAGCTG, at which AP4 is purported to bind to OGG1 promoter, is important for the OGG1 promoter activity.

In summary, these data indicate that tuberin regulates the OGG1 and this effect is at least partially through the transcription factor AP4. The transcription factor AP4 is an important regulator of the OGG1 promoter in the cell cultured and kidney tissue. Accumulation of significant levels of 8-oxodG in tumour kidney of Eker rat indicate...
that tuberin plays a significant role in protecting the cells from oxidative DNA damage. It is probably that loss of tuberin results in loss of AP4 and OGG1 expression and function in that may expose the cells to further genetic alterations and leading to accumulation of mismatched DNA base lesions, a form of genomic instability that if not repaired could accelerate further genetic alterations leading to the full-blown tumour phenotype in kidneys. These data indicate a novel role for tuberin in the regulation of DNA repair pathway through the transcription factor AP4 and provide a potential mechanism of Tsc2 and OGG1 in progression of kidney tumours.
expression of the luciferase gene was transfected into the Tsc2+/− and Tsc2−/− cells. The reporter plasmid containing OGG1 promoter (−171/−74) that drive the expression of the luciferase gene was transfected into the Tsc2−/− and Tsc2+/− cells. Forty-eight hours after transfection, luciferase activity was determined using the Luciferase Reporter Assay System. pGL3 basic vector was used as a control to normalize luciferase activity (1-fold). Mut-2 and Mut-3 show significant decrease of the OGG1 promoter activity compared with Mut-1. Histograms represent means ± SE from three experiments.

References

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