LLC-PK₁ Cell Growth Is Repressed by WT1 Inhibition of G-protein α₁-2 Protooncogene Transcription* 

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The temporal expression of the early growth response gene (EGR-1) is one molecular mechanism for both maximal activation of the Gα₁-2 gene and accelerated growth in mitotically active predifferentiated LLC-PK₁, renal cells. These events are dependent on an enhancer area in the 5'-flanking region of the Gα₁-2 gene that contains an EGR-1 motif (5'-CGCCCGCCG-3'). However, acquisition of the polarized phenotype in LLC-PK₁ cells is accompanied by loss of EGR-1 expression and occupancy of the EGR-1 site by nuclear binding proteins other than EGR-1. We now demonstrate that one of these binding proteins is the Wilms' tumor suppressor (WT1). Furthermore, the temporal expression of WT1 in LLC-PK₁ cells acquiring the polarized phenotype represses both Gα₁-2 gene activation and growth in these cells. These findings suggest the existence of differentiation-induced pathways in LLC-PK₁ cells that alternatively abrogate EGR-1 and promotes WT1 gene expression, thereby modulating a target protooncogene Gα₁-2, that is participatory for growth and differentiation in renal cells. These studies emphasize the usefulness of the LLC-PK₁ renal cell as a model to elucidate normal programs of genetic differentiation in which WT1 participates.

Wilms' tumor (WT) is a pediatric nephric neoplasm arising from the continued proliferation of embryonic blastemal cells that fail to differentiate (1). WT occurs in both sporadic and hereditary forms and as part of the WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) (2). Chromosome 11p13 contains a region encoding the Wilms' tumor (WT) gene that is commonly deleted in heritable WT (3, 4). Internal deletions and mutations of the WT1 gene are found in some WTs (reviewed in Ref. 5). Although WT1 is expressed in uterus, spinal cord, spleen, abdominal wall musculature, and the mesothelial lining of organs within the thoracic cavity, its highest expression is in the developing urogenital system (6). The WT1 gene encodes four splice variants of a transcription factor containing a glutamine/proline-rich N terminus and four carboxyl Cys₂His₂ type zinc fingers, which recognize 5'-GGGCGCGTGTG-3' and redundant 5'-TCC-3' cis motifs (7-9). Alternative splice I allows the variable insertion of exon 5 between the transactivating and DNA binding domains of WT1. Alternative splice II allows the variable insertion of nine nucleotides encoding lysine, threonine, and serine (+KTS or −KTS) between the third and fourth zinc fingers (7). WT1 cis motifs are also recognized by another Cys₂His₂ type zinc finger transcription factor EGR-1 also known as NGFI-A, Krox 24, TIS-8, and Zif 268, which is induced after mitogenic and differentiation cues in several cell types (reviewed in Ref. 10). In contrast to EGR-1, WT1 appears to predominantly function as a transcriptional repressor of several growth-related genes including EGR-1, insulin-like growth factor II, platelet-derived growth factor α chain, insulin-like growth factor receptor, colony-stimulating factor 1, transforming growth factor β1, and Pax2 (reviewed in Ref. 11). WT1 mutations prevent DNA binding or transcriptional repressive functions that are postulated to allow the constitutive expression of growth factors leading to renal neoplasia. Consistent with this possibility, suppression of colony formation occurs in WT cells transfected with wild type WT1 isoforms (12).

Further insights into molecular cascades linking WT1 to target genes in renal cells would clearly be advantageous. WT's occur with greater frequency in pediatric kidneys containing persistent renal stem cells, a condition designated nephroblastosamia or nephrogenic rests. WT1 mutations occur in these rests, suggesting they may represent a transitional cell preceding malignancy (13). Embryonal kidney cell tumors reminiscent of WT can be induced in rats given the alkylation agent N-nitroso-N'-methyl urea (14). However, cultured cells have yet to be developed from these sources. We have previously identified genes participatory for epithelial cell growth and differentiation events in LLC-PK₁, cells, an extensively characterized cultured epithelial cell line derived from juvenile male pig kidney (15). These studies indicated that as in lower eukaryotic organisms such as Dictyostelium (16-18) and Droso-philina (19), heterotrimeric guanine nucleotide binding (G) proteins are involved in signal transduction pathways required for both growth and cellular differentiation programs in renal cells.

G proteins are composed of individual α, β, and γ subunits that are encoded by gene superfamilies that have been conserved by eukaryotes throughout evolution (reviewed in Ref. 20). Most of the transducing activities of G proteins in mammalian cells are associated with the state of activation of the α subunit, which is involved in GDP/GTP exchange and GTP

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§ The abbreviations used are: WT, Wilms’ tumor; PBS, phosphate-buffered saline; bp, base pair(s).
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hydrolysis (reviewed in Refs. 21 and 22). G proteins can alter cell growth or differentiation by participation in growth factor receptor signaling pathways that converge in the nucleus to alter gene expression. Mutations in Gαi2, comparable with Ha-ras GP21, which decrease GTPase activity, are found in tumors of the adrenal cortex and ovary (23). Such mutations, which convert the Gαi2 gene into the oncogene gip2 induce increased growth and oncogenic transformation in Rat-1a cells (24). Increased growth may be a consequence of persistent activation of pathways coupled to mitogen-activated protein kinase (25). The Gαi2 subunit interacts with pathways required for differentiation of F9 teratocarcinoma cells (26). Even modest repression of Gαi2 expression is associated with renal developmental and morphologic abnormalities in transgenic mice, underscoring its important role in renal differentiation events (27). Polarized LLC-PK1 cells contain two Gα isoforms, Gαs and Gαi2, which are involved, respectively, in the regulation of hormone-stimulated adenyl cyclase and constitutive proteoglycan secretion through the Golgi complex (28, 29). The genes encoding both Gα subunits are transcriptionally activated in these cells in a coordinated manner during growth and differentiation but differ in response to glucocorticoids and cAMP (30–32). We recently determined in mitotically active predifferentiated LLC-PK1 cells that the temporal expression of EGR-1 is one molecular mechanism for both maximal activation of the Gαi2 gene and accelerated growth in these cells. These events were dependent on an enhancer area in the 5′-flanking region of the Gαi2 gene that contains an EGR-1 motif (5′-CGCCCCCCCG-3′) (33). Notably, acquisition of the proliferative phenotype in LLC-PK1 cells was accompanied by loss of EGR-1 expression and occupancy of the EGR-1 site by nuclear binding proteins other than EGR-1. In the present study we determine whether one of these binding proteins is WT1.

EXPERIMENTAL PROCEDURES

Cell Culture Cells

Wild type LLC-PK1 cells are a polarized epithelial cell line derived from pig kidney. Cells were grown as confluent monolayers and maintained in Dulbecco’s modified Eagle’s medium containing 10 or 0.1% fetal calf serum in a 5% CO2 atmosphere as described previously (15). Cells were plated at a density of 1 × 106/cm2, achieving confluence at approximately culture day 7.

Cellular Transfections

Plasmids—pRSV (WT1) and pRSV (Gαi2) are plasmids containing, respectively, the entire coding sequence of human WT1 and the entire coding sequence of rodent G-protein αi2 subunit driven by a Rous sarcoma virus promoter. Two plasmids m-14 LUC or m-4 LUC containing or lacking a putative EGR-1 binding sequence (5′-CGCCCCCCCG-3′) were generated from nested deletions of plasmid 10–4 LUC (XmalII), which contains an αi2 5′-flanking sequence fused to a firefly luciferase reporter gene as described previously (30). A third plasmid-mutated m-14 LUC that replaced the nucleotides 1–5 of the EGR-1 site with adenosines was generated by polymerase chain reaction mutagenesis as described previously (33) utilizing the mutagenizing primer, 5′-CGGCCGTACGAGATCCGCCAAAAACCGCCGTCGGGCAGCGGAG-3′ (34). These plasmids were used to generate a plasmid containing an αi2 coding sequence of rodent G-protein αi2 Protooncogene Transcription.

Transient Transfections—Plasmids were transfected in equimolar amounts into LLC-PK1 cells by calcium phosphate precipitation as described previously (30). Optimum transfection efficiency was obtained by the addition of 20 μg of total plasmid DNA/5-mm2 p10 plate (Falcon) followed by incubation for 20 h without glycerol shock. When required, this amount of DNA was achieved by the addition of “carrier plasmid” Bluescript II KS+. Transfection efficiency was normalized by co-transfection with 2.5 μg of pSV2Apop, a plasmid carrying a human placental alkaline phosphatase reporter gene driven by a Rous sarcoma virus promoter (generously provided by T. Kadesch, University of Pennsylvania).

Transfection Assays—Forty-eight to 98 h after transfection, LLC-PK1 cells were washed twice in phosphate-buffered saline (without calcium or magnesium) and then lysed by addition of 1.0 ml of lysis buffer A (1% Triton, 25 mM glycyglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, and 1% fresh dithiothreitol). Scraped lysates were transferred to Eppendorf microfuge tubes and centrifuged at 10,000 × g for 5 min at 4°C. The supernatants were transferred to fresh Eppendorf tubes and briefly vortexed prior to each assay. In some experiments the cell number of each plate was determined by direct cell count of trypsinized cells utilizing inverted phase microscopy.

Firstly Luciferase and Human Placental Alkaline Phosphatase Assays—These were performed as described previously (30). Results are expressed as percent increase ± S.E. in luciferase activity normalized for heat-insensitive alkaline phosphatase activity. Data were analyzed by the paired Student’s t test.

Protein Assay—This was performed by the dye binding assay of Bradford as described by the manufacturer (Bio-Rad).

Mobility Shift Assays

Nuclear Extract Preparation—Nuclear proteins were extracted from LLC-PK1 cells as described previously (33).

Binding Assays—6 μg of nuclear extract was preincubated for 30 min in the presence of 32P end-labeled double-stranded DNA, 4–6 μg of poly(d-lc), 140 μg KCl, 9% glycerol, 18 μg Tris, pH 7.3, and 1 μg EDTA at 4°C. Complexes were separated on 5–6% polyacrylamide gel with 0.1% SDS for 2 h at 4°C. Complexes were visualized by autoradiography. The mobility shift complexes were cut out and washed with ethanol to remove free labeled DNA bands.

RNA Gel Blots

RNA from LLC-PK1 cells was separated by electrophoresis in 1.0% formaldehydeagarose gels and then transferred to GeneScreen Plus membranes (DuPont NEN). Membranes were prehybridized for 2 h at 42°C in the presence of 1% SDS, 1 μl NaCl, 10% dextran sulfate, and 50% deionized formamide. Hybridization was performed under similar conditions for 24 h in the presence of a human WT1 plasmid labeled with [α-32P]ATP by priming with random hexamers followed by extension of these primers with the Klenow fragment of DNA polymerase (30). After hybridization, membranes were washed twice for 30 min each in 0.3 M NaCl, 0.03 M sodium citrate at 23°C, then in the same buffer with 1% SDS at 65°C for 5 min followed by 15 min NaCl, 1.5 M sodium citrate at 65°C. The membranes were dried and autoradiographed with Kodak XAR film at −80°C for 6–96 h with or without Cronex Lightning Plus intensifying screens. Quantification of hybridization signals was performed by densitometry of the autoradiograms with an LKB ultroscan XL enhanced laser densitometer.

Immunoblotting and Immunofluorescence of EGR-1 and WT1

LLC-PK1 cells were washed twice in phosphate-buffered saline (without calcium or magnesium) and then lysed by addition of 1.0 ml of lysis buffer A. Scraped lysates were solubilized by boiling in sample buffer (1% SDS, 30 μl Tris, pH 6.8, 12% glycerol) and loaded onto a 10% acrylamide gel with 150 μg of protein loaded per lane. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred onto Immobilon membrane (Millipore), and the membrane was then stained with Coomassie Blue to ensure that all lanes contained equivalent amounts of transferred protein. The destained membrane was then blocked in blotting buffer (5% nonfat dry milk in 20 μM Tris, pH 7.4, with 0.15 mM NaCl and 3% Triton X-100), incubated with either preimmune mouse IgG or mouse monoclonal H7 antibody (which detects the non-zinc finger region of the EGR-1 protein corresponding to residues 29–117) or rabbit polyclonal α6F or murine monoclonal H7 antibody (which detects the 173-residue amino-terminal non-zinc finger region of the WT1 protein) diluted 1/1000 in blotting buffer and washed. In other experiments cell lysates were initially reacted with these antisera followed by precipitation with protein A prior to electrophoresis as described previously (8). EGR-1 and WT1-bound proteins were reacted with an enhanced chemiluminescent detection system as described by the manufacturer (Amersham Corp.) followed by autoradiography.

LLC-PK1 cells plated on glass coverslips were fixed for immunofluorescent staining on Days 1–7. Cells were fixed in 4% paraformaldehyde for 1 h, permeabilized in Triton X-100 for 4 min, and then incubated in PBS containing 0.1% bovine serum albumin for 5 min to reduce non-specific background staining. The cells were incubated for 2 h in anti-EGR-1 Ab 21 (alpha 1) or anti-WT1 antibody 7H2, preimmune rabbit IgG, or non-immune murine ascites at 1:50 or 1:100 dilutions, washed three times in 0.1% bovine serum albumin in PBS, and then incubated for 1 h with goat anti-rabbit or anti-murine IgG conjugated to fluorescein.
iso-thiocyanate (Kirkegaard and Perry). Cells were washed three times in PBS, then mounted in 100 mM Tris-HCl:glycerol, 50:50, 2% isothiocyanate (Kirkegaard and Perry). Cells were washed three times (Pharmacia Biotech Inc.).

During culture, LLC-PK₁ cells differentiate from a rounded cell type to a fully polarized epithelium. Prior to their polarization and tight junction formation, these cells undergo several rounds of cell division coincident with the maximal EGR-1 expression and activation of the Gᵢ2 subunit. (31). To determine whether the WT₁ gene also participates in these events a full-length human WT₁ cDNA and a polyclonal (α6F) or monoclonal (H7) antibody to the 173-residue amino-terminal non-

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Contributing to the temporal transcriptional repression of the WT1 protein. These data suggested that the WT1 protein was intact during the activation of the G_{αi-2} gene. This region contains a binding site (5'-CGCC-CCCCGC-3') for the EGR-1 transcription factor that provides a genomic signaling pathway for mitogenesis (33). To assess whether WT1 was repressing cell growth by repressing maximal transcriptional activation of the G_{αi-2} gene, cells were co-transfected with the pRSV WT1 plasmid, and plasmids encoding firefly luciferase reporter genes fused to 5'-flanking areas of the G_{αi-2} gene with (M14) or without the EGR-1 site (mutated M14 and M4). As seen in Fig. 3, a 60% repression of G_{αi-2} transcription was only found in renal cells following their transfection with the M14 plasmid that contained both an intact EGR-1 binding site and also overexpressed a functional WT1 protein. These data suggested that the WT1 protein was contributing to the temporal transcriptional repression of the G_{αi-2} gene in LLC-PK1 cells during culture.

To determine whether the WT1 protein was directly contributing to the activation of the G_{αi-2} gene, a double-stranded 23-bp DNA segment derived from the 5'-flanking sequence of the gene, which also contained the EGR-1 consensus sequence (5'-ATCCGCC CGCCCGCCCGCGC-3'), was synthesized and end-labeled for direct binding studies in mobility shift assays. Nuclear extracts from LLC-PK1 cells that were either actively dividing and non-polarized (culture day 1) or LLC-PK1 cells that were relatively quiescent and fully polarized (culture day 7) were examined. As seen in Fig. 4, the binding patterns of nuclear extracts from culture day 1 were different from those on culture day 7. Nuclear extracts from day 7 cells consistently demonstrated an additional faster mobility complex. We previously identified the EGR-1 protein as a predominant component of nuclear binding proteins in day 1 dividing cells but not day 7 quiescent cells (33). Based on our immunochemical studies it would be anticipated that the WT1 protein should be present in nuclear extracts of quiescent fully polarized LLC-PK1 cells on culture day 7. To determine whether WT1 was one of the proteins interacting with the 23-bp probe, nuclear extracts from culture days 1 and 7 were preincubated with H7 antibody. Following electrophoresis, retarded mobility of only the additional complex in nuclear extracts from culture day 7 was found. The specificity of this interaction was demonstrated by competition with the 28-kDa protein expressing the amino-terminal 173 residues of the WT1 protein. These data demonstrate that the WT1 protein was one component of these nuclear complexes. Detectability of the WT1 protein was consistent with its pattern of maximal expression in quiescent polarized LLC-PK1 cells that also have a corresponding repression of the G_{αi-2} gene.

These findings suggest the existence of differentiation-induced pathways in LLC-PK1 cells that alternatively abrogates EGR-1 and promotes WT1 gene expression, thereby modulating a target protooncogene G_{αi-2} that is participatory for growth and differentiation in these renal cells. These studies emphasize the usefulness of the LLC-PK1 renal cell as a model to elucidate normal programs of genetic differentiation in which WT1 participates. Further examination these pathways may provide significant insights into the molecular events involved in renal hypertrophy, nephrogenesis, and oncogenesis.

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