Growth of LLC-PK1 Renal Cells Is Mediated by EGR-1 Up-regulation of G Protein α2-i-Protooncogene Transcription*

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The early growth response zinc finger transcription factor (EGR-1) and the heterotrimeric guanine nucleotide binding protein encoded by the protooncogene Gai-2 each play pivotal roles in signaling pathways that control cell growth and differentiation. The Gai-2 gene 5′-flanking region contains a putative binding site (5′-CCGCCCCGGCC-3′) for EGR-1 that may allow it to be a target gene for EGR-1 mitogenic signaling. We now demonstrate in LLC-PK1 renal cells the temporal expression of EGR-1 protein by immunoblotting and immunochemistry coincident with the maximal activation of the Gai-2 gene during cell growth. To determine whether Gai-2 or EGR-1 influence epithelial cell growth, LLC-PK1 cells were transiently transfected with plasmids encoding cDNAs for Gai-2 (pRSV Gai-2) or EGR-1 (pRSV EGR-1) driven by a viral Rous sarcoma promoter enhancer to overexpress each protein. Following transfection, cell growth was examined in media containing either 10 or 0.1% fetal bovine serum. Only cells transfected with plasmids encoding Gai-2 and EGR-1 had growth rates greater than that of serum replete cohorts. To assess whether EGR-1 was contributing to the transcriptional activation of the Gai-2 gene, cells were cotransfected with pRSV EGR-1 and plasmids encoding firefly luciferase reporter genes fused to 5′-flanking areas of the Gai-2 gene containing either the EGR-1 binding site or a mutated EGR-1 binding site (5′-AAAAACCCGC-3′). A 320% enhancement of Gai-2 transcription was found only in LLC-PK1 cells following their transfection with plasmids that contained both the EGR-1 binding site and overexpressed EGR-1 protein. Utilizing mobility shift assays, which compared nuclear extracts from cells before and after cell polarization, a probe containing the EGR-1 motif detected induced nuclear protein complexes during transcriptional activation of the Gai-2 gene. An anti-EGR-1 antibody specifically retarded the mobility of the induced nuclear complexes, indicating that the EGR-1 protein was a component of these complexes. These data provide direct evidence for a novel mitogenic signaling pathway coupling proximal signaling events that activate EGR-1 gene expression to a target protooncogene Gai-2 that is participatory for growth and differentiation in renal cells.

Heterotrimeric guanine nucleotide binding proteins (G proteins) play an essential role in transmembrane signaling by coupling receptors with enzyme and ion transport processes in mammalian cells (1). These regulatory proteins are composed of individual α, β, and γ subunits that are encoded by super gene families that have been conserved by eukaryotes throughout evolution (2). 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 hydrolysis (3).

Recently, it has been established that G proteins alter cell growth or differentiation by participation in growth factor receptor signaling pathways that converge in the nucleus to alter gene expression. Pertussis toxin, which decouples a subset of G proteins (Gαi), attenuates both Gαi inhibition of adenylyl cyclase in platelets (4) and mitogenic responses to serum, thrombin, bombesin/gastrin-activating peptide, and vasopressin (5). Both pertussis toxin effects can be mimicked by antibodies to Gαi2 subunits (6). Mutations in Gαi2 comparable with Hras G21P that decrease GTPase activity are found in tumors of the adrenocortical cortex and ovary (7). Such mutations, which convert the Gαi2 gene into the oncogene gip2, induce increased growth and oncogenic transformation in Rat-1 cells (8). Increased growth may be a consequence of persistent activation of pathways coupled to mitogen-activated protein kinase (9). In both Dicyostelium (10, 11, 12) and Drosophila (13), differentiation events are dependent on G protein-α subunit expression. Similarly, it has been recently demonstrated that the Gαi2 subunit interacts with pathways required for differentiation of F9 teratocarcinoma cells (14). Importantly, even modest repression of Gαi2 expression is associated with renal morphologic abnormalities in transgenic mice (15).

We have utilized the polarized renal epithelial cell LLC-PK, as a model to study G protein-regulated functions. These studies indicated that only two Gαi isoforms, Gαi2 and Gαi3, are detected in these cells, which, respectively, are involved in the regulation of hormone-stimulated adenylyl cyclase and constitutive proteoglycan secretion through the Golgi complex (16, 17). In polarized LLC-PK1 cells, the Gαi3 isoform is found in Golgi and apical membranes, whereas the Gαi2 isoform is found at the basolateral membrane consistent with the location of their physiological effects (16, 17). We demonstrated that the genes encoding both Gαi subunits are transcriptionally activated in these cells in a coordinated manner during growth and differentiation but differ in response to glucocorticoids and CAMP (18, 19, 20).

*This work was supported by National Institutes of Health Grant DK-42543, American Heart Association Grant 13-555-890, and Established Investigatorship award 9403090 (to L. E.) and National Institutes of Health Grants F32 DK-08838 (to J. D. F.), DK-02271 (to T. B. K.), and DK-58452 (to L. E. and D. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EGR-1 Regulation of the G Protein α-2 Subunit Gene

In the present study, we examine in mitotically active pre-differentiated LLC-PK1 cells, the molecular mechanism for the activation of the Gα-2 gene. We now demonstrate that the expression of the early growth response gene 1 (EGR-1), also known as TIS-8, Krox24, Zif268, and NGFI-A (21), is increased in dividing LLC-PK1 cells, which temporally coincides with the maximal transcriptional activation of the Gα-2 gene. An increase in Gα-2 gene growth rates during culture is the physiological consequence of overexpression of each gene product in these cells. We also demonstrate that the enhancer area in the 5′-flanking region of the Gα-2 gene contains an EGR-1 motif (5′-GGCCCGGGC-3′) (21), which is both the site for both EGR-1 binding and activation of the Gα-2 gene during renal cell growth. These studies provide evidence for the first example of a novel mitogenic signaling pathway coupling proximal signaling events that activate EGR-1 gene expression to a target protooncogene Gα-2 that is participatory for growth and differentiation in renal cells.

EXPERIMENTAL PROCEDURES

Cell Culture

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 previously described (16). Cells were plated at a density of 1 × 10⁴/cm² achieving confluence at approximately culture day 7.

Cellular Transfections

Plasmids—pRSV (EGR-1), and pRSV (Gi-2) are plasmids containing, respectively, the entire coding sequence of EGR-1 and the entire coding sequence of rodent G protein α-2 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′-GGCCCCGGC-3′) were generated from nested deletions of plasmid 10-4 LUC (XmaIII), which contains 105 5′-flanking sequence fused to a firefly luciferase reporter gene as previously described (18). A third plasmid, mutated m-14 LUC, which replaced the nucleotides 1-5 of the EGR-1 site with adenosines was generated by polymerase chain reaction mutagenesis as previously described (20) utilizing the mutagenizing primer 5′-CGGGCTACGAGATC CGCGTACGAGATC-3′ (Promega). Plasmids containing this DNA segment were confirmed by denaturation gel nucleic acid sequencing.

Transient Transfections—Plasmids were transfected in equimolar amounts into LLC-PK1 cells by the calcium phosphate precipitation as previously described (13). Optimum transfection efficiency was obtained by the addition of 20 μg of total plasmid DNA per 55 cm² p10 plate (Falcon), followed by incubation for 20 h without glycerol shock. When required, this amount of DNA was achieved by the addition of 20 pg of total plasmid DNA per 55 cm² p10 plate (Falcon), followed by incubation for 20 h without glycerol shock.

Protein Assays—These were performed as previously described (22). Results are expressed as percent increase ± S.E. in luciferase activity normalized for heat-inensitive alkaline phosphatase activity. Data were analyzed by paired Student's t test.

Mobility Shift Assays

Nuclear Extract Preparation—Nuclear proteins were extracted from LLC-PK1 cells as described by Saatcioglu et al. (22).

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(dI-dC), 140 μM KCl, 9% glycerol, 18 mg Tris, pH 7.3, and 1 mM EDTA at 4 °C. Complexes were separated on 5–6% polyacrylamide gel with 89 mM Tris borate, 89 mM boric acid, 2 mM EDTA buffer (TBE) at 0.2× concentration. The electrophoresis was carried out at 10 V/cm for 3–5 h x 0.1× TBE buffer at 4 °C.

In Vitro Translation Assays—These were performed as previously described (23) utilizing T3 polymerase to drive transcription of a 2.1-kilobase (kb) EGR-1 DNA outlined into Bluescript for use in a rabbit reticulocyte translation system as described by the manufacturer (Promega).

Immunoblotting and Immunofluorescence of EGR-1

LLC-PK1 cells were washed twice in phosphate-buffered saline (without calcium or magnesium) and then lysed by the addition of 1.0 ml of lysis buffer A. Scraped lysates were solubilized by boiling in sample buffer (1% SDS, 30 mM 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 Commassie 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 mM Tris, pH 7.4, with 0.15 mM NaCl and 1% Triton X-100), incubated with either preimmune or immune rabbit IgG anti-EGR-1 WI 21 (alpha 1) (which detects the tumor especific 48-kD protein) or preimmune rabbit IgG (which only detects Gα-2 as Gα-1 and is not expressed in LLC-PK1 cells) (DuPont NEN), diluted 1/1000 in blotting buffer, and washed. EGR-1- and Gα-2-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, permeasalized in Triton X-100 for 4 min, and then incubated in phosphate-buffered saline containing 0.1% bovine serum albumin for 5 min to reduce nonspecific background staining. The cells were incubated for 2 h in anti-EGR-1 WI 21 (alpha 1) or preimmune rabbit IgG conjugated to fluorescein isothiocyanate (Kirkegaard and Perry). Cells were washed three times in 0.1% bovine serum albumin in phosphate-buffered saline, and then incubated for 1 h with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Amersham Corp.).

 Autoradiography

For mobility shift and immunoblotting studies, the dried gels or membranes were autoradiographed with Kodak X-AR film at –80 °C for 1 week with Cronex lightning plus intensifying screens (Dupont NEN). Quantification of signals was performed by densitometry of the autoradiograms with an LKB ultroscan XL enhanced laser densitometer (Pharmacia Biotech Inc.).

RESULTS

EGR-1 is Maximally Expressed During the Growth of LLC-PK Cells—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. In other cell types, the Gα-2 protooncogene is participate in pathways essential for growth and differentiation. We have previously demonstrated maximal activation of the Gα-2 gene during growth of LLC-PK1 cells (19). However, the transactivating factors required for transcriptional activation of the Gα-2 gene during growth have yet to be identified in any cell type. The EGR-1 gene encodes a zinc finger transcription factor of the Cys²-His² subclass that is rapidly induced by a variety of agents during induction of cellular proliferation (21). The EGR-1 gene is rapidly activated in renal tissues following ischemia and hypotrophy (24, 25). Following activation, the expressed EGR-1 protein is primarily detected in the nuclei of thick ascending limbs and

1 The abbreviations used are: EGR-1, early growth response gene 1; FBS, fetal bovine serum; bp, base pair(s).
principal cells of the collecting ducts in the cortex and medulla.

To determine whether EGR-1 also participates in mitogenic events that precede the acquisition of the polarized phenotype in renal cells, we utilized an antibody raised to the carboxyterminal non-zinc finger region of the EGR-1 protein. Representative images of cell cultures at days 2 and 7 are shown. A, in polarized confluent cells from a culture at day 7, only weak nuclear staining can be seen in a few cells at the extreme right and lower corners of the field. B, at day 3, all nonconfluent cells have nuclear staining. Particularly intense nuclear staining is seen at the lower right corner of the field, which represents the edge of a sheet of nonconfluent cells. No staining was observed with preimmune rabbit IgG (data not shown).

expression of Gαi-2 proteins. Thus, the pattern of maximal expression of the EGR-1 protein coincided with the temporal maximal activation of the Gαi-2 gene during growth, which we have previously documented in this cell type (19).

**EGR-1 Increases Gαi-2 Gene Transcription in LLC-PK1 Cells**—In other cell types, the overexpression of the Gαi-2 subunit can activate signaling pathways that contribute to accelerated cell growth. To determine whether Gαi-2 or EGR-1 influence renal epithelial cell growth, LLC-PK1 cells were transiently transfected with Bluescript or plasmids encoding cDNAs for Gαi-2 or EGR-1, driven by a viral Rous sarcoma promoter-enhancer to overexpress each protein. Following transfection, growth was examined in media containing either 10 or 0.1% fetal bovine serum (FBS). As seen in Fig. 3, cells transfected with plasmids encoding Gαi-2 and EGR-1 had growth rates by day 3 that were 2.8 and 1.7 times faster, respectively, than cells transfected with Bluescript in 10% FBS. LLC-PK1 cells require 10% FBS to grow efficiently and maintain viability in culture. However, despite reducing the serum to 0.1%, cells transfected with plasmids encoding either Gαi-2 or EGR-1 had growth rates faster than serum replete cohorts transfected with Bluescript. Transfection efficiency in all of these experiments was comparable at approximately 10–20% efficiency. Despite the initial accelerated growth rates of Gαi-2 and EGR-1 transfected cells, they still became quiescent and developed a normal polarized phenotype upon achieving confluence. These data suggested that both Gαi-2 and EGR-1 activated signaling pathways that normally contribute to renal cell growth.

**EGR-1 and Gαi-2 Increase Growth in LLC-PK1 Cells**—We previously documented in rapidly dividing nonpolarized cells a 135-bp enhancer area in the −200 to −335 region of the Gαi-2 gene. This region contains a putative binding site (5′-CGCCCCCGC-3′) for the EGR-1 transcription factor that may provide a genomic signaling pathway for mitogenesis. To assess whether EGR-1 was contributing to the transcriptional activation of the Gαi-2 gene, cells were cotransfected with the pRSEV Gαi-2 plasmid and plasmids encoding firefly luciferase reporter genes fused to 5′-flanking areas of the Gαi-2 gene with (M14) or without the EGR-1 site (mutated M14 and M4). As seen in Fig. 4, a 320% enhancement of Gαi-2 transcription was only found in renal cells following their transfection with the M14 plasmid, which contained both an intact EGR-1 binding site and also overexpressed a functional EGR-1 protein. These data suggested that the EGR-1 protein was contributing to the temporal transcriptional activation of the Gαi-2 gene in LLC-

**FIG. 2. Immunoblot of LLC-PK1 cell membranes harvested from cells following trypsinization and culture days 1-7.** Quiescent LLC-PK1 cells from day 10 of culture were trypsinized (day 0) and cultured for successive days. Immunoblotting of membranes from each of these cultures was performed to compare the relative amounts of EGR-1 protein present. Immunoblotting of total cell extract from each of these cultures was performed to compare the relative amounts of EGR-1 and Gαi-2 protein present. Each lane represents 150 μg of protein quantitated as described under “Experimental Procedures” using immune rabbit IgG anti-EGR-1 Wi 21 (alpha 1) (which detects the non-zinc finger region of the EGR-1 protein) and AS 7 antibody, which detects the carboxyl terminus of Gαi-2. Autoradiograms of these proteins from the same acrylamide gel show in the upper panel EGR-1 induction (~80 kDa) on culture day 2. The lower panel shows maximal induction of Gαi-2 (~40 kDa) on culture day 2.
Identification of the EGR-1 Protein as a Component of an Induced Nuclear Transcription Complex during Transcriptional Activation of the Gai-2 Gene—To determine whether the EGR-1 protein was directly contributing to the activation of the Gai-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'-ATCCGCCGCCCCGCGC-GTCCGGG-3'), was synthesized and 32P end-labeled for direct binding studies in mobility shift assays. In vitro translated EGR-1 protein was utilized to assess its capability for binding the EGR-1 consensus sequence. As seen in Fig. 5A, this probe bound a component of the non-programmed reticulocyte lysate as we have previously described (23). However, the probe specifically bound the slower mobility EGR-1 protein. Furthermore, immune rabbit IgG anti-EGR-1 WI 21 (alpha 1) (which detects the non-zinc finger carboxyl-terminal region of the EGR-1 protein) but not preimmune rabbit IgG could specifically retard the mobility of this complex.

To confirm that the EGR-1 protein was acting directly by binding its cognate site and not the region 5' to the EGR-1 binding site in the M14 construct (see Fig. 4, lower panel), we assessed the ability of this area with the mutated EGR-1 binding site (5'-AAAAACCGC-3') to bind the EGR-1 protein. As seen in Fig. 5B, probes containing either the mutated EGR-1 binding site or the 5'-flanking area of M14 and the mutated EGR-1 binding site displayed indistinguishable binding of both the non-programmed and programmed reticulocyte lysate. These data demonstrated that both the mutated EGR-1 site and the region 5' to the EGR-1 binding site in M14 were incapable of binding the EGR-1 protein. These findings suggested that the EGR-1 protein might be acting by a direct interaction with its cognate site to activate the Gai-2 gene.

To examine this possibility, we compared nuclear extracts from LLC-PK1 cells that were either actively dividing and non-polarized (culture days 1–2) or relatively quiescent and fully polarized (culture day 8). As seen in Fig. 6, the binding patterns of nuclear extracts from culture days 1–2 were strikingly different from those on culture day 8. The 23-bp EGR-1 probe detected two prominent induced complexes in nuclear extracts from culture days 1–2. By contrast, the same probe detected three distinct complexes in nuclear extracts from culture day 8. The upper complex had slightly lower mobility as compared with the upper complex in days 1–2 nuclear extracts, and the second slower mobility complex was also detected that was not seen in nuclear extracts of culture days 1–2, and a third nuclear complex, whose mobility was comparable with that seen in nuclear extracts in days 1–2, was much less intense. These data confirmed that nuclear extracts from culture day 8 as compared with culture days 1–2 contained differing DNA binding proteins that interact with the EGR-1 consensus sequence (5'-CGCCCCGCGC-3').

Based on our immunocytochemical studies, it would be anticipated that the EGR-1 protein should be present in nuclear extracts of actively dividing LLC-PK1 cells on culture days 1–2. To determine whether EGR-1 was one of the proteins interacting with the EGR-1 probe, nuclear extracts from culture days 1–2 and day 8 were preincubated with immune rabbit IgG anti-EGR-1 WI 21 (α1). Following electrophoresis, an additional plasmid encoding EGR-1. The lower panel display cells transfected with Bluescript II ks. Cells were grown in either 10% fetal calf serum (left side) or 0.1% fetal calf serum (right side). The cell doubling time ± S.E. of six experiments is depicted below each panel. B, growth curves in 10% fetal calf serum and 0.1% fetal calf serum are depicted for these experiments. Squares depict cells transfected with Bluescript II ks, triangles depict cells transfected with plasmids encoding EGR-1, and circles depict cells transfected with plasmids encoding Gai-2.

**Fig. 3.** Inverted phase microscopy of transiently transfected LLC-PK1 cultures. LLC-PK1 cells were simultaneously transfected with Bluescript II ks or plasmids encoding Gai-2 and EGR-1 as described under "Experimental Procedures." 48 h after transfection cultures, cell number and human placental alkaline phosphatase content were determined in each plate. A, cells transfected with a plasmid encoding Gai-2. The middle panel displays cells transfected with a plasmid encoding EGR-1. The lower panel display cells transfected with Bluescript II ks. Cells were grown in either 10% fetal calf serum (left side) or 0.1% fetal calf serum (right side). The cell doubling time ± S.E. of six experiments is depicted below each panel. B, growth curves in 10% fetal calf serum and 0.1% fetal calf serum are depicted for these experiments. Squares depict cells transfected with Bluescript II ks, triangles depict cells transfected with plasmids encoding EGR-1, and circles depict cells transfected with plasmids encoding Gai-2.
stral slower mobility complex could be easily detected in extracts from culture days 1–2. By contrast, an additional slower mobility complex was barely detected in extracts from culture day 8. These data demonstrated that one component of these nuclear complexes was the EGR-1 protein. Detectability of this protein was consistent with its pattern of maximal expression in dividing LLC-PK1 cells that also have a corresponding activation of the Gai-2 gene.

**DISCUSSION**

We have previously demonstrated in renal cells that the Gαs2 gene is transcriptionally activated during growth and differentiation and is subsequently repressed to a basal state following this period (19). We had suggested that the necessity for a genetic control mechanism that prevents the strong constitutive activation of the Gai-2 gene in fully differentiated renal epithelia may be required to maintain normal intracellular signaling and also to repress normal growth pathways, thereby preventing oncogenic transformation. To directly test this possibility, LLC-PK1 cells were transiently transfected with a plasmid encoding the cDNA for Gai-2 fused to the viral Rous sarcoma promoter enhancer to constitutively overexpress Gai-2 proteins. Although only 10–20% of these cells were transiently transfected, this was sufficient to confer to the overall cell population growth rates that were, respectively, 2.8 times faster than cells transfected with Bluescript in 10% FBS. Furthermore, although LLC-PK1 cells normally require 10% FBS to grow efficiently in culture, high growth rates were maintained for cells grown in 0.1% FBS that were transfected with plasmids encoding Gai-2. These findings are consistent with other studies that indicate that Gai-2 can couple receptors and effectors in signaling pathways for growth and differentiation in fibroblasts (6) and F9 teratocarcinoma cells (14). Mutations from Arg179 to Cys or His, which decrease GTPase activity in Gαs2, have been found in tumors of the adrenal cortex and ovary, which convert this gene into the oncogene gip2 (7). Transfection of gip2 into Rat-1 cells induces their oncogenic transformation (5, 8). Increased growth and oncogenic transformation in this setting is, in part, attributable to persistent activation of downstream effectors such as mitogen-activated protein kinase (9). Importantly, stoichiometric control of Gαs2 protein is also critical for normal intracellular signaling, as overexpression of wild type Gαs2 subunits can also induce oncogenic transformation in Rat-1a cells (5). Conversely, the serum-stimulated mitogenic response of mouse Balb/c3T3 fibroblasts can be inhibited by either pertussis toxin or their direct injection with antibodies against Gαs2 subunits (6). Recent studies suggest that even modest repression of Gαs2 expression in vivo is associated with renal morphologic abnormalities in transgenic mice (15).

In our previous studies, an enhancer region (position −200 to −335) in the Gai-2 gene was identified in a deletion series used to define the gene promoter (18). Interestingly, enhancer activity was found in this area only in proliferating cells prior to cell polarization. These studies suggested that the transcription factor or factors necessary for transactivation of the gene might be induced by mitogenic signaling pathways. Inspection of the enhancer area revealed a motif (5′-CGCCCCCCGC-3′) that could serve as a perfect binding site for the EGR-1 zinc finger transcription factor. The gene encoding this transcription factor is rapidly induced by a variety of agents that induce cellular proliferation (31) and has recently been implicated in signaling pathways essential for the differentiation of macrophages (26) and cardiac myocytes (27). In renal tissues, the EGR-1 gene is rapidly activated following ischemia and hypertrophy (24). Following EGR-1 gene activation, the expressed protein is primarily detected in the nuclei of thick ascending limbs and principal cells of the collecting ducts in the cortex and medulla (25).

To determine whether this transcription factor could be playing a role in Gai-2 gene transactivation in LLC-PK1 renal cells, we first demonstrated that it was expressed in this cell type. In fully confluent quiescent LLC-PK1 cells, the Gαs1 protein was not detected either by immunoblotting or by immunocytochemistry. However, in actively dividing cells prior to polarization, the EGR-1 protein was easily detected in the nuclei of these cells in a pattern identical to that seen in renal tissues following ischemic and hypertrophy. Immunoblotting indicated that the relative content of this protein was highest in dividing cells and declined rapidly to undetectable levels in differentiated cells that had achieved polarity. Importantly, EGR-1 protein expression coincided with the same pattern of expression that we have previously documented for Gai-2 proteins in this cell type during culture. If the pattern of EGR-1 protein expression contributed to LLC-PK1 cell mitogenesis during culture, we reasoned that overexpression of this protein might affect growth in this cell type by stimulating endogenous Gai-2 gene expression. Although only 10–20% of these cells were transiently transfected, this was sufficient to confer to the overall
population growth rates that were initially 1.7 times faster than cells transfected with Bluescript in 10% FBS. As with cells transfected with plasmids to overexpress Gai-2, accelerated growth rates could be maintained despite reduction of serum in the culture media to 0.1%.

Although EGR-1 might have many potential target genes, we reasoned that EGR-1 might be acting to up-regulate Gai-2 gene expression to account for increased growth in dividing renal cells. To examine this possibility, we cotransfected plasmids to overexpress EGR-1 (pRSV EGR-1), and plasmids that contained the Gai-2 5' flanking sequence with EGR-1 binding site (5'-CGCCCCCGG-3') and a 28-hp DNA segment containing the EGR-1 consensus sequence was compared with a 28-hp mutated prohice with EGR-I programmed lysate mutnted by adenosines encompassing the 5'-end of M14 to the beginning of M4, as depicted in the lower panel of Fig. 4. Lane 1, EGR-1 probe alone; lane 2, EGR-1 probe with non-programmed reticulocyte lysate; lane 3, EGR-1 probe with EGR-1 programmed lysate; lane 4, 28-hp mutated probe alone; lane 5, 28-hp mutated probe with EGR-1 programmed lysate; lane 6, 28-hp mutated probe with non-programmed reticulocyte lysate; lane 7, 62-hp mutated probe alone; lane 8, 62-hp mutated probe with non-programmed reticulocyte lysate; lane 9, 62-hp mutated probe with EGR-1 programmed lysate.

![Fig. 5. Mobility shift assays with the 23-bp DNA segment of the Gai-2 gene containing the EGR-1 motif binds EGR-1 in vitro translated protein.](image)

![Fig. 6. Mobility shift assays with the 23-bp DNA segment of the Gai-2 gene containing the EGR-1 binding site identify EGR-1 as a component of an induced nuclear complex in dividing LLC-PK1 cells.](image)
with pRSV EGR-1 plasmids with reporter plasmids that contained the mutated EGR-1 binding site or had the site deleted. One interpretation of these findings is that EGR-1 expression was maximal in cells that were 10–20% confluent, hence additional EGR-1 protein would not appreciably contribute to further activation of the Gai-2 gene. However, as the cells became more confluent and their endogenous EGR-1 protein levels fell, there would be a corresponding decrease in Gai-2 gene transcription in all cells except those that were transfected with plasmids to overexpress EGR-1. Cells transfected with pRSV EGR-1 would maintain high EGR-1 protein levels allowing for the persistent maximal activation of the Gai-2 gene.

To verify that EGR-1 was directly contributing to the activation of the Gai-2 gene, a 23-bp DNA fragment of the 60-bp gene enhancer region containing the central EGR-1 binding motif was synthesized for use in mobility shift assays with in vitro translated EGR-1 protein. This probe was able to specifically bind EGR-1 protein. Furthermore, this complex could be recognized by rabbit antibody generated against the non-zinc finger carboxyl-terminal region of the EGR-1 protein. Two additional DNA segments were synthesized to confirm that the EGR-1 protein was only binding its cognate site in the 60-bp enhancer region. EGR-1 binds its cognate DNA site by participation of three distinct zinc fingers (28). Finger 1 binds near the 5'–end of the primary strand (5'-CGCCCCCGG–3'), finger 2 binds near the center (5'-CGCCCCCGG–3'), and finger 3 binds near the 3'-end of the primary strand (5'-CGCCCCCGG–3'). A mutated DNA segment corresponding to the EGR-1 probe was therefore synthesized, which differed by five 5'-adenosine substitutions (5'-AAAAACCGC–3') to prevent binding of fingers 1 and 2. We demonstrated that this probe was unable to bind in vitro translated EGR-1 protein in mobility shift assays. Furthermore, utilizing a primer derived from the upper strand 5'-ai-2 sequence of M14 with the bottom strand primer of the mutated EGR-1 probe, a DNA segment was derived from the M14 plasmid that was also unable to bind in vitro translated EGR-1 protein in mobility shift assays, which demonstrated that the area 5' to the EGR-1 binding site in M14 was unable to bind EGR-1. These studies complemented our functional studies, which suggested that increased transcription of the Gai-2 gene by EGR-1 overexpression was occurring specifically through its cognate binding site and not in the region 5' to this site.

To confirm that this discrete area of the gene participated in an EGR-1-mediated transcriptional response, nuclear extracts were prepared from cells 18–24 h or 8 days following culture for use in the mobility shift assay. These studies indicated that nuclear extracts from culture day 8 as compared with culture days 1–2 contained differing DNA binding proteins that interact with the EGR-1 consensus sequence (5'-CGCCCCCGG–3'). Furthermore, the preincubation of these extracts with an antibody that specifically recognizes EGR-1 confirmed that the induced complexes from cells 18–24 h contained the EGR-1 protein as evidenced by the production of a slower mobility "super shifted" nuclear complex. Detectability of this protein was consistent with its pattern of maximal expression in dividing LLC-PK1 cells that also have a corresponding activation of the Gai-2 gene.

It was also apparent from these studies that the temporal deactivation of the Gai-2 gene in fully polarized LLC-PK1 cells taken from day 8 of culture was not simply mediated by the loss of EGR-1 protein. Our studies suggested that EGR-1 was replaced with a lower molecular weight protein complex or complexes that efficiently bind the EGR-1 motif. A candidate trans-acting factor that may act as a repressor in this context is the Wilms' tumor protein (WT1), which has greater than 65% amino acid sequence similarity with EGR-1 in its zinc finger region (21). Importantly, WT1 has recently been shown to efficiently bind the EGR-1 motif (29) and act as a repressor of gene transcription either independently or in concert with the tumor suppressor factor p53 in several cell types (30). We are currently investigating whether WT1 and/or p53 can act as a repressor of Gai-2 gene expression in renal cells. We believe these studies provide evidence for a novel mitogenic pathway in renal cells that potentially link proximal signaling events that induce EGR-1 gene expression to activation of a genomic target essential for growth and differentiation in this cell type, the Gai-2 protooncogene. Upon further examination, this pathway may provide significant insights into the molecular events involved in renal hypertrophy, nephrogenesis, and oncogenesis.

REFERENCES

1. Gilman, A. G. (1997) Annu. Rev. Biochem. 56, 615-649
2. Simon, M. I., Strathmann, M. F., and Gautam, N. (1991) Science 252, 802-808
3. Birnbauer, L., Abramowitz, J., Yatani, A., Okabe, K., Mattess, R., Graf, R., Stanford, J., Codina, J., and Brown, A. M. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 225-244
4. Simonds, W. F., Goldsmith, P. K., Codina, J., Unson, C. G., and Spiegel, A. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7809-7813
5. Gupta, S. K., Gallego, C., and Johnson, G. L. (1992) Mol. Biol. Cell 3, 123-128
6. LaMorte, V. J., Goldsmith, P. K., Spiegel, A. M., Meinkoth, J. L., and Ferraisano, R. J. (1992) J. Biol. Chem. 267, 801-804
7. Lyons, L., Landis, C. A., Harash, G., Vallier, L., Grunwald, K., Feichtinger, H., Duh, Q. Y., Clark, O. H., Kawasaki, E., Bourne, R. H. et al. (1990) Science 248, 655-659
8. Pecor, A. M., Wong, Y. H., and Bourne, E. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7031-7035
9. Gupta, S. K., Gallego, C., Johnson, G. L., and Heasley, L. E. (1992) J. Biol. Chem. 267, 7967-7970
10. Hadwiger, J. A., and Firtel, R. A. (1992) Gene & Dev. 6, 38-49
11. Firtel, R. A., van Haselt, P. J. M., Kimmel, A. R., and Devreotes, P. N. (1989) Cell 58, 235-249
12. Hadwiger, J. A., Wilkie, T. M., Strathmann, M., and Firtel, R. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8215-8217
13. Parks, S., and Wieschaus, E. (1991) Cell 64, 447-458
14. Moehn, C. M., Johnson, G. L., and Malbon, C. C. (1992) Science 258, 1373-1375
15. Watkins, D. C., Hod, Y., and Malbon, C. C. (1993) Science 260, 991-995
16. Ercolani, L., Stow, A. D.,ForeColor, E. J., Lo, H. G., and Hagi, J., and Austello, D. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4635-4639
17. Stow, A. D., de Almeida, A. J. B., Narula, N., Holtzman, E. J., Ercolani, L., and Austello, D. A. (1991) J. Cell Biol. 114, 1113-1124
18. Holtzman, E. J., Soper, B. W., Stow, A. D., Austello, D. A., and Ercolani, L. (1991) J. Biol. Chem. 266, 1763-1771
19. Holtzman, E. J., Kinane, T. B., West, K., Soper, B. W., Karga, H., Austello, D. A., and Ercolani, L. (1993) J. Biol. Chem. 268, 3964-3975
20. Kinane, T. B., Shang, C., Fider, J., and Ercolani, L. (1993) J. Biol. Chem. 268, 24669-24676
21. Sukhatme, V. P. (1990) J. Am. Soc. Nephrol. 1, 859-866
22. Sastagi, M., Fify, D. J., Pasco, D. S., and Fagan, J. B. (1990) J. Biol. Chem. 265, 9551-9558
23. Cao, X., Koski, R. A., Gashe, A. M., McGinneman, M., Morris, C. P., Caffre, R., Hey, R. V., and Sukhatme, V. P. (1990) Mol. Cell. Biol. 10, 1831-1949
24. Ouerritee, A. J. M., Malt, R. A., Sukhatme, V. P., and Converse, D. V. (1990) J. Clin. Invest. 85, 755-771
25. Boverey, J. V., Sukhatme, V. P., Bamberger, M., Ouerritee, A. J., and Brown, D. W. (1991) Cell Regul. 2, 281-290
26. Nguyen, H. Q., Hoffmann-Liebermann, B., and Liebermann, D. A. (1993) Cell 72, 279-290
27. Gupta, S. K., Gupta, M., Zaki, R., and Sukhatme, V. P. (1991) J. Biol. Chem. 266, 12613-12616
28. Pavletich, N. P., and Pabo, C. O. (1991) Science 253, 809-817
29. Saclodi, M. L., Cook, D. M., Morris, J. F., Gashe, A., Sukhatme, V. P., and Rauscher, F. J. (1991) Science 253, 1550-1553
30. Maheswaran, S., Prif, S., Bernard, A., Morris, J. F., Rauscher, F. J., Hill, D. E., and Haber, D. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5100-5104