Expression and differential regulation of Id1, a dominant negative regulator of basic helix – loop – helix transcription factors, in glomerular mesangial cells

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Received June 30, 1993; Revised and Accepted October 15, 1993

ABSTRACT

Id1 is a family of dominant negative helix – loop – helix (HLH) proteins that block cell-specific transcription mediated by basic HLH (bHLH) transcription factors. We have analyzed Id1 expression in mesangial cells as a first step towards understanding the putative role of bHLH transcription factors in cell type-specific gene expression in the kidney. Glomerular mesangial cells expressed an abundant 1.1 kb mRNA transcript for Id1, but in contrast to other cell types Id1 mRNA was expressed in both randomly cycling cells and in serum-deprived, quiescent cultures. When quiescent mesangial cells were treated with serum to re-enter G₁, Id1 mRNA levels were rapidly (2–4 h) and transiently down-regulated. Down-regulation of Id1 mRNA following addition of serum to mesangial cells was cell type-specific and contrasted with induction of Id1 by serum in BHK-21 and 3T3 fibroblasts. Down-regulation of Id1 mRNA correlated with mitogenesis and occurred when quiescent cells were treated with growth factors that activate G protein-coupled receptors and receptor protein tyrosine kinases but not with a non-mitogenic cAMP analog. Down-regulation of Id1 by growth factors required *de novo* protein synthesis, suggesting that a labile protein was involved. Appearance of E-box DNA binding activity in mesangial cell extracts followed down-regulation of Id1 message. Steady state Id1 mRNA levels and E-box DNA binding activity were not tightly correlated, suggesting complex regulation of Id1 activity. mRNA transcripts for E2A gene products were also expressed in mesangial cells, but these cells failed to express mRNAs for MyoA/MyoD-related genes. Collectively, these data demonstrate that Id1 is expressed in renal mesangial cells and suggest that bHLH complexes might be important for transcriptional regulation in the kidney. In addition, the observation that Id1 mRNA is transiently down-regulated by serum in mesangial cells suggests that Id1 gene expression is more complicated than previously appreciated and is tightly regulated in a cell-specific manner.

INTRODUCTION

Mesangial cells are microvascular pericytes in the renal glomerulus that synthesize and assemble the mesangial matrix and have been postulated to regulate glomerular hemodynamics (1, 2). Mesangial cells *in vivo* are normally quiescent with a low mitotic index; however, in response to glomerular injury mesangial cells undergo a phenotypic switch to an activated state characterized by increased synthesis and reorganization of the extracellular matrix, protease secretion, processing of extracellular macromolecules such as immune complexes, and cell growth in the form of hypertrophy or hyperplasia (3, 4). A shift to the activated mesangial phenotype has been postulated to contribute to the pathogenesis of glomerulosclerosis (1, 2). However, transcriptional regulation underlying mesangial cell phenotype, and its dysregulation in glomerular disease, is poorly understood. Basic-helix–loop–helix (bHLH) transcription factors regulate cell type-specific and developmentally-important transcriptional responses (5–7). Proteins in this family include myogenic factors such as MyoD and myogenin (5, 8), and the IgG enhancer binding proteins E12 and E47 (6, 9). Other bHLH factors regulate cell type determination in *Drosophila* such as the *achaete-scute* complex, *daughterless*, *hairy*, *twist*, and *enhancer of split* [see (5, 8) for review]. bHLH proteins bind either as homodimers or heterodimers to specific DNA sequences containing the CANNTG or E-box motif. Dimerization of bHLH factors is mediated by two conserved amphipathic α helices separated by a non-helical loop. Dimerization between cell-specific (i.e., MyoD, myogenin, *achaete-scute*) and ubiquitously-expressed (i.e., E12, E47, *daughterless*) bHLH proteins is a critical step controlling transcriptional activation and cell type specificity in myogenesis, neurogenesis, sex determination, and mesoderm formation. At present it is not known whether bHLH proteins regulate cell type-specific gene expression in the kidney or contribute to transcriptional control in mesangial cell activation.

Binding of bHLH proteins to DNA is regulated in part by dominant negative proteins that dimerize with bHLH factors but lack the basic amino acids necessary for DNA binding (10). Thus, HLH proteins of this class—such as Id1, Id2, HLH462 (Id3), and *extramacrochaetae* (10–15)—prevent specific binding of bHLH factors to E-box sequences on DNA. bHLH proteins that also contain a leucine zipper motif (i.e., TFE3, c-Myc, AP-4)

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resist inactivation by Id1. Id1 blocks myogenic differentiation (10) and transcriptional activation of the IgG (16,17) and insulin enhancers (18). Id1 mRNA is transiently repressed in myeloid cells treated with granulocyte-colony stimulating factor to induce terminal differentiation, and ectopic expression of Id1 also inhibits myeloid differentiation (19). These experiments suggest that Id1 antagonizes cell differentiation by inhibiting bHLH factors required for developmental programs. Benezra et al. (10) demonstrated that Id1 is expressed at high levels in proliferating myoblasts and other cell types and that Id1 mRNA is down-regulated when cells differentiate. Others have shown that Id1 and Id-like genes are induced by mitogens (14, 20, 21), leading to the hypothesis that mitogens block differentiation in part through induction of Id1 with subsequent inactivation of bHLH proteins.

As a first step towards understanding cell-specific transcriptional regulation in mesangial cells, we have characterized expression and regulation of Id1 in cultured mesangial cell strains. Expression of Id family genes is highly cell type specific (13–15, 19); moreover, regulation of Id expression appears to be the major mechanism controlling Id activity. Because Id1 contributes to transcriptional regulation and differentiation in muscle cells, we reasoned that in mesangial cells, which also express a contractile muscle-like phenotype, Id1 might be expressed and differentially regulated. We report here that glomerular mesangial cells express abundant mRNA levels for Id1, but in contrast to other cell types Id1 mRNA is expressed in serum-deprived, quiescent cultures and is transiently down-regulated by addition of serum mitogens. These data reveal complex, cell-specific regulation of Id1 gene expression by mitogens. In addition, the observation that mesangial cells express E12 mRNA and E-box DNA binding activity suggest that bHLH proteins might contribute to transcriptional regulation in renal mesangial cells.

MATERIALS AND METHODS

Mesangial cell culture and measurements of [³H]thymidine uptake

Mesangial cell strains from male Sprague–Dawley rats were isolated and characterized as previously reported (22,23). Briefly, primary cultures were isolated from glomerular explants partially digested with bacterial collagenase to form glomerular cores. Mesangial cell strains were maintained in RPMI 1640 medium supplemented with 17% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml each of insulin and transferrin, and 5 ng/ml of selenite at 37°C in a 5% CO₂ incubator. Cells in passage 5–25 were employed in these studies.

Cultures of BHK-21 and 3T3 fibroblasts were obtained from the American Type Culture Collection. [³H]thymidine uptake in mesangial cells was measured as previously reported (24) with minor modifications. Mesangial cells at approximately 80% confluence in 24-well plates were incubated in DMEM/0.5% FBS for 24 h to induce quiescence. Agonists were then added (time=0), and after a 16 h incubation the cells were pulsed with 1.0 µCi/ml [methyl-³H]thymidine for 2 h. Radiolabeled DNA was extracted by washing twice with Dulbecco's PBS, fixing in methanol/acetic acid/water (50:10:40) for 1 h at 4°C, and solubilizing and counting in 1.0% SDS (24). The mitogenicity of endothelin and epidermal growth factor in mesangial cells was previously confirmed by cell counts (24, 25).

RNA extraction and Northern RNA blot analysis

Preparations of total mesangial cell RNA and subsequent analyses were carried out exactly as previously described (25, 26). The Northern RNA blots (10 µg/lane) were hybridized with a full-length [³²P]-labeled cDNA probes for murine Id1 or MyoA (3.0×10^7 – 1.0×10^8 cpm/µg/DNA), kindly provided by Harold Weintraub, or E12 cDNA provided by Cornelis Murre. Final stringency of washing was 0.2×SSPE at 42°C; Id1 mRNA:cDNA heteroduplexes were stable at temperatures up to 68°C. After detection of the mRNA:cDNA heteroduplex by autoradiography (8–16 h at –70°C), the filters were stripped of the [³²P]cDNA probe and rehybridized with GAPDH cDNA. Size estimates of discrete mRNA species were obtained from the 28S and 18S rRNA subunits stained with methylene blue after hybridization (26). Hybridization signals were quantified by scanning densitometry and divided by the fold change in GAPDH mRNA from the same blot.

DNA binding assays

For detection of DNA binding activity by bHLH complexes in mesangial cells, whole cell extracts were prepared exactly as described by Baichwal and coworkers (27) and used immediately. Oligodeoxynucleotide duplexes were end labeled with polynucleotide kinase and [³²P]ATP and purified using D-25 Sephadex columns. Oligodeoxynucleotide probes contained a single copy of an E-box motif from the muscle creatine kinase enhancer, muscle enhancer factor-1 (MEF-1): 5'-GAT CCCCCCAACACCTGCTGCCTGA-3' (19). All probes were synthesized by National Biosciences (Plymouth, MN). DNA binding reaction mixtures contained 5 µg protein, 1 ng labeled probe (80,000–100,000 cpm), 2 µg poly(dI-dC) · poly(dI-dC), 10 mM HEPES (pH 7.6), 22mM Tris borate (pH 8.3), 10% glycerol, 210 mM KCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.5 mM PMSF, 0.5% Trasylol in a 20 µl total volume. Following a 30 min incubation at room temperature, complexes were resolved on a 5% polyacrylamide gel at 30 mA constant current in 44.5 mM Tris-borate buffer and 1 mM EDTA. Gels were dried and complexes typically detected in overnight exposures at –70°C.

Reagents

Mitogens used were obtained from the following sources: endothelin-1, Peptide Institute (Tokyo); epidermal growth factor, Gibco (Gaithersburg, MD); fetal bovine serum, Hyclone (Logan, UT). 8-Bromo-cAMP was from Boehringer Mannheim (Indianapolis, IN).

RESULTS

Expression and differential regulation of Id1 mRNA in renal mesangial cells

Expression of the Id1 gene is tightly regulated in a cell type-specific manner or as part of distinct developmental programs. Some cell types fail to express Id1 or express only specific members of the Id family (13–15, 19). Previous experiments have shown that Id2 but not Id3 is expressed in the kidney (14, 15), but expression of Id1 in the kidney has not been investigated. Thus, to determine whether mesangial cells expressed an Id gene we prepared total RNA from cycling mesangial cells and measured Id1 mRNA levels by Northern RNA blot analysis.

Mesangial cells from two independent strains expressed abundant levels of a 1.1 kb message for Id1 (Fig. 1), similar in size to Id1 mRNA transcripts in other cell types.

Differential expression of Id1 appears to be an important mechanism regulating Id1 activity in all cell types studied. To date there is little or no evidence for post-translational modification of Id1 protein. In most differentiated, quiescent cells

Figure 1. Expression of Id1 mRNA in two mesangial cell strains analyzed by Northern RNA blotting. Total cellular RNA was prepared from randomly cycling mesangial cells, and 12 μ g RNA was denatured, fractionated on a 1.2% agarose gel, and transferred to reinforced nitrocellulose as described in Materials and Methods. Filters were probed with a 32 P-labeled cDNA encoding Id1 and washed with final stringency of $0.2 \times \text{SSPE} - 0.1\%$ SDS at 42°C . The filters were then stripped and rehybridized with 32 P-GAPDH cDNA (26). Exposure times for autoradiography ranged from 6 h with GAPDH to 16–24 h with Id1. Each lane represents independent experiments performed in different mesangial cell strains.

Id1 expression is low; however, when quiescent cells are stimulated by serum or other mitogens, Id1 levels are rapidly increased (13–15,19). To test whether the Id1 gene was differentially regulated in mesangial cells, we treated serum-starved, quiescent cells with serum and measured Id1 mRNA levels. We previously documented that serum deprivation for 24 h renders $>90\%$ of mesangial cells quiescent (24,26). Quiescent mesangial cells expressed a 1.1 kb Id1 message that was rapidly (2–4 h) down-regulated when cells were stimulated to proliferate with 17% FBS (Fig. 2A). Four hours after serum treatment Id1 levels were nearly undetectable. Down-regulation of Id1 contrasted with marked induction by serum of the immediate-early gene, *c-fos*, over the same time course (Fig. 2B). Down-regulation of Id1 was transient, however, as by 24 h Id1 mRNA levels had recovered, albeit not to the previous levels observed in quiescent cells (Fig. 2A). Transient down-regulation of Id1 mRNA was reproducible and was observed in three independent experiments (Fig. 2C). These data suggest that, in contrast to other cell types, Id1 is transiently down-regulated when quiescent mesangial cells are induced by mitogens to re-enter G_1 .

To confirm that our observation of Id1 mRNA down-regulation by serum was not an artifact of our experiments, we examined whether under the same conditions Id1 mRNA was up-regulated by serum in other cell types. In contrast to down-regulation of Id1 mRNA observed in mesangial cells, in quiescent BHK-21 and 3T3 fibroblast cell lines serum up-regulated rather than down-regulated Id1 (Fig. 3). Similar induction of Id1 by serum mitogens was observed when quiescent WI-38 fibroblast cell lines were induced to proliferate with serum (Mader *et al.*, unpublished data). Thus, down-regulation of Id1 mRNA is not observed in all cells undergoing the G_0 – G_1 transition. Indeed, previous experiments have shown that mitogens up regulate Id1 mRNA in myocytes (10,20), 3T3 fibroblasts (14), and vascular smooth

Figure 2. Transient down-regulation of Id1 mRNA in quiescent mesangial cells treated with 17% fetal bovine serum (FBS). (A) Mesangial cells were made quiescent in Dulbecco's minimal essential medium (DMEM)/0.5% FBS (Control) and then treated with FBS for the indicated times. Total RNA was extracted and Northern RNA blot analysis was performed as described in Experimental Procedures using an Id1 cDNA probe (10). (B) RNA from the same experiment was also analyzed for *c-fos* expression by sequentially hybridizing with rat *c-fos* and GAPDH cDNA probes. (C) The graph represents relative Id1 levels (mean $\pm$ SEM) from 3–4 independent experiments in different mesangial cell strains. Relative intensity of the Id1 signal (Control=1) was analyzed by scanning densitometry relative to the GAPDH signal. Note that the 8 and 16 h time points are not included in the Northern blot presented above in Fig. 2A but were included in other blots.

muscle cells (21), suggesting that down-regulation of Id1 by mitogens in mesangial cells is unusual. Transient regulation of Id1 has also been observed in myeloid precursor cells induced to terminally differentiate with granulocyte-colony stimulating factor (19). Taken together with our data showing transient regulation of Id1 when mesangial cells are stimulated to re-enter G_1 , these results suggest that in some cells Id1 gene expression is transiently regulated when cells undergo major changes in the genetic program. We conclude that regulation of Id1 gene expression by mitogens is more complicated than previously appreciated and appears to be tightly regulated in a cell-specific manner.

Down-regulation of Id1 mRNA correlates with mitogenesis

If down-regulation of Id1 is regulated by the G_0 – G_1 progression in mesangial cells, then treatment of mesangial cells with different classes of growth factors should down-regulate Id1.

Figure 3. Induction of Id1 mRNA in quiescent fibroblasts treated with FBS. Quiescent (DMEM/0.5% FBS for 48 h) BHK-21 or 3T3 fibroblasts at approximately 80% confluence were treated with 17% FBS. At the indicated times total RNA was extracted and Id1 mRNA levels were assessed by Northern RNA blot analysis as described in Fig. 1. Identical results were observed in two independent experiments.

Similarly, Id1 should not be repressed by non-mitogenic agonists. To test this, we stimulated quiescent mesangial cells with endothelin-1, a potent mesangial growth factor linked to G protein-coupled receptors (24), and epidermal growth factor, which binds to receptors with intrinsic protein tyrosine kinase (PTK) activity. Endothelin-1 (100 nM) and epidermal growth factor (100 ng/ml) induced a 3.7- and 3.2-fold increase in [3 H]thymidine uptake, respectively (Fig. 4). Both endothelin-1 and epidermal growth factor down-regulated Id1 mRNA 4 h after addition to quiescent cells, although repression was greater with epidermal growth factor (Fig. 4). Serum induced the largest increase in [3 H]thymidine uptake (Fig. 4) and down-regulation of Id1 (Fig. 1A). 8-Bromo-cAMP, which activates protein kinase A, is not mitogenic in mesangial cells (Fig. 4). 8-Bromo-cAMP also failed to down-regulate Id1 and in fact slightly increased Id1 mRNA above levels in quiescent cells (Fig. 4). It is interesting to note that induction of an Id1 related gene in fibroblasts, HLH462 (Id3), is stimulated by addition of forskolin to activate adenylate cyclase and increase cAMP levels (14). A cAMP response element-like sequence has been identified in the promoter of the Id3 gene, although it is not yet known if this site recognizes and binds the cAMP response element binding protein (14). Thus, A-kinase-dependent signaling mechanisms might act to regulate Id1 gene expression in different cell types. Taken together, the present data suggest that Id1 is down-regulated by growth factors that bind to G protein-coupled receptors and receptor PTK and that down-regulation of Id1 mRNA correlates with the G_0 – G_1 transition.

Protein synthesis is required for down-regulation of Id1 mRNA in quiescent cells

To investigate the mechanism whereby Id1 is down-regulated in mesangial cells, we asked whether down-regulation by serum mitogens required new protein synthesis. A 30 min preincubation with cycloheximide (15 μ g/ml) prevented down-regulation of Id1 mRNA by serum (Fig. 5). In fact, cycloheximide slightly superinduced Id1 mRNA above basal levels. Cycloheximide did not alter levels of GAPDH mRNA, suggesting that the increase

Figure 4. Down-regulation of Id1 mRNA in quiescent mesangial cells correlates with mitogenesis. Quiescent mesangial cells were treated for 4 h with either quiescent medium (DMEM/0.5% FBS, vehicle control) or different classes of mesangial cell growth factors including 100 nM endothelin-1 (ET-1), 100 ng/ml epidermal growth factor (EGF), and 17% FBS. The non-mitogenic protein kinase A agonist, 8-bromo-cAMP (10 μ M), was also added. Total RNA was then extracted and Id1 mRNA was analyzed as described in Fig. 1. Identical results were observed in two independent experiments. (Right) In concurrent experiments in the same mesangial cell strains [3 H]thymidine uptake in response to the same agents was measured as described in Experimental Procedures. Data are mean $\pm$ SEM for three independent experiments in triplicate.

in Id1 mRNA did not reflect global increases in RNA polymerase II activity or mRNA stability. These results suggest a molecular basis for down-regulation of Id1 by mitogens. That cycloheximide blocked down-regulation of Id1 mRNA by serum suggests that *de novo* synthesis of a labile protein is required for repression

Figure 5. Cycloheximide blocks down-regulation of Id1 mRNA by FBS and superinduces Id1 mRNA transcripts. Quiescent mesangial cells were preincubated for 30 min with DMEM/0.5% FBS plus 15 μ g/ml cycloheximide (CHX) to block *de novo* protein synthesis (26). At time zero cells were treated with 17% FBS, and at the indicated times total RNA was extracted and Id1 and GAPDH mRNA levels were analyzed as described in Fig. 1. Identical results were observed in two independent experiments.

Figure 6. Transient appearance of E-box DNA binding activity concomitant with down-regulation of Id1 message. Quiescent mesangial cells were treated with fetal bovine serum (FBS) for the times indicated, after which cell extracts were prepared and binding reactions were performed using a 32 P-oligodeoxynucleotide probe containing a MEF-1 binding site as described in Materials and Methods. Equivalent amounts of protein were added in each lane. Specificity of bands 1 and 3 were indicated by homologous competition of 18 h extracts with a 100-fold molar excess of unlabeled MEF-1 probe. In two independent experiments only the higher mobility band (i.e., band 1) was transiently up-regulated.

of Id1 mRNA by a transcriptional or post-transcriptional (i.e., mRNA stability) mechanism.

Expression of E-box DNA binding activity and bHLH protein in mesangial cells

Our results indicating that Id1 is expressed and differentially regulated in mesangial cells suggests that Id1 may regulate E-protein DNA binding activity and contribute to transcriptional regulation in the kidney. To more directly address this issue, we carried out gel mobility shift assays to analyze E-box DNA binding activity in extracts from quiescent and serum-stimulated cells. Specific target promoters regulated by E box cis-elements have not been characterized in mesangial cells, so we employed an oligodeoxynucleotide probe that carried an E-box from the muscle creatine kinase enhancer, the muscle enhancer factor-1 (MEF-1) binding site, that binds several bHLH hetero-oligomeric complexes in nuclear extracts from cardiac and skeletal muscle, and from myeloid cells (19). Gel shift analysis revealed two distinct bands (bands 2 and 3, Fig. 6) of E-box DNA binding activity in quiescent cells, but only band 3 represented specific binding as band 2 was not reduced by homologous competition with a 100-fold excess of cold MEF-1 probe (Fig. 6). The intensity of band 3 was unaffected by serum treatment for 2–24 h. In contrast, a weak E-box DNA binding activity with higher mobility was detected at 4 h and reached a maximum at 18 h before returning to basal levels at 24 h (band 1, Fig. 6). These data suggest that when Id1 levels decline to a threshold level,

Figure 7. Expression of bHLH mRNAs in mesangial cells characterized by Northern RNA blot analysis. Total RNA (12 μ g each lane) was extracted from cycling and quiescent mesangial cells and analyzed by Northern RNA blotting as described in Fig. 1 using 32 P-cDNA probes for E12 and MyoA. Exposure times were 16 h for both E12 and MyoA blots. Longer exposures or the MyoA blot (i.e., 3 days) failed to reveal detectable message levels. Identical results were observed in two independent experiments.

bHLH proteins are made available to form functional homodimers or heterodimers that bind to E-box DNA sequences. It is not yet clear why only specific E-box DNA binding activities are transiently regulated in mesangial cells (i.e., band 1 vs. band 3). The constitutively expressed E-box DNA binding activity might represent binding of bHLH-leucine zipper factors, which are known to be insensitive to down-regulation by Id1.

Another important criteria to establish a functional role for Id1 and bHLH factors in mesangial cells would be co-expression of E proteins such as the alternatively spliced products of the E2A gene, E12 and E47. A current model for Id action is that Id associates with E proteins and prevents them from forming active hetero-oligomeric complexes with cell type-specific bHLH factors such as MyoD and myogenin (10, 13, 28). We therefore reasoned that an E protein gene would be co-expressed with Id1 in mesangial cells if the function of Id1 was to form complexes with E proteins. Quiescent and serum-treated mesangial cells expressed two mRNA transcripts that hybridized under high stringency to an E12 cDNA probe (Fig. 7). E12 mRNA was constitutively expressed and was not differentially regulated by serum mitogens. We also analyzed expression of other bHLH genes in mesangial cells. Because mesangial cells have a contractile, muscle-like phenotype, we asked whether genes related to the MyoD family were expressed in these cells. A previous report demonstrated that the kidney expresses an mRNA transcript which hybridizes to a MyoD-related cDNA clone, MyoA (7). However, we were unable to identify mRNA transcripts homologous to MyoA in mesangial cells (Fig. 7), suggesting that MyoA must be expressed in different regions of the nephron. Taken together, these results demonstrate that bHLH genes (E2A gene products) are expressed in mesangial cells but that they probably form hetero-oligomeric complexes with bHLH proteins distinct from the MyoA/MyoD family.

DISCUSSION

Most hierarchical models for cell-specific gene expression in developing organs postulate a role for a small group of master regulatory genes controlled in a precise temporal pattern (5, 29, 30). The majority of these genes encode proteins (i.e., MyoD, myogenin, Pit-1, GHF-1, Oct-2, etc.) that are sequence-specific DNA binding proteins acting in concert with other factors to control expression of cell-specific genes (i.e., albumin, muscle genes, etc.). Some of these transcription factors are expressed exclusively in specific tissues (i.e., MyoD in skeletal muscle) whereas expression of other factors is not restricted (i.e., HNF-1, C/EBP, etc.), which suggests that simple restriction of transcription factor activity is insufficient to explain tissue-specific expression. Transcription factors that regulate cell type-specific gene expression are important not only for development and differentiation but also for the response to cell injury since the process of repair recapitulates many aspects of development. At present relatively little is known about transcription factors that regulate cell type-specific gene expression in the kidney. The extreme heterogeneity of cells comprising the differentiated nephron has complicated attempts to understand nephrogenesis (31). The Wilm's tumor gene (*WT1*) encodes a protein expressed predominantly in the kidney and spleen that is implicated in both nephrogenesis and tumorigenesis (32). Although the exact role of WT1 has yet to be identified, the four COOH-terminal zinc fingers with a Cys₂-His₂ motif and a proline/glutamine-rich NH₂ terminal region suggest a possible role in transcriptional

regulation. Results of gene targeting experiments in embryonic stem cells are consistent with a crucial role for the *WT1* locus in urogenital development (33). HNF-1 is a transcription factor of the POU-homeodomain family that contributes to cell-specific expression of genes in the liver, and the observation that HNF-1 is abundantly expressed in differentiated renal tissue might suggest a role for this novel class of dimerizing homeodomain proteins in transcriptional regulation in the kidney (34). Another promising development is the recent cloning of a gene, *Kid1*, that encodes a zinc-finger-containing protein expressed exclusively in the kidney and is developmentally regulated (35). Although the arrangement of zinc finger motifs suggests that Kid1 might be a DNA binding protein, sequence-specific DNA binding has not yet been demonstrated. However, the non-zinc finger NH₂ terminal region of Kid1 acts as a strong transcriptional repressor when fused to the DNA binding domain of GAL4 (35). Gorski and coworkers (36) cloned a divergent homeobox gene, *Gax*, whose expression is largely confined to adult cardiovascular tissues. *Gax* is constitutively expressed in cultured renal mesangial cells [(36), Simonson and Walsh, unpublished data], but a putative role for *Gax* in transcriptional regulation in these cells has not been defined. Clearly, further analysis of transcription factors that control the phenotype of renal cells is important for understanding the molecular basis of nephrogenesis and the response of the kidney to injury.

Our present data suggest a possible role for Id1 and bHLH proteins in transcriptional regulation in renal mesangial cells. First, we have shown that both randomly cycling and serum-starved quiescent mesangial cells express abundant levels of Id1 mRNA. Previous studies have shown that Id2 but not Id3 is expressed in kidney, but in these experiments Id2 expression was not localized to specific cell types in the kidney nor was Id2 shown to be differentially regulated (14, 15). We are unaware of other studies to examine Id1 expression in renal tissue or in specific renal cell types. The failure to observe Id1-related messages on low-stringency Northern blots suggests that Id1 is the predominant Id family gene expressed in mesangial cells. Id1 expression is low in most differentiated, quiescent cell types (13–15, 19). Surprisingly, in the present experiments employing mesangial cells strains both quiescent and randomly cycling, serum-treated mesangial cells expressed abundant levels of Id1. The observation of Id1 expression in quiescent cells is particularly relevant to mesangial cell physiology as the mitotic index of mesangial cells *in vivo* is quite low (1, 2). Because proliferation and a phenotypic switch to an activated state is a common response of mesangial cells to glomerular injury, we assessed possible regulation of Id1 expression in quiescent mesangial cells treated with serum. In marked contrast to other cell types, in quiescent mesangial cells Id1 mRNA is transiently down-regulated by serum mitogens. Our experiments confirming previous reports of up-regulation of Id1 by mitogens in fibroblasts demonstrated that the finding of transient Id1 down-regulation in mesangial cells was not a technical artifact. Moreover, Id1 was transiently repressed over the same time course in which *c-fos* was up-regulated, confirming our previous studies showing a normal immediate early gene response in mesangial cells (23, 25). This unique observation of transient down-regulation of Id1 mRNA by serum mitogens in mesangial cells might reflect different roles for Id1 in specific cell types.

To further analyze the role of mitogens to regulate Id1 expression, we asked whether mitogens that bind to specific classes of receptors and initiate distinct pathways of mitogenic

signaling could transiently repress Id1. Endothelin-1 is a vasoactive peptide that binds to G protein-coupled receptors of the ET_A subtype in mesangial cells and stimulates mitogenesis (24, 25). Mitogenic signaling from ET_A receptors in mesangial cells requires both protein kinase C and a non-receptor protein tyrosine kinase activity (25). We showed that endothelin-1 added to quiescent mesangial cells induced transient down-regulation of Id1 with a similar time course as serum treatment. Addition of epidermal growth factor, which binds to and activates a receptor protein tyrosine kinase, also down-regulated Id1 mRNA with a similar time course as serum and endothelin-1. Unlike endothelin-1, however, mitogenic signaling by epidermal growth factor receptors in mesangial cells occurs largely independent of protein kinase C (25). These data suggest that down-regulation of Id1 mRNA is a common response to mitogenic stimulation of quiescent mesangial cells and is initiated by different classes of growth factor receptors that evoke distinct pathways of mitogenic signaling. Down-regulation of Id1 mRNA in mesangial cells was specific for growth factors and was not observed with a non-mitogenic cAMP analog, suggesting that Id1 is regulated concordantly with the G₀–G₁ progression in mesangial cells. Taken together with previous experiments showing that activation of adenylate cyclase stimulates Id3 mRNA levels, and that the Id3 promoter contains a cAMP response element-like sequence (14), our data suggest that Id gene expression is up-regulated by protein kinase A-dependent signaling mechanisms. We also showed that down-regulation of Id1 by mitogens appeared to involve *de novo* synthesis of a labile protein. Although relatively little is known about down-regulation of Id1 gene expression, a previous study showed that Id1 is down-regulated in osteoblasts by vitamin D (37). But in contrast to our present data, down-regulation of Id1 by vitamin D in osteoblasts is insensitive to cycloheximide (37). These results strengthen our conclusion that down-regulation of Id1 by mitogens occurs by distinct pathways in mesangial cells.

Transient down-regulation of Id1 mRNA in quiescent mesangial cells incubated with serum suggests that E-box DNA binding activity by bHLH proteins might be transiently up-regulated over the same time course. Using an oligodeoxynucleotide probe containing a MEF-1 site, which recognizes bHLH hetero-oligomeric complexes from numerous cell types, we observed two specific E-box binding activities in whole-cell extracts. One of these activities (band 1) was absent in extracts from quiescent cells but appeared with a delayed time course following down-regulation of Id1 message. Thus, steady state levels of Id1 mRNA were not tightly correlated with appearance of MEF-1 DNA binding complexes. This apparent lack of correlation between Id1 mRNA and MEF-1 binding was also observed in myeloid cells by Kreider and coworkers (19). One explanation for these data is that Id1 protein, which in cells is mostly complexed with E proteins (28), has a slow turnover. Perhaps binding of Id1 to E proteins stabilizes Id1 and protects against proteolytic degradation. Similarly, the lag between reappearance of Id1 mRNA and disappearance of MEF-1 DNA binding complexes might simply reflect slow translation rates and the time required to form E protein:Id1 heterodimers and lower bHLH complexes below a critical threshold level required for DNA binding. Regulation of Id1 activity is likely to be complex and further experiments are required to address these questions. Given the well-characterized ability of the MEF-1 site to selectively bind bHLH complexes, we suggest that band 1 represents transient up-regulation of a bHLH complex subsequent

to Id1 reaching some threshold level at which E proteins are no longer complexed with Id1 and are free to bind other bHLH proteins and form functional, sequence-specific DNA binding complexes. Because mesangial cell-specific promoters regulated by E-box *cis*-elements have not yet been identified, we can not directly test this hypothesis. However, given the clear ability of bHLH complexes to direct transcription in other cell types, it seems likely that the formation of active bHLH complexes would contribute to transcriptional regulation in mesangial cells. The present experiments also showed that mesangial cells express an E2A gene product, most likely E12. Constitutive expression of an E2A gene product in mesangial cells is important as E2A proteins are thought to be the major binding partner for Id genes (28). Our finding that MyoA/MyoD genes are not expressed in mesangial cells is consistent with the concept that different muscle types (i.e., skeletal and cardiac) express unique families of bHLH proteins that regulate transcription of muscle-specific genes (5–7). For example, genes regulated by the MyoD family are transcriptionally active in both skeletal and cardiac muscle, but MyoD family members are found only in skeletal muscle (8). E-box *cis*-elements are thought to contribute to cell type-specific transcription in cardiac muscle, but E12 and E47 probably complex with unique bHLH proteins in the myocardium. Thus, an important goal for future studies will be to characterize the bHLH proteins that form complexes with E2A gene products that direct transcriptional responses in mesangial cells. In addition, bHLH complexes and c-Jun have recently been shown to interact and reciprocally regulate transcription directed by AP-1 and E-box *cis*-elements (38,39). Therefore, transient down-regulation of Id1 in mesangial cells and the appearance of functional bHLH complexes might also affect the activity of other transcription factors in a form of nuclear cross-talk.

ACKNOWLEDGEMENTS

We are grateful to Drs Harold Weintraub and Cornelis Murre for providing cDNA clones. We also thank Dr Ken Walsh for stimulating discussions and comments on this manuscript. Supported by a grant from the National Institutes of Health DK-46939 and by a Grant-in-Aid 93-1514 from the American Heart Association (both to M.S.S.).

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