Cyclin A Downregulation and p21cip1 Upregulation Correlate With GATA-6–Induced Growth Arrest in Glomerular Mesangial Cells

Daisuke Nagata, Etsu Suzuki, Hiroaki Nishimatsu, Masao Yoshizumi, Toshiaki Mano, Kenneth Walsh, Masataka Sata, Masao Kakoki, Atsuo Goto, Masao Omata, Yasunobu Hirata

Abstract—The GATA-6 transcription factor is reported to be expressed in vascular myocytes. Because glomerular mesangial cells (GMCs) and vascular myocytes have similar properties, we examined whether GATA-6 was expressed in cultured GMCs and whether overexpression of GATA-6 induced cell cycle arrest in GMCs, using a recombinant adenovirus that expresses GATA-6 (Ad GATA-6). GATA-6 expression in GMCs was downregulated when quiescent GMCs were stimulated by serum to reenter the cell cycle. [3H]thymidine uptake was inhibited in GMCs infected with Ad GATA-6 in a dose- and time-dependent manner. The expression of cyclin A protein was decreased and that of the cyclin-dependent kinase inhibitor p21cip1 was increased in GMCs infected with Ad GATA-6. Although the expression of p21cip1 transcripts did not change remarkably, p21cip1 protein was stabilized in GMCs infected with Ad GATA-6, suggesting a post-transcriptional regulation of p21cip1 expression. Northern blot analysis showed that expression of the cyclin A transcript was decreased in Ad GATA-6–infected cells, whereas this decrease of cyclin A was not observed in GMCs derived from p21cip1 null mice. Our results demonstrate that GATA-6 is endogenously expressed in GMCs and that overexpression of GATA-6 can induce cell cycle arrest. Our results also show that GATA-6–induced cell cycle arrest is associated with inhibition of cyclin A expression and p21cip1 upregulation. Finally, our results indicate that the GATA-6–induced suppression of cyclin A expression depends on the presence of p21cip1. (Circ Res. 2000;87:699-704.)

Key Words: cell cycle • transcription factors • kidney • gene transfer • cyclin-dependent kinases

Glomerular mesangial cells (GMCs), which are interposed among glomerular capillaries, not only support the glomerular capillary loops but also have contractile and phagocytic properties. Like vascular smooth muscle cells (VSMCs), GMCs contain both actin and myosin filaments and contract in response to vasoactive agents such as angiotensin II and vasopressin.1,2 It is well established that proliferation of GMCs and increase in the surrounding matrix are implicated in the pathophysiology of some forms of glomerulonephritis. It is also well known that persistence of glomerulonephritis often results in renal hypertension (hypertension associated with renal parenchymal disease), which is the major cause of secondary hypertension, and renal failure. However, little attempt has been made to modulate the growth of GMCs using endogenously expressed genes that may be implicated in maintenance of the differentiated phenotype or quiescence of GMCs.

The GATA transcription factors comprise 6 isoforms. Among them, the GATA-1/-2/-3 genes are mainly expressed in hematopoietic cells, whereas the GATA-4/-5/-6 genes are predominantly expressed in the heart and gastrointestinal tract.3 Gene disruption experiments of each GATA transcription factor have shown that each GATA gene has distinct functions.4–9 We have recently shown that the GATA-6 gene was expressed in cultured human and rat VSMCs and that its expression is rapidly downregulated when quiescent cultured VSMCs are stimulated by serum mitogens to initiate cell cycle reentry.10 We have also shown that overexpression of GATA-6 gene induced cell cycle arrest in VSMCs and fibroblasts.11 These results suggest that GATA-6 is a candidate gene that may be implicated in maintenance of the differentiated phenotype in VSMCs and that overexpression of GATA-6 can inhibit proliferation of VSMCs.

Cell cycle progression is regulated by cyclin-dependent kinase (cdk), the activities of which oscillate during the cell cycle. Cdks are regulated by cyclins, positive coactivators, and cdk inhibitors.12,13 In mammalian cells, cyclin D–cdk4/ cdk6, cyclin E–cdk2, cyclin A–cdk2, and cyclin B–cdk2 are the main cyclin-cdk complexes that regulate the progression of G1, G1/S, S, and G2/M phases, respectively. Cdk inhibitors
comprise 2 families, the ink4 and cip/kip families. Cdk inhibitors of the cip/kip family are of particular interest in that they inhibit the activity of a broader spectrum of cdks, including cdk2, cdk4, and cdk6. The cip/kip family is composed of p21\(^{19-27}\), p27\(^{19-21}\), and p57\(^{19-27}\).

Given that GMCs and VSMCs have similar properties, it is tempting to speculate that the GATA-6 transcription factor may participate in maintenance of the quiescent phenotype in GMCs. To test the hypothesis, we examined whether the GATA-6 gene was expressed in GMCs and whether overexpression of GATA-6 inhibited GMC proliferation using a replication-defective adenovirus (Ad) expressing GATA-6.

**Materials and Methods**

**Cell Culture**
Rat GMCs were obtained from cultures of isolated rat glomeruli as previously described.\(^{17}\) GMCs were also isolated from glomeruli of p21\(^{−/−}\) mice. Rat VSMCs were cultured from rat thoracic aortas according to the explant method, as previously described.\(^{17}\) The HepG2 cell line, which is derived from human hepatoma cells, was cultured in DMEM with 10% FBS.

**Plasmids**
Details of the subcloning of hemagglutinin (HA) epitope–tagged human GATA-6 cDNA in a mammalian expression vector (pcDNA3HA-hGATA-6), isolation of the human cyclin A cDNA, and preparation of the full-length p21\(^{19-21}\) cDNA are described elsewhere (see online-only supplementary information; data supplement available at http://www.circresaha.org).

**Construction of a Replication-Defective Ad Encoding Human GATA-6**
Construction of a replication-defective Ad that expresses HA-tagged human GATA-6 (Ad GATA-6) was performed according to the cosmid cassette and Ad DNA-terminal protein complex (COS-TPC) method as previously described.\(^{19}\) A recombinant Ad that expresses glomerular-filtered phosphate (green fluorescent protein) was obtained from Quantum Biotechnologies.

**Adenoviral Infection**
Subconfluent GMCs were infected with Ad GATA-6 or Ad GFP in low-serum medium (DMEM/0.1% FBS) with multiplicity of infection (MOI) varying from 0 to 50. Cells were incubated in the low-serum medium for 72 hours and then restimulated with growth medium (DMEM/10% FBS) for 8, 16, and 24 hours.

**RNA Extraction and Northern Blot Analysis**
Total RNA was extracted using Trizol reagent (Gibco-BRL), and poly(A) RNA was purified using SCIGEN mRNA isolation kit (SCIGEN) according to the instructions provided by the manufacturer. Northern blot analysis was performed as previously described.\(^{17}\)

**Preparation of Protein Extracts**
Protein extracts were prepared as previously described.\(^{19}\)

**Western Blot Analysis**
Western blot analysis was carried out as previously described.\(^{19}\)
Primary antibodies were used at a dilution of 1:100.

**cdk2 Kinase Assays**
The assays were performed as previously described; 75 µg of each protein extract was used for the assays.

**Results**

**GATA-6 Is Expressed in Cultured GMCs**
We first examined whether GATA-6 was expressed in rat GMCs by Northern blot analysis using a probe corresponding to the zinc finger domain of human GATA-6. A single transcript was detected in GMCs, and the size of the transcript was identical with that observed for GATA-6 in VSMCs (Figure 1). The accumulation of GATA-6 transcripts was downregulated in quiescent GMCs by stimulation with serum mitogens, and the suppression was maximal at \(\sim\)8 hours after stimulation (19% expression compared with the expression level at 0 hours). The GATA-6 expression returned to the basal level 20 hours after stimulation.

**GATA-6 Induces Growth Arrest in GMCs**
To examine whether overexpression of GATA-6 induced growth arrest in GMCs, GMCs were infected with a recombinant Ad expressing HA-tagged human GATA-6 (Ad GATA-6). A recombinant Ad expressing GFP (Ad GFP) was used as the negative control. In GMCs treated with Ad GFP or PBS, stimulation with serum mitogen increased \[^{3}H\]thymidine incorporation in a time-dependent manner (Figure 2A). Infection with Ad GATA-6, infected at an MOI of 50, completely suppressed a serum mitogen-induced increase in \[^{3}H\]thymidine incorporation in a time-dependent manner (Figure 2A). We also examined the dose dependence of Ad GATA-6–induced inhibition of \[^{3}H\]thymidine uptake. Ad GATA-6, infected at an MOI varying from 0 to 100, inhibited serum mitogen-induced increase in \[^{3}H\]thymidine uptake in a dose-dependent manner (Figure 2B). Because the effect of Ad GATA-6 on cell growth reached a plateau at 50 MOI, all subsequent experiments were performed at 50 MOI unless otherwise specified.
GATA-6–Induced Changes in the Expression of the Cell Cycle–Regulatory Factors

We next examined the effects of Ad GATA-6 infection on the protein expression levels of the cell cycle–regulatory factors in GMCs (Figure 3). Expression of HA-tagged GATA-6 protein in Ad GATA-6–infected GMCs was confirmed by Western blot analysis using anti-HA antibody. Expression of cyclin D1 was induced by serum mitogen in a time-dependent manner, and Ad GATA-6 infection did not affect the induction, suggesting that the early G1 phase progressed normally in Ad GATA-6–infected cells. In contrast, time-dependent induction of cyclin A expression by serum mitogen was substantially inhibited by Ad GATA-6 infection. Consistent with previous observations,20 expression of p21 cip1 was induced by serum mitogen in a time-dependent fashion. However, expression of p21 cip1 was significantly higher in cells infected with Ad GATA-6 compared with those infected with Ad GFP at each time point examined (Ad GFP:Ad GATA-6: 1:3.3 ± 0.9, 8 hours after stimulation [n = 4, P < 0.05]; 1:3.7 ± 0.3, 16 hours after stimulation [n = 4, P < 0.01]). Expression of cyclin E, cdk2, cdk4, and p27kip1 did not change remarkably during the time course, and Ad GATA-6 infection did not affect their expression. We next examined whether amounts of cyclin A and p21 cip1 associated with cdk2 were indeed changed. Protein extracts prepared from cells infected with Ad GATA-6 or Ad GFP were immunoprecipitated with anti-cdk2 antibody and immunoblotted with anti–cyclin A antibody or anti-p21 cip1 antibody. As shown in Figure 4, the amount of cdk2-associated cyclin A was lower in Ad GATA-6–infected cells compared with that in Ad GFP–infected cells (63% of control level), whereas the amount of cdk2-associated p21 cip1 was higher in Ad GATA-6–infected cells (340% of control level).

Overexpression of GATA-6 Inhibits cdk2 Kinase Activity

We examined whether Ad GATA-6 infection inhibited cdk2 kinase activity. Anti-cdk2 antibody was used to immunoprecipitate total cdk2, and kinase activity was measured using histone H1 as the substrate. Serum mitogen induced cdk2 kinase activity in a time-dependent manner in control cells infected with Ad GFP. In contrast, a serum mitogen–induced increase in cdk2 kinase activity was inhibited to 41% (16 hours after stimulation [n = 4, P < 0.05]; 1:3.7 ± 0.3, 16 hours after stimulation [n = 4, P < 0.01]). Expression of cyclin E, cdk2, cdk4, and p27kip1 did not change remarkably during the time course, and Ad GATA-6 infection did not affect their expression. We next examined whether amounts of cyclin A and p21 cip1 associated with cdk2 were indeed changed. Protein extracts prepared from cells infected with Ad GATA-6 or Ad GFP were immunoprecipitated with anti-cdk2 antibody and immunoblotted with anti–cyclin A antibody or anti-p21 cip1 antibody. As shown in Figure 4, the amount of cdk2-associated cyclin A was lower in Ad GATA-6–infected cells compared with that in Ad GFP–infected cells (63% of control level), whereas the amount of cdk2-associated p21 cip1 was higher in Ad GATA-6–infected cells (340% of control level).
heat-stable inhibitors of cdk2 activity that could be largely immunodepleted with anti-p21\(^{\text{cip1}}\) antibody (see online Figure 1B; online-only data supplement available at http://www.circresaha.org).

**Overexpression of GATA-6 Inhibits Accumulation of Cyclin A Transcripts and Stabilizes p21\(^{\text{cip1}}\) Protein**

We next examined whether GATA-6–induced increase of p21\(^{\text{cip1}}\) expression and decrease of cyclin A expression occurred at the level of mRNA or protein. We performed Northern blot analysis using full-length human p21\(^{\text{cip1}}\) cDNA and human cyclin A cDNA as probes. A single cyclin A transcript was detected in GMCs. The accumulation of cyclin A transcripts increased 20 hours after stimulation with serum mitogen in Ad GFP–infected cells and remained increased until 32 hours after stimulation, whereas the accumulation of cyclin A transcripts was reduced significantly in Ad GATA-6–infected cells (28±6\% compared with Ad GFP infection, 24 hours after stimulation \(n=4, P<0.05\)) (Figure 6A). In contrast, GMCs appeared to express low levels of p21\(^{\text{cip1}}\) transcripts, because p21\(^{\text{cip1}}\) transcripts were not detected when total RNA was used for the analysis (data not shown). We therefore used poly(A) RNA extracted from GMCs that were stimulated with serum mitogen for 20 hours. In these samples, infection with GATA-6 had little or no effect on p21\(^{\text{cip1}}\) transcript levels (Figure 6B). The p21\(^{\text{cip1}}\) transcript levels in Ad GATA-6–infected GMCs increased 1.2-fold at the most, compared with those in Ad GFP–infected GMCs, suggesting the post-transcriptional regulation of p21\(^{\text{cip1}}\) expression. We therefore examined the stability of p21\(^{\text{cip1}}\) protein in Ad GATA-6–infected cells (Figure 6C). After induction of p21\(^{\text{cip1}}\) protein by stimulation with serum mitogen for 16 hours, 10 \(\mu\)g/mL of cycloheximide (CHX) was added to the medium, and the time course of the expression of p21\(^{\text{cip1}}\) protein was examined. The expression of p21\(^{\text{cip1}}\) in Ad GFP–infected cells decreased rapidly (30\% of control level, 30 minutes after incubation with CHX), whereas that in Ad GATA-6–infected cells was relatively stable (85\% of control level, 30 minutes after incubation with CHX), suggesting stabilization of p21\(^{\text{cip1}}\) protein in Ad GATA-6–infected cells.

**GATA-6–Induced Inhibition of Cell Cycle Progression and the Accumulation of Cyclin A Transcripts Depend on p21\(^{\text{cip1}}\) Expression**

We finally examined whether GATA-6–induced inhibition of cell cycle progression and cyclin A gene expression depended on the presence of p21\(^{\text{cip1}}\). GMCs obtained from wild-type and p21\(^{\text{cip1}}\)/− mice were infected with Ad GATA-6 or Ad GFP, and \[^{3}\text{H}\]thymidine uptake was examined 16 hours after stimulation of quiescent GMCs with serum mitogen. Forced expression of GATA-6 significantly inhibited \[^{3}\text{H}\]thymidine uptake in GMCs obtained from wild-type mice, whereas GATA-6 overexpression did not significantly inhibit \[^{3}\text{H}\]thymidine uptake in GMCs obtained from p21\(^{\text{cip1}}\)/− mice (Figure 7A). In accordance with the results, the expression of cyclin A transcripts in wild-type GMCs was inhibited by GATA-6 overexpression (55\% expression compared with Ad GFP infection, 24 hours after stimulation), whereas that in p21\(^{\text{cip1}}\)/− GMCs was not remarkably inhibited by GATA-6 overexpression (93\% expression compared with Ad GFP infection, 24 hours after stimulation) (Figure 7B), suggesting...
that GATA-6–induced inhibition of the cyclin A expression depended on p21<sup>cip1</sup> expression.

**Discussion**

GMCs share characteristics with VSMCs, and abnormal growth of GMCs has been implicated in the pathogenesis of glomerulonephritis. In this study, we first confirmed that GATA-6 was an endogenous gene expressed in GMCs and then examined the effects of GATA-6 overexpression on the growth of GMCs using a recombinant Ad that expressed human GATA-6.

Several data presented in this study suggested that upregulation of p21<sup>cip1</sup> was implicated in growth arrest induced by GATA-6 overexpression in GMCs. We have shown previously that GATA-6-induced growth arrest in VSMCs as well as in fibroblasts and that it was associated with p21<sup>cip1</sup> upregulation. Thus, upregulation of p21<sup>cip1</sup> appears to be a general mechanism by which GATA-6 induces cell cycle arrest. Several factors, including MyoD, CCAAT/enhancer binding protein α, vitamin D<sub>3</sub> receptor, Gax, and the tumor suppressor BRCA1 reportedly induced p21<sup>cip1</sup> expression, and the induction of p21<sup>cip1</sup> correlates with growth arrest or differentiation. Most of these factors induce p21<sup>cip1</sup> upregulation by stimulating transcription of the p21<sup>cip1</sup> gene. In contrast, transcriptional regulation did not appear to play a major role in GATA-6–induced upregulation of p21<sup>cip1</sup>, because the accumulation of p21<sup>cip1</sup> transcripts did not change remarkably compared with the changes in the amounts of p21<sup>cip1</sup> protein. Furthermore, p21<sup>cip1</sup> protein was stabilized in GMCs infected with GATA-6. These data suggest that the induction of p21<sup>cip1</sup> by GATA-6 is largely regulated at a post-transcriptional level. Along these lines, it is noteworthy that CCAAT/enhancer binding protein α stabilizes p21<sup>cip1</sup> protein.

We have also demonstrated, for the first time, that expression of cyclin A, but not of cyclin D1 or cyclin E, was specifically inhibited by GATA-6 infection. In contrast with p21<sup>cip1</sup>, the level of cyclin A transcripts was remarkably influenced by GATA-6 infection. The serum mitogen–induced accumulation of cyclin A transcripts was significantly inhibited by infection with Ad GATA-6, suggesting that the suppression of cyclin A occurred at the transcriptional level. However, because it was possible that GATA-6–induced inhibition of cyclin A expression might be related to cell cycle arrest induced by p21<sup>cip1</sup> upregulation, we examined cyclin A expression in the absence of p21<sup>cip1</sup>. Our results demonstrated that GATA-6 overexpression did not inhibit cell growth or the accumulation of cyclin A transcripts in p21<sup>cip1</sup>–/– GMCs, suggesting that GATA-6–induced suppression of cyclin A expression depended on p21<sup>cip1</sup> expression.

The results also suggested that GATA-6–induced inhibition of cyclin A expression might be a secondary effect of cell cycle arrest induced by p21<sup>cip1</sup> upregulation rather than a direct effect of GATA-6 on the cyclin A expression (see online-only supplementary information; data supplement available at http://www.circresaha.org). However, it should be emphasized that GATA-6–induced suppression of cyclin A expression appears to be a specific effect of GATA-6 rather than a nonspecific result of cell cycle arrest, because expression levels of other cell cycle–regulatory factors were not affected by GATA-6 overexpression.

Several studies have shown that it is possible to modulate the proliferation of GMCs by modifying the cell cycle–regulatory genes. Here, we have shown that GATA-6 is an endogenous factor in GMCs that can modulate the proliferation of these cells. GATA-6–induced growth arrest of GMCs was associated with inhibition of cyclin A expression and p21<sup>cip1</sup> upregulation. Therefore, modulation of GATA-6 gene expression in kidney may be a useful strategy to treat the progression of mesangial proliferative glomerulonephritis.

**Acknowledgments**

This study was supported in part by Grants-in-Aid 09281206 and 10218202 from the Ministry of Education, Culture and Science of Japan (to Y.H.). We thank Etsuko Taira and Marie Morita for technical assistance.
References


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_Circ Res._ 2000;87:699-704

doi: 10.1161/01.RES.87.8.699

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/8/699

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MATERIALS AND METHODS (SUPPLEMENT)

Plasmids: To prepare hemagglutinin (HA) epitope-tagged human GATA-6 cDNA in a mammalian expression vector (pcDNA3HA-hGATA-6), pcDNA1-hGATA-6 (1) was digested with ApaLI and XbaI and the insert was ligated into pcDNA3HA vector (2) at BamHI and XbaI sites, together with a double-strand oligonucleotide encoding the amino terminal fragment of the human GATA-6. The sense and antisense strands of the double-stranded oligonucleotides were as follows: Sense strand: 5'-GATCTTACCAGACCCTCGCCGCTCTCTCCAGCCAGGTCCCGCCGCTACGACGGCGCGCCGCCGGGCTTTAGC-3', antisense strand: 5'-TGACGAAGCCGCCGGCCTCGAGGCGGCCCGACCTGGTAG-3'. The cDNA encoding human cyclin A was isolated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from Hela cells as described below. One µg of total RNA was subjected to reverse transcription using Ready To Go/You-Prime First-Strand Beads (Pharmacia Biotech, Uppsala, Sweden) as instructed by the manufacturer. The primers used for RT-PCR were as follows: Reverse transcription: 5'-TTACAGATTTAGTGTCTCTGTT-3', sense primer: 5'-GGATC-CTTGGGCAACTCTGCGCGC-3', antisense primer: 5'-GGATCCTTACGATTAC-GTGTCTCTG-3'. These primers were designed to amplify the human cyclin A corresponding to the second codon up to the stop codon. The PCR conditions were 1 min at 95 °C, 1 min at 55 °C, and 1.5 min at 72 °C for 35 cycles, with final extension for 10 min at 72 °C. The PCR-amplified product was digested with BamHI, and subcloned in the pcDNA3HA vector at the BamHI site. The nucleotide sequences of the constructs were confirmed by cycle sequencing using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster, CA). The fragment excised from the pcDNA3HA vector by BamHI digestion was used as a probe for Northern blot analysis. pBluescript SK(+) -human p21cip1 (3) was digested with NotI and the
insert encoding the full length p21cip1 was used as a probe for Northern blot analysis.

RESULTS (SUPPLEMENT)

We also used anti-cyclin A antibody for immunoprecipitation and cyclin A-associated cdk2 activity was measured. As shown in Online Figure 1A, cyclin A-associated cdk2 kinase activity was inhibited to 43% in Ad GATA-6-infected cells compared with that in Ad GFP-infected cells. To confirm that inhibition of cdk2 kinase activity in Ad GATA-6-infected cells was partly mediated by p21cip1 upregulation, protein extracts prepared from Ad GFP-infected cells were mixed with boiled protein extracts prepared from Ad GATA-6-infected cells, and cdk2 kinase assay was performed (Online Figure 1B). The boiling procedure was necessary to inactivate cyclins and cdks contained in protein extracts prepared from Ad GATA-6-infected cells. cdk2 kinase activity observed in Ad GFP-infected cells was inhibited by mixing with preboiled protein extracts prepared from Ad GATA-6-infected cells (54±9% compared with Ad GFP-infected cells, n=4, p<0.01). This inhibition was abolished by immunodepletion of p21cip1 from the boiled protein extracts before mixing, while immunodepletion with normal rabbit serum or with anti-p27kip1 antibody (data not shown) before mixing did not fully restore cdk2 kinase activity. These results suggested that heat-stable inhibitors of cdk2 activity are induced in Ad GATA-6-infected cells and that the inhibitory activity can be largely immunodepleted with anti-p21cip1 antibody.

DISCUSSION (SUPPLEMENT)

The mechanism of GATA-6 inhibition of the cyclin A expression is not clear. Several studies have shown that the activating transcription factor (ATF)/cyclic AMP responsive element (CRE) site in the cyclin A gene was responsible for the full activity of the cyclin A promoter (4, 5). The promoter activity of the cyclin A gene was downregulated in contact-inhibition in bovine aortic endothelial cells (BAEC) via the ATF/CRE site (4). Another report showed that the
ATF/CRE site was also required for the cyclin A promoter activity and that protein levels or the phosphorylation of CRE-binding proteins were regulated by cell cycle progression (6). Thus, the ATF/CRE site appears to be a major regulatory site in the cyclin A promoter. Indeed, we observed that GATA-6-induced suppression of the cyclin A promoter activity was mediated by the ATF/CRE site in cotransfection assays in which several mutants of the cyclin A promoter were used (data not shown). However, our results of electrophoretic mobility shift assays showed that GATA-6 was not included in the complex which bound to the ATF/CRE site (data not shown). The GATA-6-induced inhibition of cyclin A expression might be a secondary effect of cell cycle arrest induced by p21cip1 upregulation rather than a direct effect of GATA-6 on the cyclin A promoter.

REFERENCES (SUPPLEMENT)


FIGURE LEGENDS (SUPPLEMENT)

Online Figure 1

A) GATA-6-induced inhibition of cyclin A-associated cdk2 kinase activity. GMCs were infected with 50 M.O.I. of Ad GATA-6 (GATA6) or Ad GFP (GFP) in low serum medium and then restimulated with serum mitogen for 16 hrs. Fifty μg of each protein extract was immunoprecipitated with anti-cyclin A antibody and cdk2 kinase activity was measured using histone H1 as the substrate. The same amount of each protein extract was immunoblotted with anti-cdk2 antibody, and anti-cyclin A antibody to show their amounts used for the assay.

B) GATA-6-infected GMCs contain heat-stable cdk2 inhibitory factors. Protein extracts prepared from Ad GATA-6-infected GMCs were boiled for 10 min (Boiled G6) and mixed with those prepared from Ad GFP-infected GMCs. The mixture was subjected to cdk2 kinase assay using anti-cdk2 antibody for immunoprecipitation. In some experiments, protein extracts prepared from Ad GATA-6-infected GMCs were boiled and incubated with anti-p21cip1 antibody (p21Ab) or control normal rabbit serum (NRS) before mixing experiments in order to immunodeplete p21cip1.