The Homeobox Gene GAX Activates p21WAF1/CIP1 Expression in Vascular Endothelial Cells through Direct Interaction with Upstream AT-rich Sequences*

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Tumors secrete pro-angiogenic factors to induce the ingrowth of blood vessels from the surrounding stroma, the end targets of which are vascular endothelial cells (ECs). The homeobox gene GAX inhibits angiogenesis and induces p21WAF1/CIP1 expression in vascular ECs. To elucidate the mechanism through which GAX activates p21WAF1/CIP1 expression, we constructed GAX CDNA s with deletions of the N-terminal domain, the homeodomain, or the C-terminal domain and then assessed these constructs for their ability to activate p21WAF1/CIP1. There was an absolute requirement for the homeodomain, whereas deleting the C-terminal domain decreased but did not abolish transactivation of the p21WAF1/CIP1 promoter by GAX. Deleting the N-terminal domain did abolish transactivation. Next, we performed chromatin immunoprecipitation and found, ~15 kb upstream of the p21WAF1/CIP1 ATG codon, an ATTA-containing GAX-binding site (designated A6) with a sequence similar to that of other homeodomain-binding sites. GAX was able to bind to A6 in a homeodomain-dependent manner and thereby activate the expression of a reporter gene coupled to this sequence, and this activation was abolished by mutating specific residues in this sequence. On the basis of the sequence of A6, we were then able to locate other ATTA-containing sequences that also bound GAX and activated transcription in reporter constructs. Finally, we found that the ability of these GAX deletions to induce G0/G1 arrest correlates with their ability to transactivate the p21WAF1/CIP1 promoter. We conclude that GAX activates p21WAF1/CIP1 through multiple upstream AT-rich sequences. Given the multiple biological activities of GAX in regulating EC function, identification of a putative GAX-binding site will allow the study of how GAX activates or represses other downstream targets to inhibit angiogenesis.

Angiogenesis is critical to the growth, invasion, and metastasis of human tumors because the diffusion of oxygen and nutrients is limited to ~1 mm in aqueous solution (1). Key to the process of angiogenesis is the vascular endothelial cell (EC)2 (2). In health, ECs respond to a balance between pro- and anti-angiogenic factors secreted by various cell types to maintain blood vessel homeostasis (3, 4). Tumors hijack this process by secreting pro-angiogenic factors to supply themselves with oxygen and nutrients, a transition known as the “angiogenic switch” (3, 4). Because targeting angiogenesis has developed into a promising avenue of research and treatment for malignancies (5), understanding the transcriptional regulation of the angiogenic phenotype in ECs has become particularly important.

Although the extracellular signals and downstream signaling pathways activated by pro- and anti-angiogenic factors have been topics of intense study (6–9), less is known about the transcriptional regulators that lead to the up- and down-regulation of batteries of genes necessary for the angiogenic phenotype. Because of their ubiquitous nature and their importance in regulating morphogenesis, organogenesis, cell proliferation and migration, and tumor formation, we considered it likely that homeodomain proteins (10–14) are involved in the transcriptional regulation of angiogenesis. Indeed, several homeobox genes have now been so implicated (15–26). For example, HOXD3 expression activates the angiogenic phenotype in vascular ECs through the activation of urine plasminogen activator and integrins αvβ3 and αvβ1 (15, 16), and HOXAS, a HOX cluster gene implicated in regulating p53 expression in human breast cancer (27–29), inhibits angiogenesis by down-regulating vascular endothelial growth factor receptor-1 and ephrin A1 in vascular ECs (26).

Recently, we reported that the homeobox gene GAX (whose mouse homolog is Meox-2) (30–33) strongly influences EC phenotype (20, 25). Originally described in vascular smooth muscle cells (VSMCs) (32, 34–38), in the adult, GAX is expressed primarily but not exclusively in the cardiovascular system and kidney (32, 39, 40) and in the placenta (41, 42). Pointing to a role in regulating VSMC proliferation and phenotype is its pattern of regulation, in which mitogenic signals result in a rapid down-regulation of GAX expression, whereas growth arrest signals induce a slower up-regulation (32). Given

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2 The abbreviations used are: EC, endothelial cell; VSMCs, vascular smooth muscle cells; HUVECs, human umbilical vein endothelial cells; shRNA, short hairpin RNA; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; VEGF, vascular endothelial growth factor; EMSAs, electrophoretic mobility shift assays.
its association with the mesoderm (31, 43), we looked for GAX expression in vascular ECs, observing an expression pattern similar to that observed in VSMCs (20, 25), with mitogenic and pro-angiogenic signals rapidly down-regulating GAX expression (25). Moreover, GAX also inhibits NF-κB signaling in vascular ECs (25), and inhibition of NF-κB activity is anti-angiogenic in several systems (44–49). Finally, GAX expression also inhibits angiogenesis in both in vitro and in vivo models (20, 25).

In addition to the inhibition of NF-κB signaling, a potentially important mechanism through which GAX could inhibit tumor-induced angiogenesis is the inhibition of cell cycle progression by activating the cyclin kinase inhibitor p21WAF1/CIP1 (20, 37). In vascular ECs, GAX also inhibits proliferation and transactivates p21WAF1/CIP1 (20). However, the sequences in the p21WAF1/CIP1 promoter and the likely mechanisms through which GAX accomplishes cell cycle arrest in vascular ECs have not yet been elucidated. In this study, we dissected the GAX protein and identified its homeodomain and polyhistidine (CAX or opa) repeat as important for DNA binding and transactivation, respectively. We also identified at least two upstream sequences to which GAX binds and thereby activates p21WAF1/CIP1 expression. These findings suggest that a major component of the mechanism by which GAX inhibits angiogenesis is its ability to induce cell cycle arrest in ECs by directly activating p21WAF1/CIP1 expression and thus inhibiting the early, proliferative stage of angiogenesis. GAX may thus represent a potentially promising target for the anti-angiogenic therapy of human tumors.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Human umbilical vein endothelial cells (HUVECs) and EGM-2 medium were obtained from Cambrex Corp. (Walkersville, MD). HUVECs were cultured according to the manufacturer’s instructions.

**Expression and Reporter Constructs**—GAX deletions were constructed by PCR. First, a full-length human GAX (amino acids 1–303; referred to as hugax) cDNA clone (32, 33) was isolated from HUVEC total RNA by reverse transcription-PCR. Next, the N-terminal fragment of hugaxNT (amino acids 1–245; referred to as hugaxN) cDNA clone (32, 33) was constructed by PCR. First, a full-length human GAX (hugax) cDNA clone (32, 33) was constructed by PCR. First, a full-length human GAX (hugaxNT, amino acids 1–187), hugaxN (amino acids 1–245), and an N-terminal deletion fragment of GAX (hugaxΔNT, amino acids 188–303) were generated by PCR with the appropriate primers. Constructs in which either the GAX homeodomain (amino acids 188–245; hugaxΔHD) or the GAX CAX (opa) repeat (hugaxΔCAX) was deleted were produced by overlap PCR. All cDNA deletion constructs contained EcoRI and XhoI restriction enzyme sites and were initially cloned into the pcDNA3.1 expression vector (Invitrogen) in-frame with a FLAG tag at the N-terminal end of the peptides. Mammalian retroviruses were similarly constructed as derivatives of LZRSpBMN-Z, in which the lacZ gene had been excised to make LZRSΔ (50). All PCR-amplified cDNA constructs were sequenced completely, and protein expression was verified by Western blotting.

The construction of the GAX expression vector pCGN-GAX and the replication-deficient adenoviral vectors expressing the rat and human homologs of GAX, all conjugated to the α-he-magglutinin epitope, has been described (37). The control replication-deficient adenoviral vector expressing green fluorescent protein was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey). The p21WAF1/CIP1 promoter–luciferase plasmid was a kind gift from Dr. Kenneth Walsh (Boston University). It contains 2.4 kb of p21WAF1/CIP1 upstream sequence beginning at nucleotide 11 in the cDNA and is the same construct used (20, 37) and described in detail (51) previously. Finally, the short hairpin RNA (shRNA) gene-silencing vectors targeted at GAX (Ad.shMex2-GAX) and green fluorescent protein (Ad.shGFP, control) were a kind gift of Dr. Berislav Zlokovic (52).

**Gene Expression and Promoter Constructs**—Transfections were carried out using TransIT® Jurkat transfection reagent (Mirus Bio Corp., Madison, WI) according to a modification of the manufacturer’s instructions. In general, a 1 µl/1 µg ratio of transfection reagent to plasmid was used, after which cells were incubated 16–24 h and then harvested for experiments.

**Protein Expression**—Whole cell extracts from treated HUVECs were electrophoresed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with phosphate-buffered saline (PBS) plus 5% non-fat dry milk and 0.1% Tween 20 before being incubated with the appropriate dilution of primary antibody (mouse anti-FLAG monoclonal, mouse anti-α-tubulin monoclonal, mouse anti-p21WAF1/CIP1 monoclonal, and mouse anti-p53 monoclonal; Sigma) in blocking solution or rabbit anti-GAX polyclonal antibody (40). Blots were washed with blocking solution and incubated with either goat anti-mouse or anti-rabbit IgG secondary antibody (Pierce) and then washed again with blocking solution. Bands were visualized by chemiluminescence using ECL Plus reagent (Amersham Biosciences) and quantified by densitometry, with each band being normalized to α-tubulin.

**Chromatin Immunoprecipitation (ChIP)**—ChIP assays utilizing HUVECs expressing GAX and its truncated forms were carried out as follows. HUVECs were infected with LZRSΔ vector, LZRSΔ–FLAG–hugax, or LZRSΔ–hugax for 2 days and then pelleted by centrifugation at 14,000 rpm. Pelleted nuclei were incubated with 0.125 M glycine was added, and the nuclei were further incubated for 5 min to stop the cross-linking reaction. For the remaining steps of protein isolation, all buffers used to isolate the proteins contained phenylmethylsulfonyl fluoride and protease inhibitor mixture. Cells were rinsed twice with cold PBS, harvested by gentle scraping, and pelleted by centrifugation at 2000 rpm for 4 min at 4 °C, after which the pellets were washed once with 1× PBS. The cell pellets were resuspended in 300 µl of cell lysis buffer and incubated on ice for 10 min to release the nuclei. Following that, nuclei were pelleted by centrifugation at 5000 rpm for 5 min at 4 °C. Lysis buffer (300 µl) was added to the pelleted nuclei, and the mixture was incubated on ice for 10 min to lyse the nuclei and to release the chromatin.

Chromatin samples were sonicated on ice to an average length of 600 bp and then pelleted by centrifugation at 14,000...
endogenous GAX to the p21\textsuperscript{WAF1/CIP1} promoter using previously generated rabbit polyclonal antibodies to the GAX protein (40).

Gene Promoter Assays—To verify regulation of p21\textsuperscript{WAF1/CIP1} transcription by genomic fragment A, in which positive binding for the GAX protein in the ChIP assay was observed, 1.5-kb fragments containing p21\textsuperscript{WAF1/CIP1} ChIP-A and p21\textsuperscript{WAF1/CIP1} ChIP-C were cloned and inserted into pGGL3. Cotransfection assays of reporter plasmid DNA and pcDNA3.1-FLAG-hugax and pcDNA3.1-FLAG-hugaxΔHD were performed as described above to determine which domains of the GAX protein are important for regulation of p21\textsuperscript{WAF1/CIP1} transcription. Four DNA fragments (A–D) were extended from ChIP probes A–D (each ~1.5 kb) and inserted into pGGL3-Basic luciferase reporter plasmid-inserted fragment p21\textsuperscript{WAF1/CIP1}.

Promoter activities were measured using constructs with the relevant regulatory sequences placed upstream of luciferase using a plasmid containing luciferase from \textit{Renilla reniformis} under the control of the SV40 promoter (pRL-SV) as a normalization control for transfection efficiency. Firefly and \textit{Renilla} luciferase activities were measured using the Dual-Luciferase assay kit (Promega Corp., Madison, WI), and firefly luciferase activity from the p21\textsuperscript{WAF1/CIP1} luciferase promoter construct was normalized to constitutive \textit{Renilla} luciferase activity. For each experiment, HUVECs at ~80% confluence in 6-well plates were transfected with different amounts of plasmid as described for individual experiments. HUVECs were then incubated with transfection reagents for 3 h and refreshed overnight with fresh endothelial cell basal medium and supplements (Cambrex Corp.). Empty pcDNA3.1-FLAG vector was used to equalize the total plasmid DNA transfected for each well.

Quantitative Real-time Reverse Transcription-PCR—After each ChIP assay, the resuspended chromatin immunoprecipitate and flow-through were subjected to quantitative real-time PCR utilizing TaqMan probes (53) to determine whether the immunoprecipitate was enriched in the p21\textsuperscript{WAF1/CIP1} upstream chromatin sequences of interest. Quantitative reverse transcription-PCR was carried out using a Cepheid SmartCycler thermocycler, with the associated SmartCycler Version 2.0 software used to analyze the data and to determine the threshold count (C\textsubscript{T}) for each reaction. The fluorophore used was SYBR Green, and the sequences of the primers and probes were the same as those used in the initial ChIP reaction described above. Real-time PCR cycles started with an initial 1.5-min denaturation step at 95 °C, followed by 30–40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s. Each sample was run in triplicate, and C\textsubscript{T} was determined for the target gene. To normalize the signal for each ChIP target and to identify which targets were enriched in the chromatin by GAX expression, immunoprecipitate and flow-through target gene levels were normalized to \textit{β}-actin sequence levels using the ΔΔC\textsubscript{T} method (54) as described previously (55, 56), and the results are presented as the ratio of chromatin-bound sequence to unbound sequence. Differences in the target/\textit{α}-actin ratio were evaluated using one-way analysis of variance, followed by Bonferroni’s post-test.

**Electrophoretic Mobility Shift and Supershift Assays**—To explore the possible direct binding sites of GAX on p21\textsuperscript{WAF1/CIP1}
GAX Activates p21<sup>WAF1/CIP1</sup>

**GAX Activates p21<sup>WAF1/CIP1</sup>** ChIP positive binding fragment A was analyzed by probes ~30 bp long as listed in Table 2. Purified PCR products (100 ng) were end-labeled using [γ-<sup>32P</sup>]ATP and T4 kinase. The resulting labeled probes were then purified on G-50 micro columns (Amersham Biosciences). Binding reactions were carried out in 1× binding buffer (50 mM Tris (pH 7.5), 25 mM NaCl, 3.5 μM MgCl<sub>2</sub>, 0.5 μM EDTA, 5% glycerol, 0.05% Nonidet P-40, and 0.25 mg/ml bovine serum albumin), 5 μM dithiothreitol, and 50 ng/μl poly(dI-dC) using 50,000 cpm labeled probe and 0, 100, or 200 ng of protein in 20 μl of reaction mixture at room temperature for 20 min. Binding complexes were separated from unbound probe on a 4% non-denaturing acrylamide gel. Gels were dried for 45 min and exposed to film at ~80 °C. For the supershift assay, the binding reaction was as follows: 1× binding buffer, 5 μM dithiothreitol, 50 ng/μl poly(dI-dC), 100–500 ng of protein, and 2 μl of anti-FLAG antibody in a total volume of 20 μl. Reactions were incubated on ice for 20 min, after which labeled probe (50,000 cpm) was added, and the mixture was incubated at room temperature for 10 min prior to gel electrophoresis.

**Flow Cytometry/Cell Cycle Analysis**—Flow cytometry and cell cycle analysis were performed using HUVECs as described previously (37). In brief, sparsely plated, randomly cycling HUVECs were transfected with pcDNA3.1-GAX or its truncated forms and incubated overnight in normal growth medium supplemented with 5 ng/ml vascular endothelial growth factor (VEGF). Cells were then harvested and resuspended in cold PBS. Approximately 1×10<sup>6</sup> cells were fixed with 3 ml of −20 °C cold absolute ethanol for 1 h at 4 °C, washed twice, and incubated with 1 ml of 50 μg/ml propidium iodide staining solution supplemented with 50 μl of 10 μg/ml RNase A for 3 h at 4 °C. Cells were pelleted and washed twice with PBS before flow analysis on a Beckman Coulter Cytomars FC500 flow cytometer.

**RESULTS**

**Activation of p21<sup>WAF1/CIP1</sup> Expression by GAX Requires the GAX Homeodomain and N-terminal Domain**—To test which domains of the GAX protein are important for the activation of p21<sup>WAF1/CIP1</sup> expression by GAX, we constructed multiple truncated GAX forms, inserted them into expression vectors, and tagged them with FLAG at the N terminus. These constructs (Fig. 1A) included FLAG-hugax (full-length), FLAG-hugaxNT (N-terminal domain lacking the homeodomain and C-terminal domain), FLAG-hugaxΔCT (deletion of the C-terminal domain), FLAG-hugaxΔNT (deletion of the N-terminal domain), and FLAG-hugaxΔHD (deletion of the homeodomain). To verify the production and activity of the full-length GAX protein produced by the expression construct, we transduced HUVECs with vectors expressing FLAG-hugax for 24 h (see “Experimental Procedures”) and then harvested the cells for protein for Western blotting using anti-FLAG antibody. GAX expression by this vector induced p21<sup>WAF1/CIP1</sup> expression by 2-fold (Fig. 1B), consistent with our previous findings (57). In addition, expression of GAX, even when driven by adenoviral vectors, did not alter detectably the level of p53 (data not shown), also consistent with previous observations in fibroblasts that GAX can still induce p21<sup>WAF1/CIP1</sup> in p53<sup>−/−</sup> cells (37). Protein expression for the remaining deletion constructs was verified by Western blotting using anti-FLAG antibody (Fig. 1C).

Finally, to determine which domains of the GAX protein are involved in activating p21<sup>WAF1/CIP1</sup> expression, we cotransfected our GAX deletion constructs with a reporter construct containing 2.4 kb of the p21<sup>WAF1/CIP1</sup> promoter immediately upstream of the transcriptional start site coupled to a luciferase reporter (p21<sup>WAF1/CIP1</sup> promoter-luciferase) (37, 51). We observed that, as expected, deletion of the homeodomain completely abolished the ability of GAX to transactivate the p21<sup>WAF1/CIP1</sup> promoter (Fig. 1D). In contrast, deleting the C-terminal domain decreased but did not abolish transactivation of the p21<sup>WAF1/CIP1</sup> promoter, but deleting the N-terminal domain had a stronger effect, decreasing the ability of GAX to transactivate the p21<sup>WAF1/CIP1</sup> promoter almost as much as deleting the homeodomain (Fig. 1D).

**Deletion of the GAX Repeat Coding for a Polyhistidine/Glutamate Region Abolishes Transactivation**—Having observed the effect of deleting the N-terminal domain of the GAX protein on transactivation of the p21<sup>WAF1/CIP1</sup> promoter, we noted that contained within the N-terminal domain of GAX is a (CA)<sub>n</sub> repeat motif, also known as an opa or M repeat (58). This motif is frequently found in developmentally regulated genes in Drosophila. It is also found in other homeobox genes such as HOXA1 (59), Dfd (60), and Antp and is believed to be an important transcriptional regulatory domain (61). An opa repeat near the N terminus of the GAX protein codes for an 18-amino acid polyhistidine/glutamate tract composed of 12 straight histidine residues, followed by six residues consisting of four glutamates and two histidines (32). We wished to determine whether this domain functions in transactivation of the p21<sup>WAF1/CIP1</sup> promoter (Fig. 2A). To this end, we constructed another GAX deletion construct, this time lacking only the opa repeat, and tested its ability to transactivate the p21<sup>WAF1/CIP1</sup> promoter by cotransfecting pcDNA3.1-hugaxΔCAX with the p21<sup>WAF1/CIP1</sup> promoter-luciferase reporter construct. Deleting the GAX repeat completely abolished the ability of GAX to activate the p21<sup>WAF1/CIP1</sup> promoter (Fig. 2B), indicating the importance of this motif for transcriptional activation by GAX.

**GAX Binds to a Sequence 15 kb Upstream of the p21<sup>WAF1/CIP1</sup> Start Codon**—Because we did not know a priori where GAX binds in the p21<sup>WAF1/CIP1</sup> promoter, we performed ChIP using primers designed to sample the chromatin at regular intervals using 200-bp amplicons, beginning at the start codon and proceeding to ~15 kb upstream (Fig. 3A). Surprisingly, the first sequence to which GAX could bind that we identified was located ~15 kb upstream of the p21<sup>WAF1/CIP1</sup> start codon (sequence A) (Fig. 3, A and B) and was not within the 2.4-kb p21<sup>WAF1/CIP1</sup> promoter (51). We compared the level of sequence A with those of all other sequences using quantitative reverse transcription-PCR normalized to the α-actin sequence and found considerable sequence A enrichment in the FLAG immunoprecipitate. None of the other sequences was enriched in chromatin, and sequence A was not detected above any of the other sequences as determined by ChIP carried out with vectors expressing GAX without the FLAG tag (Fig. 3C). These results confirm that there is an in vivo binding site for GAX ~15 kb upstream of the p21<sup>WAF1/CIP1</sup> start codon (sequence A).
upstream from the transcriptional start site of the p21WAF1/CIP1 gene.

Given these findings, we next wished to verify that GAX binds this upstream chromatin sequence through its DNA-binding homeodomain. Consequently, we repeated the ChIP assay; only this time, we used the FLAG-tagged GAX deletion constructs that we had made initially (Fig. 1A) and performed quantitative real-time PCR as described above (Fig. 3C) to determine which of these constructs produced an immunoprecipitate enriched in sequence A. We found that neither of the constructs that lacked the GAX homeodomain (hugaxNT and hugax/H9004 HD) resulted in enrichment of sequence A, whereas constructs containing the GAX homeodomain (wild-type hugax, hugax/H9004 NT, and hugax/H9004 CT) did (Fig. 3D). These results indicate that GAX binds to this upstream chromatin sequence (sequence A) through its homeodomain.

Identification of Multiple ATTA-containing Core Binding Sites for GAX—Because sequence A is 200 bp long, we wished to identify where in sequence A GAX binds. To this end, we designed several overlapping probes for use in electrophoretic mobility shift assays. The various pcDNA3.1-GAX constructs were cotransfected with a p21WAF1/CIP1 promoter-luciferase construct previously used to demonstrate that GAX transactivates the p21WAF1/CIP1 promoter. Deleting the homeodomain abolished transactivation. Similarly, deleting the N-terminal domain dramatically decreased transactivation, whereas deleting the C-terminal domain had only a minor effect. Each experiment was performed at least three times.
mobility shift assays (EMSAs) to determine which sequence was bound by GAX (Fig. 4A and Table 2). We found that A6 strongly bound GAX in EMSAs using recombinant GAX protein (Fig. 4B), and supershifts using anti-GAX antibody (40) demonstrated that the GAX protein was bound in this complex (Fig. 4C). The sequence of A6 contains an AT-rich sequence, with ATTACAATTA at its core. Because this resembles the DNA-binding sites of other homeodomain proteins (62), we systematically mutated residues beginning one residue to the 5' - and 3'-ends of this core sequence (named A6Mt1 through A6Mt11) and repeated the EMSAs. All of the mutations resulted in a significant decrease in GAX binding, but mutating residues in the second ATTA sequence in essence eliminated GAX binding altogether. Indeed, in particular, mutating the first T (A6Mt9) abolished binding completely (Fig. 4D).

To study the functional consequences of altering the sequence of this AT-rich site, we constructed three expression vectors (Fig. 5A). One (p21A6-Luc) contained A6 inserted upstream of a minimal promoter and a luciferase reporter. The others contained A6Mt1 inserted upstream of luciferase (p21A6Mt1-Luc), because this mutation resulted in the least diminution of GAX binding compared with the control, and A6Mt11 (p21A6Mt11-Luc), because this mutant completely abolished GAX binding (Fig. 4D). In cotransfection experiments, we observed that GAX effectively transactivated the reporter containing A6 (p21A6-Luc). In contrast, neither mutant (p21A6Mt1-Luc or p21A6Mt11-Luc) could be transactivated by GAX (Fig. 5B). From this, coupled with our initial ChIP results, we conclude that the AT-rich site that we have identified at −15 kb is likely to be an important regulatory ele-
ment contributing to the activation of p21WAF1/CIP1 expression by GAX and may be an enhancer.

Next, we asked whether other AT-rich sequences resembling the AT-rich sequence in A6 exist in p21WAF1/CIP1 chromatin and could also be bound and transactivated by GAX. Four additional such sequences were identified in the 15 kb of upstream chromatin previously surveyed, two near fragment B (named B1 and B2), one in fragment C (named C1), and one near fragment F (named F1). All were able to bind GAX in vitro in EMSAs (Fig. 6A).

Because it was the only such sequence in the 2.4-kb p21WAF1/CIP1 promoter (Fig. 6A), we were most interested in mutating the A6 sequence to determine whether it contributed to the activation of p21WAF1/CIP1 expression by GAX and may be an enhancer.

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GAX Activates p21WAF1/CIP1

A

AT-rich sites in upstream p21WAF1/CIP1 chromatin resembling A6

GAX

DNA probe

B

C

D

FIGURE 6. Identification of an additional AT-rich sequence to which GAX can bind and activate transcription. A, additional AT-rich sequences in the upstream chromatin of p21WAF1/CIP1. Additional AT-rich sequences containing ATTA repeats resembling the one identified by ChIP were located on the upstream chromatin of p21WAF1/CIP1. EMSAs were then performed using probes containing these sequences as well as the sequence identified by ChIP (sequence A6) as a GAX-binding site. B, effect of deleting the ATTA repeat in the core p21WAF1/CIP1 promoter. The AT-rich sequence containing the ATTA repeat was deleted from sequence C, and then the wild-type (p21C) and mutant sequences (p21C-mut) were extended to 1.5 kb by PCR and placed upstream of luciferase in the pGL3 plasmid to produce p21C-Luc and p21C-mut-Luc, respectively. These constructs were cotransfected with pcDNA3.1-FLAG-hugax at different ratios as described under "Experimental Procedures." Deleting the sequence containing two ATTA elements nearly completely abolished transactivation of p21C-Luc by GAX. C, ChIP assays for additional ATTA-containing sequences identified in p21WAF1/CIP1 chromatin. ChIP assay was carried out using the same methodology as described in the legend to Fig. 4B using the primer sets listed, which were targeted for sequences B1, B2, C1, and F1 (see "Results" and A for descriptions) and exogenously expressed FLAG-hugax. F, forward; R, reverse; IP, immunoprecipitate. D, ChIP assay for binding of endogenous GAX. ChIP assays were carried out on untransduced HUVECs in either normal growth medium (2% fetal bovine serum (FBS)) containing 5 ng/ml VEGF or low serum medium (0.1% fetal bovine serum) using rabbit anti-GAX polyclonal antibody. Sequence B was used as a negative control, as no enrichment of GAX immunoprecipitate, with a slight decrease in GAX binding to some of the sequences in HUVECs incubated in 2% serum plus 5 ng/ml VEGF compared with HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40). In all of these sequences, we were able to detect enrichment of chromatin containing these sequences in the GAX immunoprecipitate, with a slight decrease in GAX binding to some of the sequences in HUVECs incubated in 2% serum plus 5 ng/ml VEGF compared with HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40). In all of these sequences, we were able to detect enrichment of chromatin containing these sequences in the GAX immunoprecipitate, with a slight decrease in GAX binding to some of the sequences in HUVECs incubated in 2% serum plus 5 ng/ml VEGF compared with HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40).

Effect of Deletions of the Homeo-domain or the CAX Repeat on GAX Function—We have reported previously that GAX inhibits proliferation of VSMCs and that this effect depends upon p21WAF1/CIP1, as shown by the observation that p21−/− fibroblasts are not susceptible to GAX-induced cell cycle arrest (37). Consequently, we wished to determine the functional consequences of silencing endogenous GAX expression on p21WAF1/CIP1 expression in ECs and the ability of truncated GAX proteins to induce cell cycle arrest. First, we examined GAX and p21WAF1/CIP1 expression in HUVECs incubated in either normal growth medium (containing 2% fetal bovine serum) or low serum medium (0.1% fetal bovine serum) containing 5 ng/ml VEGF. In all cases, GAX binding to chromatin containing these sequences was detected (Fig. 6C). Finally, because all of our ChIP assays had been performed using HUVECs expressing GAX via an exogenous vector, we wished to determine whether endogenously expressed GAX binds the putative GAX-binding sites identified thus far as well. We therefore repeated the ChIP assays with the primer sets for sequences A6, B1, B2, C1, and F1, only this time in HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40). In all of these sequences, we were able to detect enrichment of chromatin containing these sequences in the GAX immunoprecipitate, with a slight decrease in GAX binding to some of the sequences in HUVECs incubated in 2% serum plus 5 ng/ml VEGF compared with HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40). In all of these sequences, we were able to detect enrichment of chromatin containing these sequences in the GAX immunoprecipitate, with a slight decrease in GAX binding to some of the sequences in HUVECs incubated in 2% serum plus 5 ng/ml VEGF compared with HUVECs not expressing exogenous GAX and using rabbit anti-GAX polyclonal antibody (40).
Finally, to correlate GAX-dependent up-regulation of p21WAF1/CIP1 expression with function, we determined the effect of expressing GAX and its truncated forms on the cell cycle of HUVECs. Sparsely plated, randomly cycling HUVECs were transfected with GAX or its truncated forms using empty vector as a control, incubated overnight in normal growth medium, and then harvested for cell cycle analysis. From the 30–40% fraction of HUVECs that started in G0/G1 phase under these conditions, GAX expression significantly increased the G0/G1 fraction by ~70% (Fig. 7C). Deleting either the CAX repeat-containing N-terminal domain or the homeodomain of GAX abrogated or greatly diminished the GAX-dependent increase in the G0/G1 fraction, indicating that these domains are both critical for this effect. Taken together with previous results showing that GAX-induced cell cycle arrest does not occur in p21−/− fibroblasts (37), these results suggest that both the homeodomain and N-terminal domain are required for p21WAF1/CIP1 induction and subsequent cell cycle arrest in ECs.

**DISCUSSION**

Interactions between tumors and their surrounding stroma, particularly in angiogenesis, are critical in regulating the growth and metastasis of tumors. One of the early phenotypic changes that ECs undergo during angiogenesis is re-entry into the cell cycle. Thus, identifying the factors that regulate EC proliferation is critical to understanding the process of angiogenesis and to developing therapeutic strategies to block it. One such strategy is to target EC proliferation in response to pro-angiogenic factors such as VEGF (8) and basic fibroblast growth factor (63). Another strategy is to target EC proliferation as part of anti-angiogenic therapy, often by targeting signaling pathways downstream from the binding of pro-angiogenic factors to cell-surface receptors, including the transcriptional programs that they activate. Because of the importance of EC proliferation in angiogenesis, the purpose of this study was to elucidate in more detail how GAX induces p21WAF1/CIP1 expression and thereby induces EC cell cycle arrest. Specifically, we were interested in 1) mapping the major domains of the GAX protein that are important in regulating this activity and 2) identifying potential DNA-binding sites.

![FIGURE 7. GAX expression causes G0/G1 cell cycle arrest in HUVECs associated with down-regulation of p21WAF1/CIP1.](image-url)
responsible for the activation of the p21WAF1/CIP1 promoter by GAX.

Our results indicate that the homeodomain is critical for the activation of p21WAF1/CIP1 expression by GAX. Moreover, the CAX (opa) repeat (58) in the N-terminal domain is also likely involved in transcriptional activation by GAX. This finding is compatible with the observation that the expansion of the CAX repeats of other homeodomain genes has been associated with neurodegenerative diseases and autism (59) even though the molecular function of the polyaminoc acid stretches encoded by these repeats remains unclear. Our results suggest one possible function for this domain in at least one homeobox gene, GAX. Moreover, the hypothesis that cell cycle arrest due to GAX depends upon its ability to induce p21WAF1/CIP1 expression is supported by our observation that truncated GAX proteins in which the homeodomain or N-terminal domain containing the CAX repeat is deleted have lost the ability to induce both p21WAF1/CIP1 and G0/G1 cell cycle arrest. These results suggest an important role for GAX in regulating EC proliferation. We acknowledge that many of our results were obtained using exogenous vectors to drive the expression of GAX. However, the observation that silencing endogenous GAX with shRNA also results in the down-regulation of p21WAF1/CIP1 (Fig. 7B) suggests that this result is indeed physiologically relevant, as does the observation that the sequences identified can also bind endogenous GAX in the ChIP assay (Fig. 6D).

We were also able to identify putative GAX-binding sites in the chromatin upstream of the p21WAF1/CIP1 transcriptional start site. The results of this assay identified multiple ATTA-containing sequences to which GAX can bind. The first sequence identified (A6) strongly resembles a universal homeobox DNA consensus sequence (Fig. 4) (62, 64). The results of our experiments in which individual residues within this binding site were mutated agree with results showing the importance of a CAATTA core sequence in homeodomain-binding sites (62). On the basis of the identification of A6, we identified other ATTA-containing sites in p21WAF1/CIP1 upstream chromatin that can also be bound by GAX (Fig. 6), and these also resemble homeobox gene-binding sites. Of these, only one (sequence C1) is located within the 2.4-kb p21WAF1/CIP1 promoter, but both sequences A6 and C1 could drive p21WAF1/CIP1 promoter activity in cotransfection experiments (Figs. 5B and 6B).

The importance of homeobox genes in the regulation of EC phenotype during tumor-induced angiogenesis is becoming increasingly more apparent with the recent descriptions of several homeobox genes that promote (15–19, 21, 22, 52) or inhibit (20, 23–26) the angiogenic phenotype. We have shown here that the homeobox gene GAX up-regulates p21WAF1/CIP1 expression through at least two CAATTA-containing sites, a site in the p21WAF1/CIP1 promoter and a site 15 kb upstream of the transcriptional start site. Moreover, we have shown that it is the homeodomain that is responsible for binding to this site and that the GAX polyhistidine (opa) repeat is involved in mediating transcriptional activation of the p21WAF1/CIP1 gene. Given that it is known that HOXA10 can bind to the p21WAF1/CIP1 promoter through the recruitment of its trimeric partners PBX1 and MEIS1 in myelomonocytic cells and thus induce differentiation (65), it is tempting to speculate that GAX may activate p21WAF1/CIP1 through a similar mechanism and/or be involved in the regulation of EC differentiation. Future studies will investigate these possibilities.

The results of this study are consistent with our previously published data on GAX activity in vascular cells indicating that it inhibits VSMC and EC proliferation (20, 25, 32, 37). However, we must note that Wu et al. (52) reported recently that, in the cerebral vasculature, GAX appears to have more pro-angiogenic effects. In brain ECs, downstream effects include transcriptionally suppressing AFX1 forkhead-mediated apoptosis and silencing GAX results in reductions in brain capillary density and attenuation of the angiogenic response to hypoxia. Potential explanations for this difference could include cell type-specific differences in GAX activity or more complex function in regulating the state of EC differentiation than our current understanding. More work will be needed to determine which of these is responsible for the apparently different activities of GAX in two different vascular beds or whether it is a combination of the two.

Taken together, however, our data coupled with other studies suggest that GAX is an important regulator of EC proliferation and angiogenesis. Moreover, given how GAX inhibits NF-κB signaling, the identification of a putative GAX-binding site responsible for the activation of p21WAF1/CIP1 expression will permit us to narrow down candidate chromatin sequences in NF-κB-dependent genes that might mediate the ability of GAX to block activation of target genes by NF-κB as well as other GAX-dependent promoters. These studies will allow us to determine the molecular mechanisms by which GAX inhibits EC activation and angiogenesis as well as suggest potential strategies for inhibiting angiogenesis by modulating GAX activity.

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The Homeobox Gene GAX Activates p21WAF1/CIP1 Expression in Vascular Endothelial Cells through Direct Interaction with Upstream AT-rich Sequences

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