Interaction between Hex and GATA Transcription Factors in Vascular Endothelial Cells Inhibits flk-1/KDR-mediated Vascular Endothelial Growth Factor Signaling

Received for publication, August 7, 2003, and in revised form, March 10, 2004
Published, JBC Papers in Press, March 10, 2004, DOI 10.1074/jbc.M308730200

Recent evidence supports a role for GATA transcription factors as important signal intermediates in differentiated endothelial cells. The goal of this study was to identify proteins that interact with endothelial-derived GATA transcription factors. Using yeast two-hybrid screening, we identified hematopoietically expressed homeobox (Hex) as a GATA-binding partner in endothelial cells. The physical association between Hex and GATA was confirmed with immunoprecipitation in cultured cells. Hex overexpression resulted in decreased flk-1/KDR expression, both at the level of the promoter and the endogenous gene, and attenuated vascular endothelial growth factor-mediated tubule formation in primary endothelial cell cultures. In electrophoretic mobility shift assays, Hex inhibited the binding of GATA-2 to the flk-1/KDR 5′-untranslated region GATA motif. Finally, in RNase protection assays, transforming growth factor β1, which has been previously shown to decrease flk-1 expression by interfering with GATA binding activity, was shown to increase Hex expression in endothelial cells. Taken together, the present study provides evidence for a novel association between Hex and GATA and suggests that transforming growth factor β-mediated repression of flk-1/KDR and vascular endothelial growth factor signaling involves the inducible formation of inhibitory Hex-GATA complexes.

The endothelium displays remarkable diversity in health and disease. Endothelial cell phenotypes are modulated in space and time by mechanisms that involve the highly coordinated combinatorial action of transcriptional modules (1). The GATA family of transcription factors consists of six members (GATA-1 to -6). Of the various GATA factors, GATA-2, -3, and -6 are expressed in vascular endothelial cells (2). Initially, these transacting factors were believed to play a singular role in the differentiation of endothelial cells during development (3–5). However, more recently, studies have provided evidence that GATA factors also function as “immediate early genes,” coupling changes within the extracellular environment to changes in downstream target gene expression (6, 7). In addition to binding directly to their consensus motif (A/T)GATA(A/T), GATA factors have been shown to participate in a wide array of protein-protein interactions with Zinc finger type transcription factors, including SP1, EKLF, RBTN2, ER, and GATA itself (8–13); homeobox transcription factors such as HNF-1α, Nkx-2.5, Pit-1, and TTF-1 (14–17); Ets family PU.1 (18); MADS box protein SRF (19); RUNT domain protein AML1 (20); AP1 (21); NF-ATc (22). During cell differentiation, GATA factors may be associated with cofactors, such as basal transcription factors, FOG-1, FOG-2, CBP/p300, and histone deacetylase-3 (23–29). Taken together, these data suggest that GATA transcription factors are involved in highly complex regulatory pathways and that the dissection of these networks may provide valuable insight into the transcriptional control of endothelial phenotypes.

To that end, the goal of the present study was to identify partner proteins that interact with GATA factors in endothelial cells. Using a yeast two-hybrid system, we describe a physical interaction between GATA family of transcription factors and the homeobox protein Hex. The interaction between Hex-GATA is associated with reduced GATA binding activity and transcriptional activity, decreased expression of the GATA-2 target gene, flk-1/KDR, and secondary attenuation of VEGF-medi- ed signaling. Finally, we present data supporting the notion that TGF-β exerts its anti-angiogenesis effect by a Hex-GATA-flk-1/KDR-dependent mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells (HUVEC) (Clonetics, La Jolla, CA) were cultured in EGM-2 MV medium (Clonetics). Human embryonic kidney (HEK)-293 cells (ATCC CRL-1573) and COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. HUVEC were used within the first eight passages. Plasmids—The construction of the flk-1/KDR-luc, flk-1/KDR (GATA mut), and flk-1/KDR (SP1 mut) plasmids were previously described (30). Human GATA-2 expression plasmid (pMT1-GATA2) was a kind gift from Dr. Tatsuo Kodama (31). The abbreviations used are: VEGF, vascular endothelial growth factor; TGF, transforming growth factor; HUVEC, human umbilical vein endothelial cell(s); HEK, human embryonic kidney; GBD, Gal4 DNA-binding domain; GAD, Gal4 activation domain; UTR, untranslated region; EGFP, enhanced green fluorescence protein; IRES, internal ribosome entry sites; GAFDH, glyceraldehyde-3-phosphate dehydrogenase.
Human GATA-3 and -6 and Hex cDNA fragments were amplified using PCR from reverse-transcribed HUVEC total RNA. To generate the Hex expression vector, Hex cDNA was subcloned into the pcDNA3 vector (Invitrogen). To generate the plasmids expressing Gal4 DNA-binding domain (GBD) fused with GATA-2, -3, and -6, each of the three GATA cDNA fragments was inserted into pGBK7 (Clontech, Palo Alto, CA). The resulting mixture was then transformed into yeast (Saccharomyces cerevisiae) for 1 h at 4°C. The membrane with 1 ml of cell lysis buffer containing 1.5% IGEPAL CA-630. Each plasmid was mixed and incubated with protein G-Sepharose (Amersham Biosciences) for 1 h at 4°C overnight at 4°C. Colonies complemented by histidine autotrophy were isolated and confirmed to be positive by β-galactosidase assay according to the manufacturer's instruction (Clontech; yeast protocols handbook). The plasmids from the positive colonies were purified, and the inserts were subsequently sequenced. To analyze the protein-protein interaction, Y190 yeast were co-transfected with pGAD-Hex and a plasmid in which GBD was fused either with GATA-2, -3, and -6. Similarly, Y190 yeast were co-transfected with pGBD-GATA2 and a plasmid containing GAD fused with Hex. The cells were spread and incubated on the synthesized dropout medium plate without tryptophan and leucine.

**Transfection of COS-7 Cells and Immunoprecipitation Assays**—COS-7 cells were co-transfected with either pFLAG-Hex and pMT2-GATA2, or pFLAG-GATA2 and pcDNA3-Hex expression plasmids using TransGene 6 reagent (Roche Applied Science) as instructed by the manufacturer. Two days later, the transfected cells were freeze-thawed three times and incubated for 30 min on ice with cell lysis buffer (0.1% IGEPAL CA-630, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5 µg/ml pepstatin A, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 200 µM phenylmethylsulfonyl fluoride, pH 7.5), followed by centrifugation at 20,000 × g for 5 min. The supernatant was incubated with anti-FLAG M5 monoclonal antibody (Sigma), anti-Myc monoclonal antibody (Invitrogen), or anti-GATA-2 antibody (Santa Cruz, CA).

Alternatively, subconfluent HUVEC (2 × 10^7 cells) were harvested for nuclear extracts according to the mild nitrogen cavitation method (31) to keep intact the protein-protein interactions. 1 µg of nuclear extracts was precleared by centrifugation with 4 µg of control IgG (Santa Cruz). The resulting supernatant was incubated with 30 µg of agarose-conjugated anti-GATA-2 monoclonal antibody (Santa Cruz, sc-267 AC) or an identical amount of isotype-matched mouse control IgG (Santa Cruz, sc-2145) overnight at 4°C. The immunobilized samples were separated and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was then probed for Hex and GATA-2 by Western blot analysis, using anti-Hex antibody (generated by Dr. Tsumi Noguchi, Japan) and anti-GATA-2 antibody (Santa Cruz, sc-16044, and generated by Dr. Stuart H. Orkin, Harvard Medical School, Boston), respectively. The complexes were visualized with an ECL advance Western blotting detection kit (Amersham Biosciences).

**RNA Isolation and RNA Protection Assays**—HUVEC were serum-starved in EBM-2 medium containing 0.5% fetal bovine serum. 18 h later, HUVEC were treated with 2.5–10 ng/ml TGF-β1 (Peprotec, Rocky Hill, NJ) for 18 h (for Hex) or 24 h (for fliK/KDR). Alternatively, HUVEC were infected with adenoviruses encoding the IRES-mediated green fluorescence protein (EGFP) (Adeno-Blank) or IRES coupled to Hex and EGFP (Adeno-Hex). Adeno-Blank and Adeno-Hex were generated with a ligation into adenovirus cosmid vector and a co-transfection into HEK-293 (32). Infections were carried out at a multiplicity of infection of 20 for 24 h. HUVEC were harvested for total RNA at the times indicated, using the Isogen reagent (Nippon Gene). For *in vitro* transcription, fliK/KDR-, Hex-, and GAPDH-specific 32P-labeled riboprobes were synthesized from pGEM-mflk, pGEM-hHex, and pGEM-hGAPDH, respectively. Riboprobes were synthesized using SP6 (for fliK/KDR and GAPDH) or T7 (for Hex) RNA polymerase (Ambion, Austin, TX) and purified with a G-50 spin column (Amersham Biosciences). RNase protection assays were performed with a RPA III kit (Ambion) according to the manufacturer’s instructions.

**Transfections of HEK-293 Cells or HUVEC and Analysis of Luciferase Activity**—HEK-293 cells and HUVEC were transfected as described previously (30). Briefly, either 2 × 10^5 cells/well of HEK-293 cells or 1 × 10^5 cells/well of HUVEC were seeded onto 12-well plates 18–24 h before transfection. 0.05 pmol of the luciferase reporter plasmid (either KDR-, KDR (GATA mut)-, or KDR (SP1 mut)-luc), 50 ng of pRL-CMV (Promega), 0.075 pmol of the GATA expression vector and either 0.0375 or 0.075 pmol of Hex expression vector were incubated with 2 µl of FuGENE 6. As a negative control, empty vector (pMT2 and pcDNA3) was transfected instead of GATA-2 and Hex expression vector, respectively. 24 h later for HEK-293 cells and 48 h later for HUVEC, the cells were washed with phosphate-buffered saline, lysed, and assayed for luciferase activity using the dual luciferase reporter assay system (Promega) and a Lumat LB 9507 luminometer (Berthold, Gaithersburg, MD).
for 1 h at 37 °C. HUVEC infected with Adeno-Blank or Adeno-Hex were seeded at 1 × 10^5 cells/well and incubated for 24 h in 5% CO_2. The medium was removed, and the HUVEC were covered with 400 μl of medium was removed, and the HUVEC were covered with 400 μl of EGM-2 MV media in the absence of basic fibroblast growth factor, in the presence or absence of SU1498 (Calbiochem, San Diego, CA). Two days later, a branched capillary network was visualized under a microscope. Images from at least three different areas in each well were captured by a digital camera under a microscope.

**Nuclear Extracts and Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared as previously described (30, 33). Double-stranded oligonucleotides were labeled with [α-^32^P]dCTP and Klenow fragment and purified by spin column (Amersham Biosciences). 10 μg of HUVEC nuclear extracts were incubated with 10 fmol of ^32^P-labeled probe, 1 μg of poly(dI-dC), and 3 μl of 10× binding buffer (100 mM Tris HCl, pH 7.5, 50% glycerol, 10 mM dithiothreitol, 10 mM EDTA) for 30 min at the room temperature, followed by 30 min at 4 °C. The following oligonucleotides sequences were used for probes: 5′-UTR GATA motifs, 5′-GGCAAGCCTGAGGATCTCCTGGCTTCTCCTGCA-3′; GATA-mut motifs, 5′-GGCA-AGGTTGGCGAAGCTTCTCCTGCA-3′; flk-1 SP1 motifs, 5′-GGTGAGGG-CTCCTGCA-3′; and flk-1 SP1-mut motifs, 5′-GGTGAGGGCTCCTGCA-3′. To test the effect of antibodies on DNA-protein binding, the nuclear extracts were preincubated with monoclonal antibody to GATA-2 (we prepared from the antigen GATA-2 protein binding, the nuclear extracts were preincubated with nonimmune antibody (lane 7) and then separated by 8% SDS-PAGE. The transferred membrane was immunoblotted with anti-GATA2 (upper panel) or FLAG (lower panel) antibody. The closed arrowhead indicates the specific GATA-2 and FLAG-tagged Hex complex. B, expression vectors either pFLAG (lanes 2 and 5), pFLAG-GATA2 (lanes 1, 3, 4, and 6), pMyc (lanes 1 and 4), or pMyc-GATA2 (lanes 2, 3, 5, and 6) were co-transfected into COS-7 cells. Extracted proteins from the cells were untreated (lanes 1–3) or precipitated by either anti-FLAG antibody (lanes 4–6) and then separated by 8% SDS-PAGE. The transferred membrane was immunoblotted with anti-Myc (upper panel) or GATA-2 (lower panel) antibody. The closed arrowhead indicates the specific FLAG-tagged GATA-2 and Myc-tagged Hex complex. The faster migration complex (open arrowhead) represents nonspecific immunoreactants. The results are representative of three independent experiments. C, 80 μg of COS-7 (lanes 1 and 3) and HUVEC (lanes 2 and 4) were separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with anti-Hex antibody (left panel) and anti-GATA-2 antibody (right panel). D, nuclear extracts from HUVEC were immunoprecipitated by either anti-GATA2 antibody or control mouse IgG, then separated according to supernatant (sup., lane 1) and precipitate (ppt., lanes 2 and 3). The samples were electrophoresed and immunoblotted with anti-Hex and anti-GATA-2 antibodies. The arrow indicates the specific Hex and GATA protein.
FIG. 3. Hex suppresses GATA-2-mediated flk-1/KDR promoter activity. A, HEK-293 cells were transiently transfected with luciferase reporter plasmid containing the flk-1/KDR promoter fragment (+115 to +296) (KDR-luc), either human GATA2 expression plasmid (pMT2-GATA2) or vector alone (pMT2), and either human Hex expression plasmid (pcDNA3-HEX) or vector alone (pcDNA3). After 24 h of incubation, the cells were lysed and assayed for luciferase activity. *, p < 0.01 compared with the activity from co-transfection with KDR promoter, pMT2-GATA2, and pcDNA3. B, HUVEC were transiently transfected as above. After 48 h of incubation, the cells were lysed and assayed for luciferase activity. *, p < 0.002 compared with the activity from co-transfection with KDR-luc, pMT2-GATA2, and pcDNA3; §, p = 0.002 compared from co-transfection with KDR (SP1 mut)-luc, pMT2, and pcDNA3; ‡, p = 0.08 (not significant) compared from co-transfection with KDR (GATA mut)-luc, pMT2, and pcDNA3. The expression levels were normalized to pRL-SV40 activity and expressed as fold induction relative to co-transfection with vector alone. The means and standard deviations were derived from at least three separate experiments performed in triplicate.

FIG. 4. Hex suppresses endogenous flk-1/KDR mRNA in human endothelial cells. A, HUVEC were infected with adenovirus expressing IRES-mediated EGFP (Blank) or IRES-mediated rat Hex and EGFP (HEX) at a multiplicity of infection of 20 plaque-forming units/cells and then incubated for 48 h. The cell extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with anti-HEX antibody. M.W., molecular mass. B, HUVEC were infected with adenovirus expressing IRES-mediated EGFP (Blank) or expressing IRES-mediated rat Hex and EGFP (HEX) at a multiplicity of infection of 20 plaque-forming units/cells for 48 h, at which time total RNA was isolated. In RNase protection assays, an [α-32P]UTP-labeled 384-bp human flk-1/KDR riboprobe was incubated with no RNA (lane 1), 10 μg of yeast tRNA (lane 3), 10 μg of total RNA from control (Blank) HUVEC (lane 4), or Hex-overexpressed (Hex) HUVEC (lane 5). The protected fragment (266 bp) represents the human flk-1/KDR transcript. An [α-32P]UTP-labeled GAPDH riboprobe was hybridized with total RNA as an internal control.
FIG. 5. Hex reduces binding of GATA motif on the flk-1/KDR and GATA-binding proteins. A, electrophoretic mobility shift assays were performed with 32P-labeled 5′-UTR GATA probe and 10 µg of nuclear extract from HUVEC infected with adenovirus expressing IRES-mediated EGFP (Adeno-Blank) or adenovirus expressing IRES-mediated Hex and EGFP (Adeno-Hex). The open and closed arrows indicate specific DNA-protein complexes. In competition assays, 10-, 100-, and 500-fold molar excesses of unlabeled self-probe and mutant 5′-UTR GATA probe...
Identification of Hematopoietically Expressed Hex as a GATA-interacting Protein by Yeast Two-hybrid Screening—The GATA family of transcription factors has been implicated not only in the early differentiation of endothelial cells but also in the transduction of extracellular signals in the adult endothelium. Several members of the GATA family have been identified in endothelial cells, including GATA-2, -3, and -6. Previous studies have shown that GATA-1 and -2 interact with partner proteins in erythroid and megakaryocyte cells (34). Our goal was to identify the proteins that interact with GATA transcription factors in endothelial cells. To that end, we employed a yeast two-hybrid system in which full-length human GATA-6 served as bait. From a screen of 8.6 \times 10^5 clones, a total of 58 clones demonstrated histidine auxotrophy. After elimination of false positives, 16 clones were selected, one of which encoded a fragment (amino acids 75–270) of Hex (Fig. 1A). To confirm the specificity of interaction between Hex and GATA-6, constructs containing the Gal4-activating domain fused with full-length Hex (pGAD-Hex) and either the Gal4-binding domain fused with GATA-2 (pGAD-GATA2) or the GATA-6-binding domain alone (pGBD) were co-expressed in yeast AH109. As shown in Fig. 1B, co-expression of pGAD-Hex and pGBD-GATA6 resulted in a 4.2-fold increase in \( \beta \)-galactosidase activity compared with co-expression of pGBD and pGAD-Hex.

We next wished to determine whether Hex interacts with human GATA-2 and -3. To that end, pGAD-Hex was co-expressed in yeast AH109 with constructs containing the Gal4-binding domain fused either with GATA-2 or -3 (pGBD-GATA2 or GATA3, respectively) As shown in Fig. 1C, co-expression with human GATA-2 and -3 resulted in 5.8- and 4.7-fold induction of the \( \beta \)-galactosidase activity, respectively, compared with co-expression with Gal4-binding domain alone (pGBD). Taken together, these results suggest that Hex interacts with GATA-2, -3, and -6.

Physical Interaction between Hex and GATA-2 in Mammalian Cells—Having identified the interaction between GATA and Hex in the yeast two-hybrid system, we wished to determine whether this interaction occurs in mammalian cells. Of the various members of the GATA family of transcription factors, GATA-2 is expressed most abundantly in cultured endothelial cells (34). Several members of the GATA family have been identified in endothelial cells, including GATA-2, -3, and -6. Previous studies have shown that GATA-2, -3, and -6 interact with partner proteins in erythroid and megakaryocyte cells (34). Our goal was to identify the proteins that interact with GATA transcription factors in endothelial cells. To that end, we employed a yeast two-hybrid system in which full-length human GATA-6 served as bait. From a screen of 8.6 \times 10^5 clones, a total of 58 clones demonstrated histidine auxotrophy. After elimination of false positives, 16 clones were selected, one of which encoded a fragment (amino acids 75–270) of Hex (Fig. 1A). To confirm the specificity of interaction between Hex and GATA-6, constructs containing the Gal4-activating domain fused with full-length Hex (pGAD-Hex) and either the Gal4-binding domain fused with GATA-2 (pGAD-GATA2) or the GATA-6-binding domain alone (pGBD) were co-expressed in yeast AH109. As shown in Fig. 1B, co-expression of pGAD-Hex and pGBD-GATA6 resulted in a 4.2-fold increase in \( \beta \)-galactosidase activity compared with co-expression of pGBD and pGAD-Hex.

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Hex Inhibits GATA-2-mediated flk-1/KDR Promoter Activity—We next wished to study the functional relevance of the interaction between GATA-2 and Hex. We have previously shown that GATA-2 binds to a GATA motif in the 5′-UTR region of the flk-1/KDR promoter and that this effect is necessary for full expression (35). To determine the effect of Hex on GATA-2-mediated activation of flk-1/KDR, transactivation assays were carried out in HEK-293 cells (nonendothelial cells) and HUVEC (endothelial cells) co-transfected with KDR-luc (−115 and +296 flk-1/KDR coupled to luciferase) and an expression plasmid containing either human GATA-2 (pMT2-GATA2) or Hex (pcDNA3-Hex). As a negative control, the cells were co-transfected with vector alone (pMT1, or pcDNA3). Consistent with our previous findings, the basal level of flk-1/KDR promoter activity in HEK-293 cells was significantly transactivated by 4.2-fold with overexpression of GATA-2 (Fig. 3A). GATA-2-mediated stimulation of promoter activity was completely abrogated by co-expression of Hex in a dose-dependent manner. Moreover, overexpression of Hex did not change the basal level of the promoter activity (Fig. 3A). In HUVEC, a high level of flk-1/KDR promoter activity (8.7-fold higher than SV40 promoter plus enhancer construct (pGL2-control), not shown) occurred, and overexpression of GATA-2 resulted in 2.8-fold transactivation of the promoter (Fig. 3B). More importantly, overexpression of Hex resulted in huge reduction of the flk-1/KDR promoter activity (15.9-fold compared without expression of GATA-2 and Hex). Co-expression of GATA-2 failed to recover the Hex-mediated attenuation of the flk-1/KDR promoter activity (2.3-fold induction compared Hex expression alone) (Fig. 3B). To confirm that the Hex-mediated down-regulation of the promoter activity was mediated by the 5′-UTR GATA motif on the flk-1/KDR gene, either the GATA element or the SP1 element (as a control) point-mutated plasmid was transfected into HUVEC. KDR promoter activity from SP1 point-mutated plasmid was down to 63.1% and markedly reduced to 3.4% by the co-expression with Hex. In contrast, the promoter activity from GATA point-mutated plasmid was down to 33.1%, whereas there was no significant reduction in the presence of Hex (Fig. 3B). Previous studies have shown that Hex directly binds to a
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Hex Supresses flk-1/KDR mRNA Expression in Primary Human Endothelial Cells—To determine whether Hex modulates the expression of the endogenous flk-1/KDR gene, HUVEC were infected with adenovirus expressing either IRES-mediated EGFP (Blank) or IRES-mediated-rat Hex and EGFP (Hex) at a multiplicity of infection of 20. Using this approach, over 80% of the cells were infected as determined by EGFP expression. Western blot assays of infected cells demonstrated high levels of Hex protein (Fig. 4A). More importantly, overexpression of Hex in HUVEC resulted in a 85% reduction (mean of three independent experiments) in flk-1/KDR mRNA by RNase protection assay (Fig. 4B, compare lanes 4 and 5). These findings indicate that Hex suppresses flk-1/KDR activity not only at the level of the promoter but also at the level of the endogenous gene.

Hex Inhibits Binding of GATA-2 to the flk-1/KDR 5′-UTR GATA Motif—Based on the above findings, we hypothesized that Hex inhibits flk-1/KDR expression by interfering with GATA binding to the 5′-UTR. To test this hypothesis, we performed electrophoretic mobility shift assays in which nuclear extracts derived from HUVEC either expressing IRES-mediated EGFP (Adeno-Blank) or IRES-mediated Hex and EGFP (Adeno-Hex) were incubated with a radiolabeled probe spanning the 5′-UTR GATA motif (+98 to +122). As shown in Fig. 5 (A and B), incubation of nuclear extract from Adeno-Blank infected HUVEC with the 32P-labeled probe resulted in the appearance of two specific DNA-protein complexes (closed and open arrows). These DNA-protein complexes were inhibited by the addition of 10-, 100-, and 500-fold molar excess unlabeled self-competitor (Fig. 5A, lanes 3–5) but not by 500-fold molar excess GATA mutant competitor (Fig. 5A, lane 6). The mobility shift pattern was identical in Hex-overexpressing cells (the more slowly migrated complexes designated with the open arrows appeared with longer exposure time). However, Hex overexpression resulted in a significant reduction (54.2% reduction by densitometry) in the intensity of the GATA-binding complexes (Fig. 5, A compare lanes 3–5 and 9–11, and B, compare lanes 1 and 2). As previously reported, the DNA-protein complexes were inhibited by preincubation with the anti-GATA-2 antibody (34). In contrast, the complex was not inhibited by preincubation with anti-p65 antibody (Fig. 5B, lanes 3 and 4). Compared with control HUVEC, Hex overexpression did not significantly alter SP1 DNA binding activity (Fig. 5C). Together, these results suggest that Hex-mediated down-regulation of the flk-1/KDR is associated with an inhibition of GATA-2 binding.

Hex Suppresses VEGF-mediated Tube Formation in Primary Human Endothelial Cell Cultures—VEGF interaction with Flk-1/KDR is a critical determinant of endothelial cell proliferation and angiogenesis (37). In the next set of experiments, we wished to determine whether Hex inhibits the VEGF-Flk-1/KDR signaling axis. To that end, we carried out sandwich tube formation assays on collagen gel. In the absence of basic fibroblast growth factor, VEGF stimulated tube formation of HUVEC, after 24 h HUVEC were seeded on the collagen gel in the presence or absence of flk-1 tyrosine kinase inhibitor SU1498 (10 μM). Tube formations were observed by phase contrast microscopy at 40-fold optical magnification. The results were representative of four independent experiments. B, shown in the quantification of the tube area. *p < 0.03 compared with the activity from HUVEC infected with Adeno-Blank. The means and standard deviations were calculated with NIH Image from four independent experiments.

SU1498 (Adeno-Hex + SU1498) (Fig. 6). These findings suggest that Hex-mediated inhibition of flk-1/KDR gene expression results in a down-regulation of VEGF signaling.
FIG. 7. TGF-β down-regulates endogenous flk-1/KDR and up-regulates endogenous Hex expression in human endothelial cells. A, a confluent HUVEC were serum-starved and then incubated in the absence or presence of 2.5 or 10 ng/ml TGF-β1 for 18 h, at which time total RNA was isolated. In RNase protection assays, an [α-32P]UTP-labeled 414-bp human Hex riboprobe was incubated with 10 μg of yeast tRNA (lane 2), 10 μg of total RNA from control HUVEC (lane 3), 2.5 ng/ml TGF-β1-treated HUVEC (lane 4), or 10 ng/ml TGF-β1-treated HUVEC (lane 5). The protected fragment (296 bp) represents the human Hex transcript. An [α-32P]UTP-labeled GAPDH riboprobe was hybridized with total RNA as an internal control. B, shown is the quantification of RNase protection assays. Densitometry was used to calculate the ratio of Hex and GAPDH signals (arbitrary expression level). The data represent the averages from two independent experiments. C, a confluent HUVEC were serum-starved and then incubated in the absence or presence of 2.5 or 10 ng/ml TGF-β1 for 24 h, at which time total RNA was isolated. In RNase protection assays, an [α-32P]UTP-labeled 393-bp human flk-1/KDR riboprobe was incubated with 10 μg of yeast tRNA (lane 2), 10 μg of total RNA from control HUVEC (lane 3), 2.5 ng/ml TGF-β1-treated HUVEC (lane 4), or 10 ng/ml TGF-β1-treated HUVEC (lane 5). The protected fragment (266 bp) represents the human flk-1/KDR transcript. An [α-32P]UTP-labeled GAPDH riboprobe was hybridized with total RNA as an internal control. M.W., molecular mass. D, shown is the quantification of RNase protection assays. Densitometry was used to calculate the ratio of flk-1/KDR and GAPDH signals (arbitrary expression level). The data represent the averages from two independent experiments.
Hex Induces TGF-β Expression in Primary Human Endothelial Cells—Previous studies have shown that whereas tumor necrosis factor α, VEGF, and thrombin induce flk-1/KDR expression in endothelial cells (38–40), TGF-β1 has the opposite effect (41). In keeping with these findings, the addition of 2.5 and 10 ng/ml TGF-β1 to HUVEC for 24 h resulted in 49 and 58% reduction of flk-1/KDR mRNA, respectively (Fig. 7, A, lanes 3–5, and B). Interestingly, incubation of HUVEC with 2.5 and 10 ng/ml TGF-β1 for 18 h resulted in 2.1- and 2.4-fold stimulation of Hex mRNA, respectively. Taken together, these results suggest that TGF-β1 may exert its inhibitory effects through a Hex-GATA-2-dependent pathway.

DISCUSSION

The GATA family of transcription factors has been implicated not only in the early differentiation of the endothelial cells but also in the transduction of extracellular signals. For example, insulin-like growth factor 1, tumor necrosis factor α, and thrombin have each been shown to induce GATA-2 activity, whereas estrogens and TGF-β may actually inhibit GATA binding (6, 30, 35, 42). Together, these studies suggest that GATA transcription factors may behave like immediate early genes, serving to couple short changes in the extracellular environment to long term changes in downstream gene expression.

An important clue to understanding the multifaceted role of a transcription factor is found in its repertoire of protein-protein interactions. Indeed, previous studies have uncovered an array of partner proteins that interact with GATA transcription factors to regulate gene transcription (8, 14–18, 20, 22, 23, 25, 27). In the present study, we have extended the list of protein partners by demonstrating a novel interaction between GATA proteins and the homeobox protein Hex in primary human endothelial cells. Hex is a member of the homeobox transcription factors and has been shown to bind to a consensus motif (5′-CAATTAAT-3′) in the promoter region of downstream target genes, resulting in repression of gene transcription (43). In addition, Hex may indirectly modulate gene expression through protein-protein interactions. For example, Hex has been shown to associate with cAMP-responsive element-binding protein to induce the SMemb/nonmuscle myosin heavy chain B gene in vascular smooth muscle cells (44). Moreover, Hex-Jun protein interactions have been reported to suppress c-Jun, JunB, and JunD-mediated gene activation (45).

In the present study, we have demonstrated GATA-2, -3, and -6 that have been identified in endothelial cells were physically interacted with Hex in cultured cells by immunoprecipitation analysis. GATA-2, -3, and -6 have highly conserved zinc finger domains and the region involved the usual protein-protein interactions (2, 10, 13, 15, 18, 19, 20, 29, 37, 39). Further studies will be required to determine whether these motifs are responsible for mediating the binding of Hex to GATA-2, GATA-3, and GATA-6.

In a previous study, we demonstrated that TGF-β inhibits flk-1/KDR expression through a mechanism that involves reduced binding of GATA-2 to a palindromic GATA site in the 5′-UTR (35). In keeping with the role for GATA-2 in mediating flk-1/KDR expression, Hex overexpression resulted in reduced binding of GATA-2 to 5′-UTR GATA motif and a dose-dependent inhibition of flk-1/KDR promoter activity. It is interesting to speculate that the specific association between Hex and GATA-2 interferes with zinc finger domain-mediated DNA binding activity. Adenovirally mediated overexpression of Hex did not completely abrogate (54.2% inhibition) GATA-2 binding activity but did result in a more profound reduction of flk-1/KDR mRNA and the promoter activity (Figs. 3 and 4). It has been shown that Hex and Jun interaction inhibits Jun-mediated transactivation without interfering with the Jun-DNA binding activity (45). In addition, Hex contains the transrepression domain at the N terminus. These observations suggest that Hex may exert its inhibitory effect through two mechanisms, namely through cumulative inhibition of GATA-2 DNA to the flk-1/KDR promoter and direct repression of transcription.

The results of the present study provide new insight into how TGF-β mediates its inhibitory effect on the GATA-2–flk-1/KDR signaling axis. The observation that TGF-β treatment of endothelial cells resulted in concomitant up-regulation of Hex mRNA and down-regulation of flk-1/KDR in endothelial cells suggests that TGF-β signaling promotes transcriptionally inactive or inhibitory complexes between GATA-2 and Hex. The functional consequence of this interaction was borne out in vitro studies of angiogenesis, in which the overexpression of Hex abrogated VEGF-flk-1/KDR-dependent endothelial cell tube formation.

The mechanism by which TGF-β1 induces Hex expression remains to be elucidated. TGF-β1 is known to induce Smad-2 and -5 activity in endothelial cells (46). Moreover, Smad-mediated signaling has been implicated in the control of Hex expression (47). Mice that are null for Smad-2 deficiency are embryonic lethal at E7.5 and lack detectable levels of Hex (48). Taken together, these observations raise the possibility that TGF-β1 signaling is coupled to Hex-GATA-mediated inhibition of flk-1/KDR through a Smad-2-dependent pathway.

Most recently, Nakagawa et al. (49) reported that Hex acts as a negative regulator of angiogenesis. In the latter study, Hex was shown to completely abrogate the VEGF-mediated proliferation, migration, and invasion of HUVEC (49). Although our data are consistent with those of Nakagawa et al., they are novel in that they: 1) provide a link between a natural inhibitor of angiogenesis (TGF-β) and Hex and 2) reveal a mechanistic connection between Hex, GATA-2, and flk-1/KDR expression. Indeed, based on these findings, we propose that Hex, as well as GATA-2, represent new therapeutic targets for anti-angiogenesis therapy.

Acknowledgments—We are grateful to Stuart. H. Orkin (Harvard Medical School, Boston, MA) for kindly providing the human GATA-2 expression plasmid (pMT-GATA2) and anti-GATA-2 antibody. We also thank Drs. Ryuichiro Sato and Juro Sakai (The University of Tokyo, Japan) for technical help with co-immunoprecipitation assays.

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Interaction between Hex and GATA Transcription Factors in Vascular Endothelial Cells Inhibits \(\text{flk-1/KDR}\)-mediated Vascular Endothelial Growth Factor Signaling
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*J. Biol. Chem.* 2004, 279:20626-20635.
doi: 10.1074/jbc.M308730200 originally published online March 10, 2004

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