

Glucocorticoid and Growth Factor Synergism Requirement for *Notch4* Chromatin Domain Activation[∇]

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The Notch signaling pathway modulates cell fate in diverse contexts, including vascular development. *Notch4* is selectively expressed in vascular endothelium and regulates vascular remodeling. The signal-dependent transcription factor activator protein 1 (AP-1) activates *Notch4* transcription in endothelial cells, but other factors/signals that regulate *Notch4* are largely unknown. We demonstrate that, unlike the established trans-repression mechanism in which the glucocorticoid receptor (GR) antagonizes AP-1, AP-1 and GR synergistically activated *Notch4* transcription in endothelial cells. Fibroblast growth factor 2 (FGF-2) and cortisol induced AP-1 and GR occupancy, respectively, at a *Notch4* promoter composite response element consisting of an imperfect half-glucocorticoid response element and an AP-1 motif, which mediated signal-dependent activation. Analysis of *Notch4* promoter complex assembly provided evidence that GR and AP-1 independently occupy the composite response element, but AP-1 stabilizes GR occupancy. In multipotent 10T1/2 cells, FGF-2 and cortisol induced a histone modification pattern at the *Notch4* locus mimicking that present in endothelial cells and reprogrammed *Notch4* from a repressed to an active state. These results establish the molecular basis for a novel AP-1/GR-*Notch4* axis in vascular endothelium.

The conserved Notch signaling pathway regulates cell proliferation, differentiation, and cell fate in diverse tissues (33). Four mammalian Notch receptors (Notch1 to Notch4), which have distinct and overlapping expression patterns, share a common signaling mechanism. Binding of transmembrane Notch ligands to Notch receptors on adjacent cells induces proteolytic cleavages, liberating the Notch intracellular domain (NIC) from the plasma membrane (61). NIC translocates into the nucleus and binds the repressor CBF1/Su(H)suppressor of hairless/Lag-1 (CSL) (24, 46, 59), thus converting CSL into an activator (20, 24, 36, 74). Through this canonical pathway and CSL-independent signaling (50), Notch regulates target genes as a key step in controlling developmental processes.

Vascular development and remodeling represent important Notch-regulated biological processes (23, 57). Both *Notch1* and *Notch4* are expressed in vascular endothelium, but *Notch4* has an almost exclusively vascular expression pattern, whereas *Notch1* is expressed more broadly (71). *Notch1*^{-/-} mice exhibit embryonic lethality, due to extensive developmental defects, including impaired vascular remodeling (35, 63). *Notch4*^{-/-} mice do not exhibit overt phenotypes, but vascular remodeling is more severely disrupted in *Notch1*^{-/-}/*Notch4*^{-/-} versus *Notch1*^{-/-} mice (35). Endothelial expression of constitutively active NIC4 disrupts vascular development and is embryonic lethal (70). NIC4 overexpression in adult mouse endothelium induces arteriovenous malformations and lethality (4), indicating that Notch4 might regulate determination of arterial-venous fate, consistent with zebra fish Notch5 regulating arterial-venous differentiation (38). NIC1 or NIC4 overexpression in

cultured endothelial cells either inhibits (39, 45) or promotes (41, 64, 69) vascular morphogenesis.

The endothelium-specific *Notch4* expression (71) and genetic evidence indicate that normal vascular development requires physiological levels of Notch4 signaling. Thus, elucidation of mechanisms regulating *Notch4* expression is particularly important with regard to understanding physiological and pathological functions of Notch4. Vascular endothelium consists of functionally distinct endothelial cell subtypes (5), and *Notch4* is selectively expressed at certain vascular sites (23), including mouse aorta, intersomitic vessels, arterial capillaries, pulmonary artery, pulmonary capillaries, and cardinal/subcardinal veins (35, 42, 71, 72). *Notch4* transcripts are highest in the aorta and pulmonary artery in E9 and E13.5 mouse embryos, are detectable in adult pulmonary capillaries (71), and are restricted to arterial endothelium in E13.5 embryos (72). *Notch4* expression in mouse heart and liver is maximal by 2 weeks and 12 weeks of postnatal development, respectively (42).

The differential *Notch4* regulation at distinct vascular sites and during development might be related to unique signaling environments. Vascular endothelial growth factor 121 (VEGF₁₂₁) and fibroblast growth factor 2 (FGF-2) expression in human umbilical vein endothelial cells (HUVECs) modestly increases *Notch1* and *Notch4* mRNA (41). The antiangiogenic factor cerivastatin downregulates *Notch4* mRNA in FGF-2-treated endothelial cells (73). In arthritic, but not normal, synovial fibroblasts, tumor necrosis factor induces *Notch4* mRNA (1). However, mechanisms underlying signal-dependent, endothelium-specific transcription of *Notch4* are largely unknown.

Previously, we demonstrated that the *Notch4* locus assembles an endothelial cell-specific histone modification pattern in HUVECs and a conserved activator protein 1 (AP-1) motif is required for endothelial cell-specific *Notch4* promoter activity (78). While, in principle, AP-1 motifs can mediate signal-de-

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pendent transcriptional responses, whether signaling mechanisms target the *Notch4* promoter AP-1 motif in endothelial cells is unknown. Herein, we tested whether extracellular signals regulate *Notch4* transcription via AP-1 and whether signals target other *Notch4* locus components. Despite multiple studies demonstrating antagonism between glucocorticoid receptor (GR) and AP-1 (28, 29, 31, 34, 47, 51, 60, 79), we demonstrate that GR and AP-1 synergistically activate *Notch4* in endothelial cells and reprogram *Notch4* from a repressed to an active state in a multipotent nonendothelial cell line. Molecular analyses established a novel mechanism in which AP-1 and GR function via a composite response element, consisting of an imperfect half-glucocorticoid response element (half-GRE) and an AP-1 motif, with AP-1 stabilizing GR occupancy at this element. These studies provide the molecular basis for a model in which *Notch4* expression requires dual growth factor-glucocorticoid signaling. Perturbation of this multicomponent signaling mechanism in pathophysiological states would be expected to deregulate *Notch4* expression in endothelial cells and/or ectopically induce *Notch4* in cells that normally lack the capacity to respond to Notch4 ligands.

MATERIALS AND METHODS

Cell culture. Mouse yolk sac endothelial cells (YSECs) were derived from a hypervascular transgenic mouse expressing the *fps/fes* proto-oncogene (43). YSECs were maintained in Medium 200 (Cascade Biologics) containing low-serum growth supplement (2% fetal bovine serum, 1 μ g/ml cortisol, 10 ng/ml epidermal growth factor [EGF], 3 ng/ml FGF-2, and 10 μ g/ml heparin [Cascade Biologics]) and 1% penicillin-streptomycin (Gibco/BRL). Mouse aortic endothelial cells (MAEs), mouse heart microvascular endothelial cells (MHECs), and mouse embryonic fibroblast 10T1/2 cells were maintained in Dulbecco's modified Eagle's medium (Biofluids) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco/BRL).

Antibodies. Anti-Notch4 antibody was from Upstate Biotechnology. Anti-NIC1 is a rabbit polyclonal antiserum directed against amino acids 1759 to 2095 of human Notch1 as described previously (26). Rabbit anti-diacetylated histone H3 (06-599), anti-tetraacetylated H4 (06-866), and anti-dimethylated H3 at K4 (07-030) antibodies were from Upstate Biotechnology. Mouse immunoglobulin G (IgG) (Upstate Biotechnology) and preimmune serum (Covance) were used as chromatin immunoprecipitation (ChIP) controls. Rabbit anti-c-Fos (K-25) was from Santa Cruz Biotechnology. Mouse monoclonal anti-GR antibody (BuGR) was described previously (2), and anti- α -tubulin was from Oncogene Research Products. The secondary antibodies goat anti-rabbit IgG conjugated with horseradish peroxidase and goat anti-mouse IgG-horseradish peroxidase were from Santa Cruz Biotechnology.

Reagents. Cortisol, cycloheximide, and heparin were from Sigma-Aldrich. Recombinant human EGF was from Calbiochem. Recombinant human FGF-2 was from Alan Rapraeger, University of Wisconsin, Madison. Dose-response curves were generated with EGF (10, 30, or 100 ng/ml), FGF-2 (3, 10, or 30 ng/ml), and cortisol (0.03, 0.3 or 3 μ M) to determine the ability of individual factors to induce *Notch4* expression in YSECs (see Fig. 2A). None of the factors alone induced *Notch4*. In subsequent experiments, EGF (10 ng/ml), FGF-2 (10 ng/ml), and cortisol (1 μ M) were used. As an FGF-2 cofactor, 10 μ g/ml heparin was always included with FGF-2. All factor treatments were conducted in serum-free medium containing 0.2% bovine serum albumin. Bovine serum albumin alone had no effect on *Notch4* transcription (data not shown). Cycloheximide was used at final concentration of 20 μ g/ml, which quantitatively blocked supplement-induced c-Fos protein expression, while c-Fos mRNA induction was unaffected (data not shown).

Plasmids. For pGL3/N4pro1000, the mouse *Notch4* promoter ~1-kb fragment (-905 to +78) was amplified by PCR using the primer pair (5'→3') AAGGCC AAGCCTCCAGACTC and CAGTCAAGCTTCAGGCAGGGACCCTC. For pGL3/N4pro500, the mouse *Notch4* promoter 461-bp fragment (-383 to +78) was amplified by PCR using the primer pair (5'→3') GGTTTCAGTTCAAGA CACGTTGC and CAGTCAAGCTTCAGGCAGGGACCCTC. For pGL3/N4pro500(mGRE), the mouse *Notch4* promoter with the GRE site mutated was amplified by two-step PCR using the primer pairs (5'→3') CACAAGCGAGA

GGACACCCTACTGATGG and AGGGTGTCTCTCGCTTGTGTGACTC AGGAAACAGC and GGTTTCAGTTCAAGACACGTTGC and CAGTCAA GCTTCAGGCAGGGACCCTC. For pGL3/N4pro(mAP1), the mouse *Notch4* promoter with the AP-1 motif mutated was amplified by two-step PCR using the primer pairs (5'→3') TTGTGGCTAGACGGAAACAGCTCAGACTG and CAGTCTGAGCTGTTTCCGTCTAGCCACAA and GGTTTCAGTTCAAG ACACGTTGC and CAGTCAAGCTTCAGGCAGGGACCCTC. For pGL3/N4pro(mGRE/AP1), the mouse *Notch4* promoter with the imperfect half-GRE and AP-1 motif mutated was amplified by two-step PCR using the primer pairs (5'→3') CACAAGCGAGAGGACACCCTACTGATGG and AGGGTGTCTCTCGCTTGTGGCTAGACGGAAACAGC and GGTTTCAGTTCAAGACA CGTTGC and CAGTCAAGCTTCAGGCAGGGACCCTC. The PCR products were digested with HindIII and cloned into pGL3basic.

The 4xAP1/Luc reporter containing four AP-1 motifs was from Nancy Colburn, National Cancer Institute. The GRE2/Luc reporter containing two copies of the GRE sequence from the tyrosine aminotransferase gene was from John Cidlowski, National Institute of Environmental Health sciences (44).

Quantitative real-time RT-PCR. Total RNA was purified using Trizol (Invitrogen). cDNA was synthesized by standard procedures and quantitated by real-time reverse transcription-PCR (RT-PCR) (ABI Prism 7000). Primers were designed to amplify regions of 50 to 150 bp. Real-time RT-PCR mixtures (25 μ l) contained 2 μ l of cDNA, 12.5 μ l of SYBR green (Applied Biosystems), and the indicated primers. Product accumulation was monitored by SYBR green fluorescence. Relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Analysis of product denaturation curves post-amplification showed that primer pairs generated single products. The forward and reverse primers for real-time RT-PCR (5'→3') were as follows: *Notch4*, GAGGACCTGGTTGAAGAATTGATC and TGCAGTTTTTCCCTTTTAT CCC; *Notch1*, TATGGCCACGAGGAAGAGCT and TAGACAATGGAGCC ACGGATC; and *Gapdh*, TGCCCCATGTTTGTGATG and TGTGGTCATG AGCCCTCC.

Cell surface protein biotinylation. YSEC and 10T1/2 cell surface proteins were biotinylated as described previously (62). Cells were washed with phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺ (pH 7.4) and incubated with 0.5 mg/ml EZ-link sulfo-NHS-SS-biotin (Pierce) in PBS for 30 min at 4°C. Excess biotin was quenched by incubation with 100 mM glycine in PBS for 15 min at 4°C, followed by two washes with PBS. Cells were lysed in lysis buffer (300 mM NaCl, 50 mM Tris [pH 8.0], 0.5% NP40, 0.5% deoxycholate, 1 mM CaCl₂, 1 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 μ g/ml leupeptin) for 30 min at 4°C. Lysate was incubated overnight with streptavidin-conjugated beads (Sigma). Beads were washed three times with wash buffer (50 mM Tris [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) and eluted in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromophenol blue). Purified cell surface proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore), detected by immunoblotting with anti-Notch4 antibody (Upstate; #07-189), and visualized with the ECL-Plus enhanced chemiluminescence system (Amersham). Antibody specificity was confirmed with expressed hemagglutinin (HA)-tagged Notch4. Anti-Notch4 and anti-HA antibodies yielded similar results in immunoblotting and immunostaining assays (data not shown).

siRNA knockdown. Control and Notch4 small interfering RNAs (siRNAs) were from Dharmacon. RNA was transfected into YSECs via Lipofectamine (Invitrogen). YSECs were plated in six-well plates 1 day before transfection and were ~50% confluent when transfected. RNA (200 pmol) was incubated with 8 μ l Lipofectamine in 400 μ l of Opti-MEM (Gibco/BRL) for 30 min at room temperature and was then added to cells that were washed with Opti-MEM. The cells were incubated with the transfection mixture for 6 h, fresh medium was added, and cells were harvested 60 h posttransfection. Total RNA was analyzed by real-time RT-PCR. Cells were biotinylated for analysis of cell surface proteins.

Quantitative ChIP analysis. Real-time PCR-based ChIP analysis was performed as described previously (21, 78). Cells were incubated with medium containing 0.4% (for histone modifications) or 1% (for GR and AP-1) formaldehyde for 10 min at room temperature. Sonicated chromatin fragments averaged ~300 to 500 bp. DNA was quantitated by real-time PCR. The amounts of products were determined relative to a standard curve generated from a titration of input chromatin. Measurements were made under conditions in which signals were in the linear range, and denaturation curves showed that primer pairs yielded single products. The following forward and reverse primers (5'→3') were used for real-time PCR-based ChIP assay: N4 up, AAGGCCAAGCTCCAG ACTC and GCTTCCCGTGTCTACTACTGATACTT; N4 pro, CCATCAGTA GGGTGTCCAGGA and GCCTCAGTCTGAGCTGTTTC; N4 pro2, GGTT

TCAGTTCAAGACACGTTGC and GGTGTGACACACCTCTTTTCAA; N4 in1, CATTGCTGGCCTTTCCTGAA and TGGGATGTGAATGGCTGAGA; N4 in1-2, TGGTCAGTGGCCTGTAAAGGAG and TCCAGATGGCTTGCCC TTC; N4 ex3, GATCGATGCCAAACCCATCT and CCCCGTTGGAACAGA AAGAA; N4 in3, CAATCCACACACAAGCCTCT and CGTCTCCACCT GTTTTCTCA; N4 ex22, TCCGAGATCACTTCCACAA and TTCAGCGTT ATTGCAGCCTTT; *RPII215* pro, GCGAATCTATAAAGGGCGTCACT and TCGGCGCTTCTGAGGAGA; β *maj* pro, AGTGCCAGAAGAGCCAAGGA and CAGGGTGAGGTCTAAGTGATGACA; *Sgk1* up, GCATGCAAGAGCT TCTTCCCT and AATCGAAACACACGACAGG; *Sgk1* pro, GCAAGGCTC AAAATTTATGCG and TTCCAATCTCCGAGAACATTC; and *Sgk1* in2, TAGCAGCGAAGACTTCATGGG and GGAAAGATCTCAGCTCCA GCA.

Nuclear extract preparation. YSEC or 10T1/2 cells were harvested by scraping and collected by centrifugation at $400 \times g$ for 8 min. Cells were washed once with ice-cold PBS and resuspended in 1.5 volumes of nuclei lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl₂, and 0.2% Nonidet P-40) on ice for 5 min. Nuclei were collected by centrifugation for 5 min at $400 \times g$. Nuclei were washed by gentle resuspension in 1.5 volumes of nuclei wash buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, and 3 mM MgCl₂) and collected by centrifugation for 4 min at $400 \times g$. Nuclei were immediately resuspended in an equal volume of low-KCl extract buffer (20 mM HEPES [pH 7.5], 20 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol), and 1.33 volumes of the same buffer containing 1.2 M KCl was added dropwise. Nuclei were extracted for 45 min at 4°C with constant rotating. The suspension was centrifuged for 30 min at $150,000 \times g$. The supernatant was dialyzed against 500 volumes of a mixture of 20 mM HEPES (pH 7.4), 20% glycerol, 60 mM KCl, and 0.2 mM EDTA overnight at 4°C and centrifuged for 30 min at $150,000 \times g$. Aliquots of the supernatant were frozen on dry ice and stored at -80°C . The protein concentration was measured by the Bradford assay with gamma globulin as a standard. Dithiothreitol (10 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (20 $\mu\text{g}/\text{ml}$), and β -glycerophosphate (1 mM) were included in all buffers.

In vitro promoter complex assembly assay. Biotinylated double-stranded oligonucleotides (Operon) were immobilized on streptavidin-conjugated Dynabeads M-280 (Dyna). Nuclear extract (300 μg total protein) was incubated with DNA-bound Dynabeads (0.2 mg) in a mixture of 10 mM HEPES (pH 7.5), 60 mM KCl, 10% glycerol, 1 mM MgCl₂, and 1 $\mu\text{g}/\mu\text{l}$ poly(dI-dC) for 45 min at 4°C with rotation. After magnetic separation, beads were washed three times with 10 mM HEPES (pH 7.5), 60 or 150 mM KCl, 10% glycerol, and 1 mM MgCl₂. Adsorbed proteins were eluted in SDS-sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis on an 8% acrylamide gel. Proteins were transferred to Immobilon P membrane, detected by immunoblotting with anti-GR or anti-Fos antibody, and visualized with ECL-Plus.

Transient transfection assay. 10T1/2 cells were plated in 24-well plates 1 day before transfection and were $\sim 90\%$ confluent when transfected. Samples of plasmid DNA (0.4 μg) were added to 50 μl of Opti-MEM (Gibco/BRL), incubated with Lipofectamine 2000 reagent (2.5 $\mu\text{l}/1 \mu\text{g}$ DNA; Invitrogen) for 30 min at room temperature, and then added to cells. Cells were incubated with the mixture for 6 h and then treated with cortisol and/or FGF-2 for 16 h. Cells were harvested, and lysates were assayed for luciferase activity (Promega). The luciferase activity was normalized by the protein content of the lysates, as determined by Bradford assay (Bio-Rad) using gamma globulin as a standard.

RESULTS AND DISCUSSION

Dual growth factor-glucocorticoid signaling requirement for *Notch4* transcription in endothelial cells. Despite the strict temporospatial *Notch4* expression, *Notch4* transcriptional regulation is poorly understood. Growth factors and hormones are crucial for maintaining cultured endothelial cell phenotypes. *Notch4*-expressing mouse YSECs are cultured in medium with 2% FBS and endothelial cell supplement containing cortisol, EGF, FGF-2, and heparin, an FGF-2 cofactor. We asked whether *Notch4* expression requires FBS and the supplement. Starvation of YSECs for 24 h without FBS and the supplement strongly decreased *Notch4* mRNA (~ 10 -fold) (Fig. 1A, left). To assess whether downregulation is reversible, FBS and the supplement were added back to starved cells. Whereas FBS did not rescue expression, FBS/supplement re-

established normal *Notch4* mRNA levels (Fig. 1A, left). FBS/supplement did not regulate *Notch1* expression (Fig. 1B, left). *Notch4*, but not *Notch1*, expression was also signal dependent in MAEs and MHECs (Fig. 1A and B, middle and right). Thus, the signaling mechanism that regulates *Notch4* expression operates in diverse endothelial cell subtypes.

To determine if signal-dependent changes in *Notch4* mRNA are accompanied by altered endogenous Notch4 protein levels, Notch4 was measured by Western blotting. Due to the low abundance of Notch4, cell surface proteins were biotinylated and enriched via adsorption to streptavidin-conjugated beads (62). Due to the reactivity of available anti-Notch4 antibodies with both Notch4 and cross-reactive proteins, Notch4 was knocked down in YSECs to identify the specific Notch4 band on the blot. siRNA-mediated knockdown of Notch4 reduced *Notch4* mRNA by $\sim 90\%$ (Fig. 1C, left) and downregulated a 245 ± 6 -kDa band (Fig. 1C, middle and right), consistent with the size of full-length Notch4. An apparently identical ~ 250 -kDa Notch4 band was reported previously as a biotinylated cell surface protein representing the unprocessed form of Notch4 (62). Consistent with this, it was reported that unprocessed form of Notch receptors is functionally expressed on the cell surface (3, 32). Neither *Notch1* mRNA (Fig. 1C, left) nor protein (Fig. 1C, middle and right) was significantly affected by *Notch4* siRNA (Fig. 1C). Notch1 was detected with an antibody generated against the Notch1 C terminus, which recognizes an ~ 110 -kDa band, representing the Notch1 intracellular domain (6, 26). As a control, α -tubulin expression was unaffected. Consistent with the mRNA analysis of Fig. 1A and B, starvation downregulated the ~ 250 -kDa Notch4 band, and the supplement rescued Notch4 expression (Fig. 1D); levels of Notch1 and α -tubulin expression were constant (Fig. 1D). The syntheses of both *Notch4* mRNA and protein are therefore regulated via cell signaling in YSECs.

The endothelial supplement contains cortisol, EGF, FGF-2, and heparin. We tested whether these components are required for *Notch4* transcription. After 24 h of starvation, YSECs were treated with different concentrations of EGF, FGF-2, or cortisol. While the combined components activated *Notch4* expression to a level equivalent to that in the supplement, the individual components did not rescue expression, even at the maximal concentrations (Fig. 2A). However, cortisol combined with EGF or FGF-2 strongly induced *Notch4* expression (Fig. 2A), indicating that expression requires synergism between a growth factor, either EGF or FGF-2, and cortisol. Furthermore, identical to cortisol, the highly specific synthetic glucocorticoid agonist dexamethasone (30 nM and 300 nM) synergized with FGF-2 to induce *Notch4* expression in YSECs (data not shown).

Growth factor-glucocorticoid synergism reprograms *Notch4* from a repressed to an active state in a multipotent cell line. *Notch4* was identified as the *int3* oncogene in mouse mammary tumors (12, 14). Thereafter, *Notch4* expression was described in certain nonendothelial cells (1, 22). Overexpression of constitutively active Notch4/Int3, in which the extracellular region of Notch4 is deleted, is oncogenic (13, 27, 54, 56). Based on these results, and restricted *Notch4* expression by endothelial cells, we tested whether *Notch4* expression is ectopically activated in nonendothelial cells and whether ectopic expression involves a mechanism similar to that of endothelial cells.

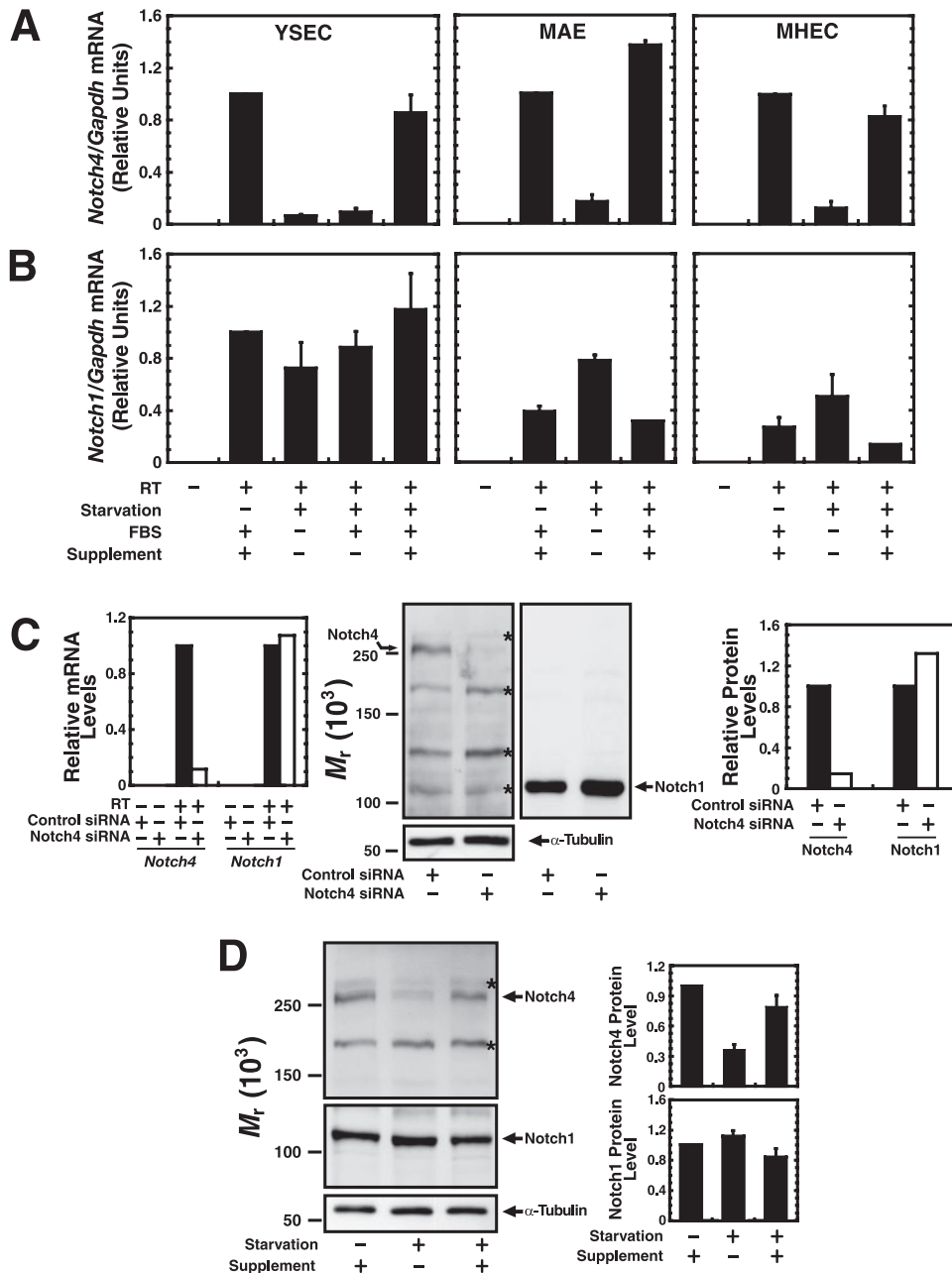


FIG. 1. Signal-dependent *Notch4* expression in endothelial cells. YSECs, MAEs, or MHECs were starved for 24 h and treated with or without FBS and/or endothelial supplement for 24 h. *Notch4* (A) and *Notch1* (B) mRNA levels were quantitated by real-time RT-PCR and normalized to *Gapdh* mRNA (mean \pm standard error of the mean for at least three independent experiments). (C) YSECs were transfected with control siRNA or *Notch4* siRNA. Total RNA was isolated 60 h posttransfection. *Notch4* and *Notch1* mRNA levels were quantitated by real-time RT-PCR and normalized to *Gapdh* mRNA (left; two independent experiments). The *Gapdh* mRNA level was unaffected by *Notch4* siRNA. Cell surface proteins were biotinylated, adsorbed to streptavidin-conjugated beads, and analyzed by Western blotting with anti-*Notch4* and anti-*Notch1* antibodies. The α -tubulin levels in the input lysates served as a loading control (middle; representative pictures from two independent experiments). The density of the specific bands was quantitated and plotted with the values obtained from cells transfected with control siRNA designated 1.0 (right; two independent experiments). (D) YSECs were starved for 24 h and treated with or without supplement for 24 h. Biotinylated cell surface proteins were analyzed by Western blotting, and the α -tubulin levels in the input lysates served as a loading control (left; representative pictures from at least three independent experiments). The density of the specific bands was quantitated and plotted with control cells designated 1.0 (right; mean \pm standard error of the mean for three independent experiments). The asterisks denote nonspecific bands present under all conditions. RT, reverse transcriptase.

Multipotent mouse embryonic 10T1/2 cells (55, 66) can be induced to express neuronal, adipocyte, smooth muscle cell, myoblast, and osteoblast markers (7, 10, 15, 19, 37, 65). In 10T1/2 cells treated for 24 h with EGF, FGF-2, or cortisol,

individually or in combinations, FGF-2/cortisol strongly activated *Notch4* expression (Fig. 2B). Unlike YSECs, EGF/cortisol did not induce *Notch4* expression. Unlike YSECs, which were starved prior to supplement addition, 10T1/2 cells were

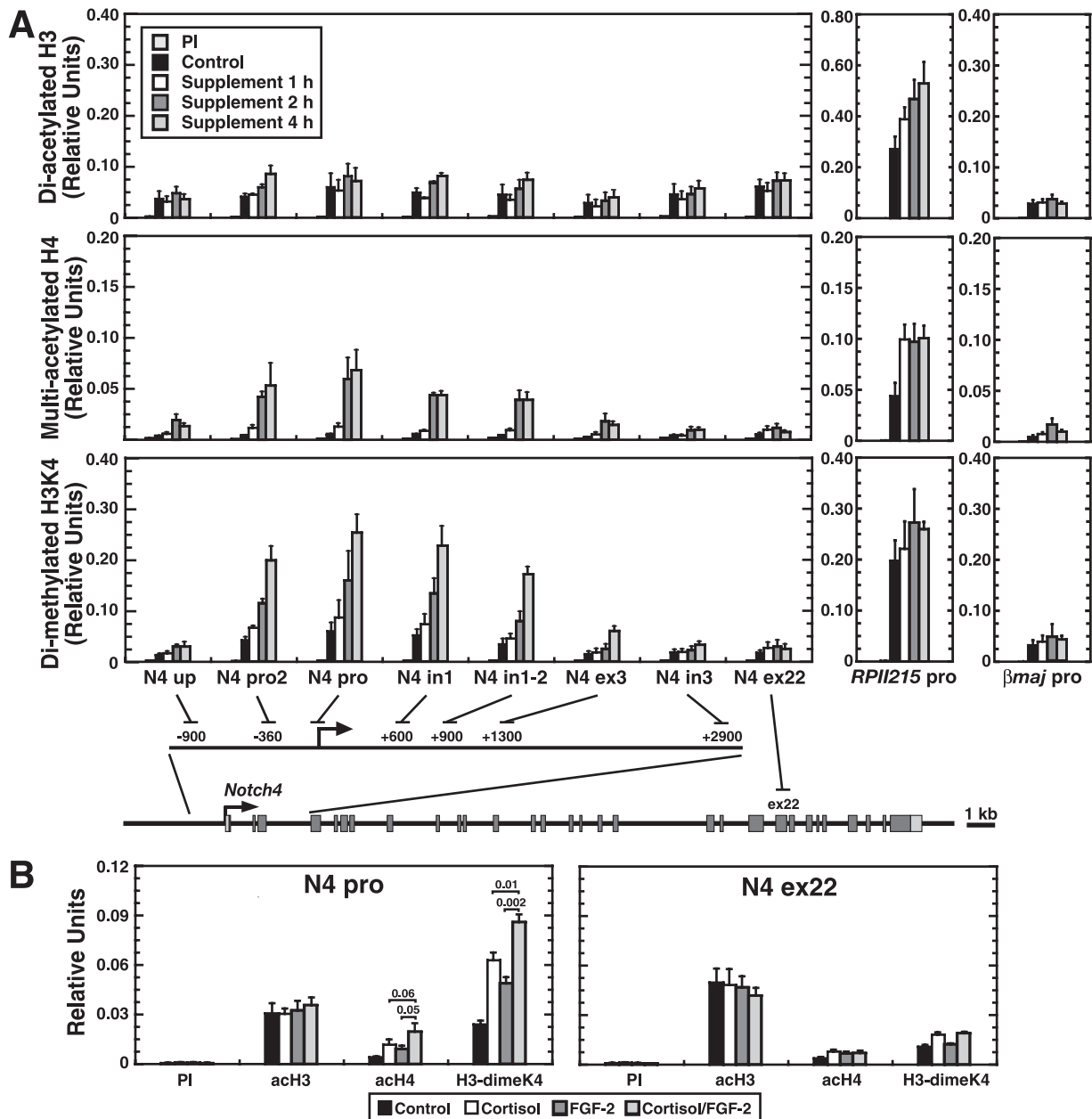


FIG. 4. Signal-dependent *Notch4* chromatin domain activation. (A) Quantitative ChIP analysis of diacetylated histone H3 (acH3), multiacetylated histone H4 (acH4), and dimethylated histone H3 at lysine 4 (H3-dimeK4) at the *Notch4* locus in 10T1/2 cells. Cells were treated with the supplement for 0, 1, 2, or 4 h. Histone modifications were analyzed at the constitutively active *RPII215* promoter and erythroid cell-specific β major promoter as controls (mean \pm standard error of the mean for three independent experiments). The positions of the amplicons analyzed by ChIP assay at the *Notch4* locus are indicated at the bottom of the figure (dark gray boxes, exons; light gray boxes, untranslated regions). (B) ChIP analysis of histone modifications at the *Notch4* promoter and exon 22 in 10T1/2 cells. Cells were treated with or without cortisol and/or FGF-2 for 4 h (mean \pm standard error of the mean for three independent experiments). PI, preimmune sera; up, upstream; pro, promoter; in, intron; ex, exon.

pro). Similar increases occurred \sim 400 bp upstream (N4 pro2) and within intron 1 (N4 in1; N4 in1-2), whereas little to no changes occurred \sim 900 bp upstream (N4 up) at exon 3 (N4 ex3), intron 3 (N4 in3), and exon 22 (N4 ex22) (Fig. 4A, left). In untreated 10T1/2 cells, the acH3 level was comparable to that detected previously in HUVECs (\sim 0.05; Fig. 4A, upper), indicating that acH3 is preestablished at the repressed *Notch4* locus in 10T1/2 cells. The induced acH4 and H3-dimeK4 levels establish a pattern resembling the HUVEC pattern. The sup-

plement did not affect epigenetic marks at the active *RPII215* promoter and the repressed β -globin promoter (Fig. 4A, right).

We tested whether cortisol and FGF-2 are both required or if a single component suffices to induce acH4 and H3-dimeK4 at *Notch4*. 10T1/2 cells were treated with cortisol, FGF-2, or cortisol/FGF-2 (Fig. 4B). Although cortisol and FGF-2 individually elevated acH4 and H3-dimeK4 at the promoter, cortisol/FGF-2 maximally increased acH4 and H3-dimeK4 with-

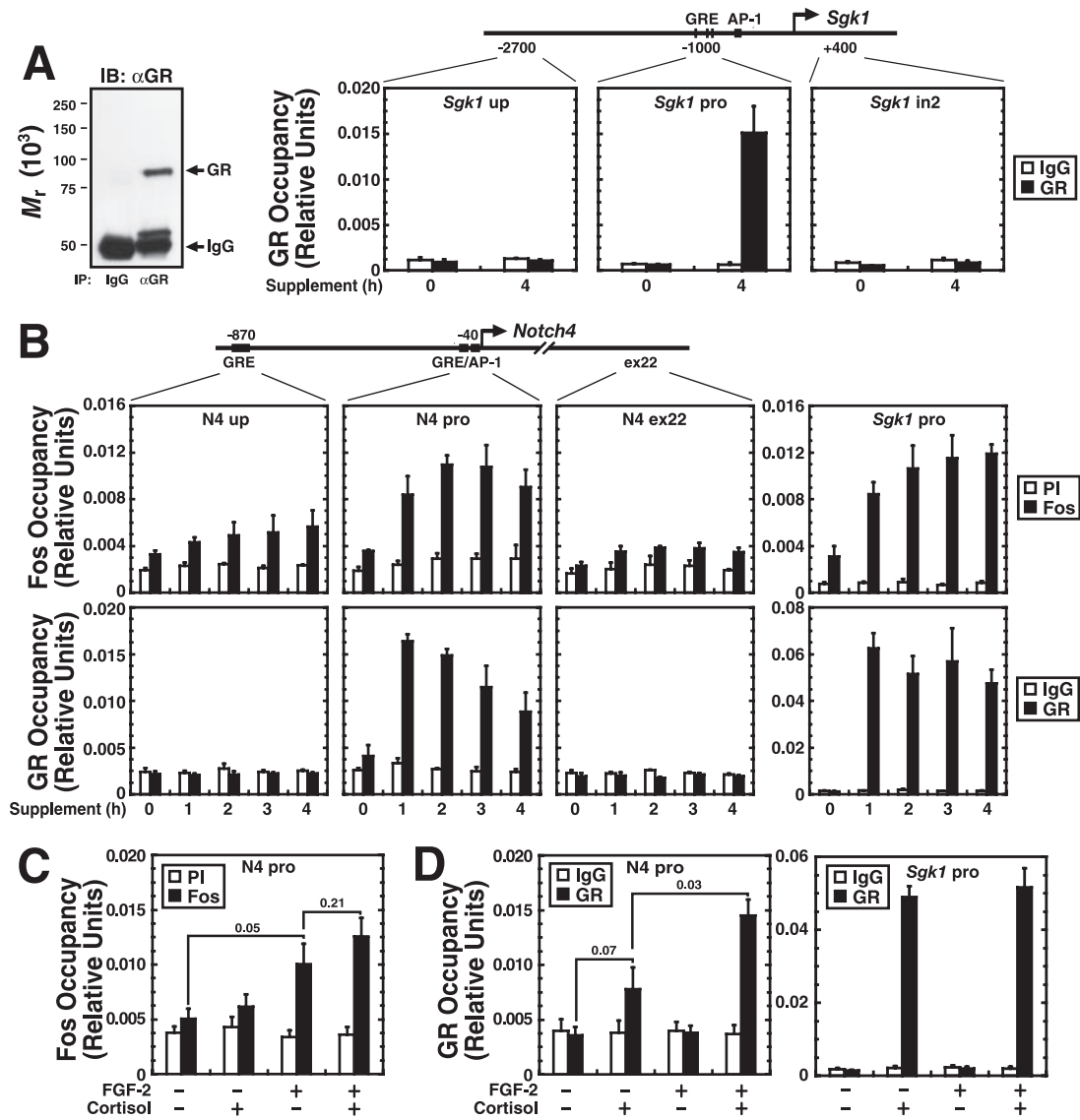


FIG. 5. Signal-dependent AP-1 and GR complex assembly at the *Notch4* promoter. (A) The Western blot on the left demonstrates the specific immunoprecipitation (IP) of endogenous GR from 10T1/2 whole-cell lysates. Shown are the results of ChIP analysis of GR occupancy at the *Sgk1* locus in 10T1/2 cells. Cells were treated with supplement for 4 h. GR was specifically recruited to the *Sgk1* promoter GREs. IB, immunoblot; α GR, anti-GR antibody. (B) ChIP analysis of Fos subunits (upper) and GR (lower) occupancy at the *Notch4* locus in 10T1/2 cells. Cells were treated with supplement for 0, 1, 2, 3, or 4 h. Fos or GR occupancy at the *Sgk1* promoter was used as a positive control (mean \pm standard error of the mean for three independent experiments). (C) ChIP analysis of Fos occupancy at the *Notch4* promoter. Cells were treated with or without cortisol and/or FGF-2 for 4 h (mean \pm standard error of the mean for three independent experiments). (D) ChIP analysis of GR occupancy at the *Notch4* promoter (left) and *Sgk1* promoter (right). Cells were treated with or without cortisol and/or FGF-2 for 1 h (mean \pm standard error of the mean for three independent experiments). PI, preimmune sera; up, upstream; pro, promoter; ex, exon.

out affecting acH3 (Fig. 4B, left). Ach4 and H3-dimeK4 were constant at exon 22 (Fig. 4B, right). These results indicate that establishment of the endothelial cell-like histone modification pattern requires cortisol and FGF-2.

Signal-dependent GR/AP-1 complex assembly at the endogenous *Notch4* locus. Cortisol activates the GR, but can activate the mineralocorticoid receptor in certain contexts (11). Similar to cortisol, the highly specific GR agonist dexamethasone, which does not activate the mineralocorticoid receptor at the concentrations used, synergized with FGF-2 to activate *Notch4* expression (data not shown), strongly implicating the GR.

FGF-2 signals through plasma membrane FGF receptor tyrosine kinases, which activate signaling components, such as the mitogen-activated protein kinase pathway (75). A conserved AP-1 motif at the *Notch4* promoter is required for endothelial cell-specific transcription (78). AP-1 subunits are induced and activated by receptor tyrosine kinase signaling (9), and FGF-2 induces and activates AP-1 subunits (48, 49). Under conditions in which supplement starvation of YSECs downregulates *Notch4* expression, c-Fos, Fra-1, and JunB were also downregulated (data not shown). The cycloheximide sensitivity of *Notch4* expression in YSECs (Fig. 3A) is consistent

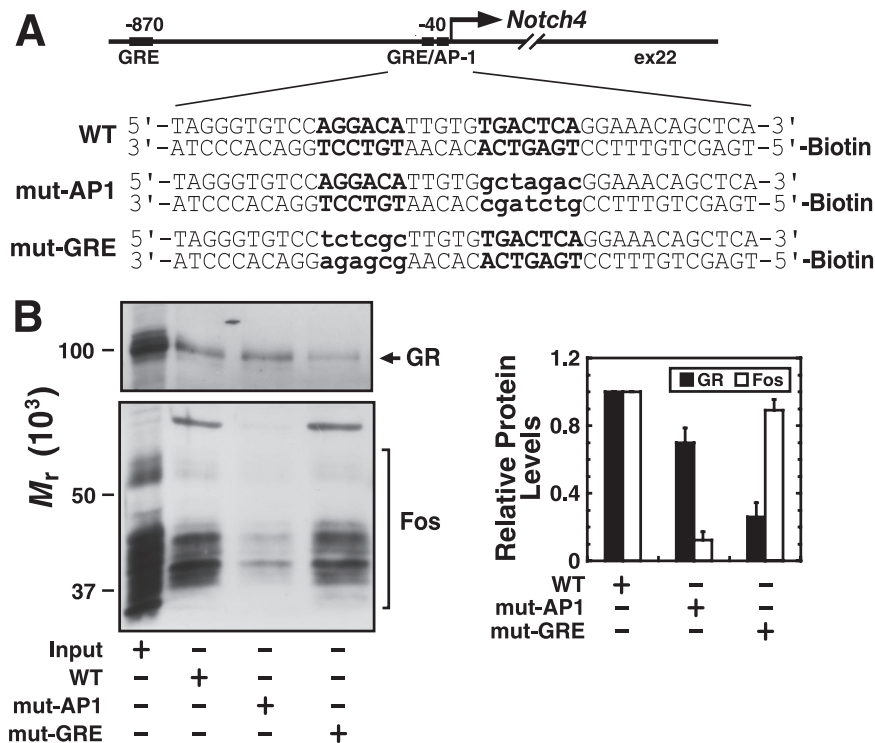


FIG. 6. GR and AP-1 independently occupy a composite response element, consisting of an imperfect half-GRE and a neighboring AP-1 motif. (A) Sequences of 5'-biotinylated double-strand oligonucleotides used in the in vitro promoter complex assembly assay: WT, 40-bp *Notch4* promoter oligonucleotides with wild-type GRE and AP-1 motifs; mut-AP1, 40-bp *Notch4* promoter oligonucleotides with mutated AP-1 motif; mut-GRE, 40-bp *Notch4* promoter oligonucleotides with mutated GRE site. The GRE and AP-1 motifs are indicated in uppercase boldface type. The mutated nucleotides are indicated in lowercase boldface type. (B) Western blotting analysis of proteins adsorbed to biotinylated oligonucleotides (representative pictures from at least three independent experiments). Monoclonal anti-GR antibody and polyclonal antibody against all Fos subunits were used for immunoblotting as indicated. The density of the specific bands was quantitated, and the value obtained for the WT condition was designated 1.0 (mean \pm standard error of the mean for three independent experiments).

with a mechanism in which growth factor signaling is required to establish normal levels of AP-1 subunit(s) prior to signal-dependent AP-1 activation (see Fig. 8).

To determine whether GR and AP-1 synergistically induce *Notch4* expression and to dissect the underlying mechanisms, we examined the *Notch4* promoter for GRE and AP-1 motifs. The promoter region contains an AP-1 motif (78) and a consensus half-GRE, \sim 40 bp and 900 bp upstream of the start site, respectively (Fig. 5B). Quantitative ChIP analysis was conducted to determine whether endogenous GR and AP-1 occupy the *Notch4* locus in 10T1/2 cells. Antibodies against Fos, but not Jun, subunits, were sufficiently specific and efficacious for ChIP (data not shown) (78), and the anti-GR antibody specifically immunoprecipitated only the GR (Fig. 5A, left). The serum- and glucocorticoid-inducible kinase 1 (*Sgk1*) promoter, which contains three consensus GREs and an AP-1 motif and is activated by both glucocorticoid and growth factors (77), was a positive control (Fig. 5A). The supplement induced rapid Fos and GR occupancy at the *Sgk1* promoter (Fig. 5A and B). At the *Notch4* locus, the supplement induced Fos occupancy at the promoter by 1 h, which persisted until 4 h (Fig. 5B, upper). Little to no Fos occupancy was detected at exon 22. Similarly, only small enrichments were detected at the upstream GRE ($P = 0.08$; 0.12, 0.17, and 0.09 for 1-, 2-, 3-, and 4-h supplement treatments, respectively, relative to time zero),

and this region lacks AP-1 motifs. No GR occupancy was detected at the upstream GRE and exon 22 with or without the supplement. However, the GR occupied the promoter region containing the AP-1 motif (Fig. 5B, lower). GR occupancy was maximal by 1 h and subsequently declined slightly. No GR occupancy was detected at other consensus GREs (at -6316 , -4360 , -3691 , $+2834$, and $+4138$; data not shown) at the locus. Five base pairs away from the AP-1 motif resides an imperfect half-GRE (AGGACA), differing by one nucleotide from the consensus half-GRE (AGAACA) (Fig. 6A, wild-type oligonucleotide [WT oligo]). Thus, the GR might bind the imperfect half-GRE, or might be tethered to the *Notch4* promoter via binding AP-1, since GR and AP-1 physically interact (28, 79).

To investigate how the GR is recruited to the promoter, we asked whether AP-1 and GR occupy the promoter independently. 10T1/2 cells were treated with cortisol, FGF-2, FGF-2/cortisol. The levels of AP-1 occupancy in FGF-2- and cortisol- and FGF-2-treated cells were similar (Fig. 5C) ($P = 0.21$). Cortisol-mediated GR activation therefore does not affect AP-1 occupancy. Cortisol induced a twofold increase of GR occupancy, which was significantly ($P = 0.03$) less than that achieved with cortisol/FGF-2 (Fig. 5D, left). Thus, FGF-2 facilitates cortisol-mediated GR occupancy. Since cortisol alone induced GR occupancy at the promoter without AP-1 occu-

pancy (Fig. 5D), this suggests that GR directly binds the promoter, inconsistent with a model in which GR is solely tethered to AP-1. However, AP-1-mediated enhancement of GR occupancy supports the notion that AP-1 stabilizes GR occupancy at the imperfect half-GRE. Importantly, AP-1 activation did not affect GR occupancy at the *Sgk1* promoter containing consensus GREs (Fig. 5D, right).

We further tested whether the GR occupies the imperfect half-GRE by analyzing whether GR and AP-1 assemble on the *Notch4* promoter in vitro. Double-stranded oligonucleotides containing 40 bp of the promoter (wild type [WT]), the AP-1 motif-mutated promoter (mut-AP1), and the imperfect half-GRE site-mutated promoter (mut-GRE) were biotin labeled (Fig. 6A), immobilized on streptavidin-conjugated beads, and incubated with nuclear extract from supplement-treated 10T1/2 cells. Western blotting indicated that AP-1 and GR absorbed to the WT oligonucleotide (Fig. 6B). Mutation of the AP-1 motif significantly reduced AP-1 binding (88% decrease; $P = 0.002$), while GR binding persisted, and mutation of the GRE significantly decreased GR binding (74% decrease; $P = 0.008$), while AP-1 binding persisted (Fig. 6B). These results provide further evidence that AP-1 and GR directly occupy the *Notch4* promoter, and the AP-1 motif and imperfect half-GRE mediate AP-1 occupancy and GR occupancy, respectively.

A conserved composite response element, consisting of an imperfect half-GRE and an AP-1 motif, mediates synergistic activation. Cortisol and FGF-2 synergistically activate *Notch4* transcription, and signals recruit GR and AP-1 to the *Notch4* promoter imperfect half-GRE and AP-1 motif, respectively. As the *Notch4* promoter has endothelial cell-specific activity that requires the AP-1 motif (78), the *Notch4* reporter might have signal-dependent activity which requires these motifs. *Notch4* promoter-luciferase constructs (Fig. 7A) were transiently transfected into 10T1/2 cells, and cells were treated with cortisol, FGF-2, or FGF-2/cortisol. Cortisol and/or FGF-2 did not affect activity of the promoterless reporter (Basic/Luc) nor that of the simian virus 40 (SV40) promoter reporter (SV40pro/Luc). Cortisol or FGF-2 alone activated the ~1-kb *Notch4* reporter (N4-pro1000/Luc) less than twofold ($P = 0.02$ and 0.002 , respectively). In contrast, cortisol and FGF-2 activated N4-pro1000/Luc more than fivefold ($P = 0.0015$) (Fig. 7B), indicating that cortisol and FGF-2 synergistically activate the promoter. This synergism is specific to the *Notch4* promoter, since cortisol and FGF-2 did not synergistically activate the AP-1 reporter (4xAP1/Luc) containing four AP-1 motifs nor the GRE reporter (GRE2/Luc) containing two GREs (Fig. 7C).

N4-pro500/Luc differed from N4-pro1000/Luc in that the upstream ~500 bp containing the consensus GRE was deleted (Fig. 7A). Both constructs responded similarly (Fig. 7B), indicating that the consensus GRE is not required and consistent with the ChIP analysis in which GR did not occupy the consensus GRE. Mutation of the imperfect half-GRE of N4-pro500/Luc [N4-pro500(mGRE)/Luc] abolished its cortisol responsiveness, while FGF-2 responsiveness persisted ($P = 0.036$). FGF-2/cortisol had a similar activity to FGF-2 alone ($P = 0.212$) (Fig. 7B). Mutation of the AP-1 motif of N4-pro500/Luc [N4-pro500(mAP1)/Luc] abrogated its cortisol and FGF-2 responsiveness. Mutation of both the imperfect half-GRE and AP-1 motif [N4-pro500(mGRE/AP1)/Luc] yielded activity equivalent to that of N4-pro500(mAP1)/Luc (Fig. 7B).

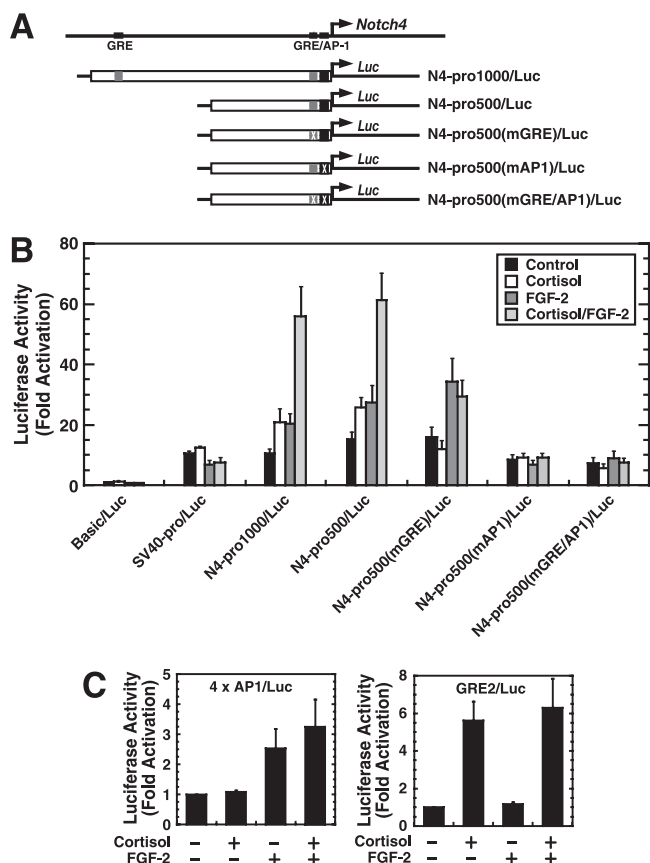


FIG. 7. The composite response element mediates synergistic transcriptional activation. (A) *Notch4* promoter luciferase reporter constructs. (B) 10T1/2 cells were transiently transfected with the following luciferase reporter constructs, respectively: pGL3/Basic vector (Basic/Luc), SV40-pro/Luc, the ~1-kb *Notch4* promoter N4-pro1000/Luc, the ~500-bp *Notch4* promoter (N4-pro500/Luc), the imperfect half-GRE-mutated *Notch4* promoter [N4-pro500(mGRE)/Luc], the AP-1 motif-mutated promoter [N4-pro500(mAP1)/Luc], and the imperfect half-GRE and AP-1 motif-mutated promoter [N4-pro500(mGRE/AP1)/Luc]. Transfected cells were treated with or without cortisol and/or FGF-2 for 16 h. Luciferase activities are shown, with pGL3/Basic activity designated 1.0 (mean \pm standard error of the mean for at least three independent experiments). (C) 10T1/2 cells were transiently transfected with the AP-1 reporter (4xAP1/Luc) containing four synthetic AP-1 motifs (left) or the GRE reporter (GRE2/Luc) containing two copies of the GRE sequence from the tyrosine aminotransferase gene (right). Luciferase activities are shown, with the untreated condition designated 1.0 (mean \pm standard error of the mean for at least three independent experiments).

The results of Fig. 7 indicate that cortisol and FGF-2 synergistically activate the *Notch4* promoter. The imperfect half-GRE mediates the cortisol response, while the AP-1 motif mediates cortisol and FGF-2 responses. Similarly, FGF-2 alone stimulates AP-1 occupancy at the promoter, while FGF-2 significantly enhanced cortisol-mediated GR occupancy (Fig. 5C and D). Thus, mutation of the AP-1 motif abolished *Notch4* promoter responsiveness to both FGF-2 and cortisol, even though the imperfect half-GRE remained intact. Conversion of the imperfect half-GRE to a perfect half-GRE yielded an activity and signal responsiveness identical to that of the wild-type construct (data not shown).

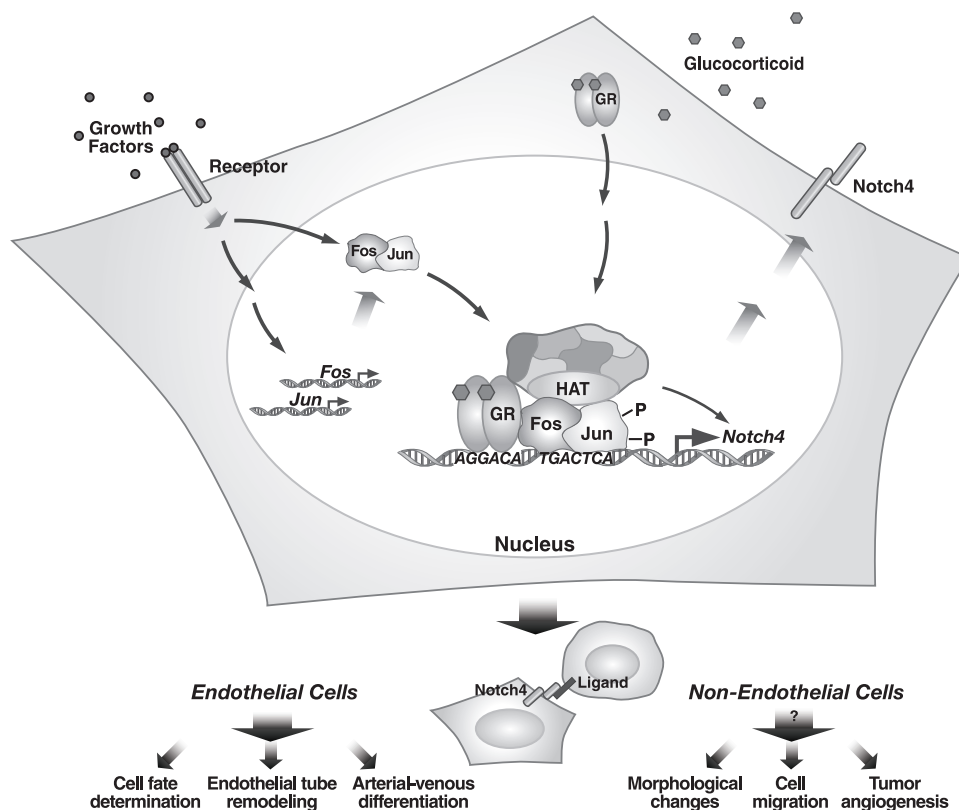


FIG. 8. Growth factor-glucocorticoid/Notch4 signaling axis. The model depicts growth factor- and glucocorticoid-mediated synergistic activation of *Notch4* transcription. The angiogenic factor FGF-2 induces genes encoding AP-1 subunits and activates AP-1 via posttranslational modifications, thereby increasing AP-1-mediated transactivation. Activated AP-1 occupies the *Notch4* promoter composite response element and enhances cortisol-bound GR occupancy at the imperfect half-GRE. The AP-1/GR complex at the composite response element recruits coactivators that increase acH4 and H3-dimeK4 at restricted regions of the locus and synergistically activate *Notch4* transcription. Notch4 is engaged by ligands on the surface of neighboring cells to stimulate angiogenic vascular remodeling. The integrity of this network is predicted to be crucial for establishment of physiological levels of Notch4 signaling in vascular endothelium. Since synergistic signaling reprograms the repressed *Notch4* locus to an active locus in a nonendothelial cell, we hypothesize that the growth factor-glucocorticoid/Notch4 axis can be engaged in pathophysiological scenarios (e.g., in malignancies in which AP-1 and GR are deregulated, thereby ectopically activating *Notch4*). As deregulated Notch signaling is oncogenic, derailing the growth factor-glucocorticoid/Notch4 axis would likely contribute to tumor progression.

Notch4 regulation via synergistic growth factor-glucocorticoid signaling: mechanistic insights. We describe herein growth factor and glucocorticoid synergism to establish an active *Notch4* locus in endothelial and nonendothelial cells. Although Notch signaling controls numerous biological processes, mechanisms regulating Notch receptor expression are poorly understood. Previously, we implicated AP-1 in mediating endothelial cell-specific *Notch4* expression, but other important factors/signals were not determined (78). As FGF-2, which activates AP-1, is a crucial proangiogenic factor that regulates vascular remodeling and tumor angiogenesis (25, 30, 76), an FGF-2-AP-1-*Notch4* pathway is likely to be important in both physiological and pathophysiological contexts. Endothelial cells express FGFR1 and/or FGFR2, and FGF-2 signaling modulates diverse endothelial cell functions, including proliferation, migration, morphology, and transcription (25). Glucocorticoids exert extensive physiological and pathological activities, including the direct regulation of endothelial cell functions (16, 17, 40, 53, 68). It was reported that cortisol induced *Notch1* and *Notch2*, but not *Notch4*, mRNA in osteoblast MC3T3 cells (52). However, mechanisms of cortisol-induced *Notch1* and *Notch2* expression are unknown. Furthermore, during dexa-

methasone-induced osteoblast differentiation, *Notch1* and *Notch2* mRNAs are downregulated in differentiating cells, whereas *Notch4* mRNA was slightly upregulated (58). It is unclear whether the apparent regulation of Notch receptor expression in these systems represents a direct action of glucocorticoids or is secondary to osteoblast differentiation. Cortisol did not induce *Notch1* expression in the endothelial cells that we have studied (Fig. 1B), and cortisol alone is insufficient to induce *Notch4* expression (Fig. 2A).

Almost all reports of GR-AP-1 interactions involve GR antagonism of AP-1-mediated transactivation (28, 60, 67, 79), and this mechanism is important for glucocorticoid anti-inflammatory activity. The GR binds AP-1, preventing AP-1 DNA binding, or the GR-AP-1 complex recruits corepressors at a target site. In a transient transfection assay with synthetic reporter constructs, the GR potentiates AP-1-mediated transcription, dependent upon AP-1 subunit composition and the distance between response elements (8, 18, 47, 51). GR positively regulates AP-1-mediated transactivation on a 25-bp composite response element, consisting of a GRE and an AP-1 motif, but only when the Jun level exceeds that of Fos (8). When the center-to-center distance between the GRE and the

AP-1 motif is >26 bp, the GR positively regulates both Fos and Jun-mediated transcription (51). At the *Notch4* promoter, the composite response element that mediates synergism consists of an imperfect half-GRE and an AP-1 motif with a 5-bp spacer, which is the first example that GR and AP-1 synergistically activate an endogenous locus.

Our results indicate that growth factor/glucocorticoid signaling converges upon AP-1 and GR to synergistically activate *Notch4* transcription. Activation is mediated by a promoter-localized imperfect half-GRE and a neighboring AP-1 motif. The imperfect half-GRE is insufficient for maximal GR occupancy and function when AP-1 is not promoter bound, and AP-1 enhances GR occupancy (Fig. 5D) and transcriptional activation (Fig. 7B). In aggregate (Fig. 8), the results support a model in which AP-1 stabilizes GR at the imperfect half-GRE, which constitutes a novel mode of GR function. It will be of considerable interest to determine whether perturbations of the growth factor-glucocorticoid axis deregulate *Notch4* expression in pathological states, such as tumor angiogenesis, and ectopically activate *Notch4* in cells that normally lack the capacity to respond to Notch4 ligand-expressing cells in the microenvironment.

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