Regulation of NF-κB-dependent Gene Expression by the POU Domain Transcription Factor Oct-1*

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Nathaniel G. dela Paz1, Simos Simeonidis2, Christopher Leo3, David W. Rose4,5, and Tucker Collins4,5

From the †Molecular Pathology Graduate Program, School of Medicine, University of California, San Diego, La Jolla, California 92093-0673, the ‡Department of Pathology, Children’s Hospital and Harvard Medical School, Boston, Massachusetts 02115, the §Department of Medicine and University of California San Diego Cancer Center, University of California, San Diego, La Jolla, California 92093-0673

Maintenance of the cells of the vessel wall in a quiescent state is an important aspect of normal vascular physiology. Transcriptional repressors are widely believed to regulate this process, yet the exact factors involved and the mechanism of repression are not known. Here, we report that the POU domain transcription factor Oct-1 represses the expression of E-selectin and vascular cell adhesion molecule (VCAM-1), two cytokine-inducible, NF-κB-dependent endothelial-leukocyte adhesion molecules that participate in the leukocyte recruitment phase of the inflammatory response. Co-transfection and microinjection studies demonstrate that Oct-1 blocks tumor necrosis factor α-stimulated E-selectin and VCAM-1 expression. Gene expression arrays indicate that control of tumor necrosis factor α-induced, NF-κB-dependent gene expression by Oct-1 is promoter-specific. A DNA-binding mutant of Oct-1 represses NF-κB-dependent reporter gene expression. Biochemically, Oct-1 interacts with p65, suggesting that Oct-1 is involved in the regulation of NF-κB transactivation function. NF-κB-dependent gene expression is more pronounced in Oct-1-deficient than in wild-type murine embryonic fibroblasts, and reintroduction of human Oct-1 abolishes these differences. Finally, the cytokine interleukin-6 induces Oct-1 gene expression, providing a biologically relevant means by which NF-κB-dependent gene expression can be selectively reverted by Oct-1 to quiescent levels.

At sites of vascular dysfunction, multiple signaling pathways are activated that can modulate gene expression. Rapid and transient induction of a select set of transcription factors, such as nuclear factor-κB (NF-κB), is followed by the expression of a tissue-specific group of target genes. For NF-κB, these genes include the endothelial-leukocyte adhesion molecules (E-selectin, VCAM-1, and ICAM-1), proinflammatory cytokines, chemokines, enzymes, and survival factors (1). Collectively, these products activate the vascular cells, resulting in the recruitment of circulating leukocytes, and stimulation of smooth muscle cell proliferation and migration, ultimately causing pathology in the vessel wall.

The NF-κB/Rel family of proteins consist of homo- or heterodimers (2). Subunits of NF-κB include NF-κB1 (p50), NF-κB2 (p52, p49, and p50B), RelA (p65), RelB, and c-Rel. NF-κB subunits share a Rel homology domain, which is a 300-amino acid N-terminal region that mediates several key functions, including dimerization, DNA binding, nuclear translocation, and binding of inhibitors. The C-terminal region of most subunits contains a transactivation domain, responsible for the recruitment of coactivators. In quiescent cells, NF-κB is localized in the cytoplasm, where it is retained through association with an inhibitor (IκB), such as IκBα. IκBs bind preferentially to different NF-κB dimers and sterically hinder recognition of the nuclear localization sequence of NF-κB subunits. Diverse stimuli can activate NF-κB, through phosphorylation and activation of the IκB kinase complex (3–5). Activated IκB kinases phosphorylate IκBs, leading to the polyubiquitination and degradation of the inhibitor by the 26 S proteasome. NF-κB dimers are transported to the nucleus, where they transactivate gene expression through interactions with other transcription factors and coactivators (6–9).

Under physiologic conditions, the cells of the vessel wall are quiescent. In this state, endothelial cells do not proliferate and have antithrombotic, vasodilator, and growth-inhibiting effects on the smooth muscle cells of the vessel wall. It is widely believed that repressors of gene transcription are important for maintaining vascular cells in the quiescent state. In the case of NF-κB activity, self-induction and expression of the inhibitor IκBα contributes to its autoregulation (10). Similarly, other transcription factors, such as Egr-1 and NFAT, are autoregulated by the NABs (11) and the Down syndrome critical region (DSCR-1 or calcipressin 1) gene products (12), respectively.
Members of the POU domain family of transcription factors interact specifically with octamer motifs and can act constitutively or in response to signaling events to either activate or repress expression of specific genes. Octamer sites are found in the promoters of a series of genes that are important in immune responses (e.g. Ig, interleukin (IL)-2, and IL-4), as well as genes that play a role in development (13, 14). Oct-1 is a prototypical member of the POU domain family, and one of its functions is to activate S phase-specific expression of the histone H2B gene (15). Additionally, Oct-1 can interact with nuclear hormone receptors, such as retinoid X receptor, thyroid hormone receptor, and glucocorticoid receptor, and influence their transcriptional activity (16, 17). Oct-1 can also repress expression of certain genes, including the von Willebrand factor promoter (18), prolactin gene promoter (19), and growth hormone promoter (16). Although the mechanism of repression by Oct is not well established, recent studies suggest that it can interact with SMRT (silencing mediator for retinoid and thyroid hormone receptor) (17).

Oct-2 is preferentially expressed in B lymphocytes and interacts with the octamer site found in the promoters of immunoglobulin genes. Oct-1 and Oct-2 recruit a B-cell-specific coactivator, designated OBF-1 (or OCA-B or Bob-1), providing an example of cell type-specific gene expression in which the critical component is a coactivator, rather than a DNA-binding protein (20). The activity of another founding member of the POU family, Pit-1, is determined by a regulated balance between a co-repressor complex that contains nuclear receptor co-repressor and histone deacetylases and a co-activator complex that contains CREB-binding protein and p/CAF (21). In response to specific signals, Pit-1 recruits the co-activator complex and displaces the repressor complex, stimulating Pit-1 function (22).

Here, we demonstrate that Oct-1 represses the expression of E-selectin and VCAM-1, two cytokine-inducible, endothelial-leukocyte adhesion molecules that mediate leukocyte recruitment during an inflammatory response. The observation that Oct-1 is a powerful repressor of two distinct NF-κB-dependent genes raises the possibility that this octamer-binding protein may have a more general role in the regulation of κB-dependent gene expression and in the subsequent production of inflammatory mediators.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human TNFα and IL-6 were purchased from R&D Systems and used at 10 and 100 ng/ml, respectively. DHT, E2, and RA were each used at 1 μM. The –578 E-selectin chloramphenicol acetyltransferase (CAT) reporter construct was previously described (23). The VCAM-1/lacZ reporter construct was generated from the VCAM-1 CAT reporter construct described and characterized earlier (24). pS2, retinoic acid receptor β, and prostate-specific antigen/lacZ reporters were previously described (25, 26). The pCG Oct-1 and pCG Oct-1 H-AAA expression plasmids, as described (27), were generously provided by W. Herr (University of Lausanne, Switzerland). Oct-1 deletion mutants were generated by PCR amplification and cloned into the pCMX expression vector. The Oct-2 expression plasmid was derived from pGEM4 Oct-2 (28). NF-κB p65 and p50 expression constructs were generously provided by D. Thanos (University of Crete, Greece). Mouse monoclonal anti-human E-selectin was purchased from Dako. Fluorescein isothiocyanate-conjugated anti-mouse IgG and tetramethylrhodamine-conjugated dextran were obtained from Jackson ImmunoResearch Laboratories, Inc. The following antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): normal rabbit IgG (SC-2027), anti-NF-κB p65 (SC-109 and SC-372-G), anti-NF-κB p50 (SC-7178), anti-GAL4 (SC-510), anti-Oct-1 (SC-232), anti-Oct-2 (SC-233), and anti-mSin3B (SC-768). Anti-β-tubulin was generously provided by N. W. Chi (University of California, San Diego). Horseradish peroxidase-conjugated secondary antibodies were obtained from either Santa Cruz Biotechnology or Amersham Biosciences.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were either isolated from fresh human umbilical cords or purchased from Clonetics/Cambrex and cultured in endothelial growth medium also obtained from Cambrex. Cells from passage four were used for the microinjection studies. In these experiments, cells were treated with TNFα for 2 h after microinjection of cells. Rat-1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium (Mediatech) supplemented with 2 mM GlutaMax (Invitrogen), 500 nM methotrexate (Sigma), 1% penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (Omega Scientific). COS-7 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 2 mM L-glutamine, penicillin/streptomycin, and 10% fetal calf serum. All immortalized murine embryonic fibroblast (MEF) cell lines (Oct-1(+)+), Oct-1(−/−), and Oct-1(−/−) infected with retroviruses carrying human Oct-1 or empty vector), described in Ref. 29, were generously provided by D. Tantin (Sharp Laboratory, MIT, Boston, MA) and were maintained in Dulbecco’s modified Eagle’s medium–low glucose (Mediatech) containing 50 μM 2-mercaptoethanol (Sigma), 1% penicillin/streptomycin, and 10% fetal bovine serum.

Transient Transfections and Reporter Assays—COS-7 cells were grown on 12-well dishes and cultured at 37 °C in the presence of 5% CO2. The COS-7 cells were transiently transfected with 1 μg of –578 E-selectin promoter-CAT or VCAM-1 promoter-CAT and 100 ng of a human cytomegalovirus-p65 expression vector by a modified calcium phosphate method. Varying concentrations of the indicated expression plasmids were transfected. Samples were balanced for total DNA content with the empty expression vector pCR3 (Invitrogen). After 24 h of incubation, cells were harvested in reporter lysis buffer (Promega), and CAT activity was determined as previously described (6, 30) and normalized to β-galactosidase activity.

Single Cell Nuclear Microinjection Assays—HUVECs were seeded at subconfluent density on acid-washed glass coverslips that were pretreated with 0.1% gelatin and were grown in endothelial growth medium. Expression plasmids encoding the appropriate genes were injected at 100 μg/ml. Cells were co-injected with tetramethylrhodamine-conjugated dextran to unambiguously identify them by immunofluorescence microscopy. After overnight incubation, the cells were treated with the appropriate cytokine, fixed, immunostained to detect E-selectin, and analyzed by confocal microscopy.
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tin expression, and mounted using gelvitol that contains 4′,6-
diamidino-2-phenylindole. Rat-1 fibroblasts were seeded on
acid-washed glass coverslips at subconfluent density and main-
tained in Dulbecco’s modified Eagle’s medium/F-12 medium
supplemented with 2 mM GlutaMax, 500 mM methotrexate, 1%
penicillin/streptomycin, and 10% fetal bovine serum. Before
injection, cells were rendered quiescent by incubation in serum-
free medium for 16–24 h. Each experiment was performed on
three independent coverslips with >300 injected cells.

**RNA Isolation, RT-PCR Analysis, and RNA Profiling**—
Total RNA was isolated using either the RNeasy Mini Kit (Qia-
gen), RNAqueous-Micro (Ambion), or RNA-Be (Tel-Test,
Inc.) according to each manufacturer’s protocol. First strand
cDNA synthesis was performed using the reverse transcriptase
and primer. StrataScript reverse transcriptase (1.25 units/sam-
piece) and TaqPlus Precision DNA polymerase (2.5 units/sample)
were added together prior to thermal cycling. 40 cycles of
amplification were typically performed. For analysis, 25 µl of
each PCR product was loaded on a 1% agarose gel. The primers
were designed as follows: E-selectin (forward), 5′-GTGAAAGCTG-
TGAGATGCGATG-3′; E-selectin (reverse), 5′-TTACACTCT-
GCGAGGAAAGAA-3′; VCAM-1 (forward), 5′-CTGGAGGAG-
ATGAGACAGAGAAG-3′; VCAM-1 (reverse), 5′-TTGAAAC-
AGGTCTAGTGTCAAC-3′; Oct-1 (forward), 5′-GAAGGCTT-
TGAACCTCAGCTTT-3′; Oct-1 (reverse), 5′-TTCCTATGC-TGGTGGCTTTCT-3′; GAPDH (forward), 5′-GAGTCAACG-
GATTTGGTCGTA-3′; GAPDH (reverse), 5′-TTCCATTGAG-
GCAGGAGCTCTCC-3′. The primers were custom ordered from
either GenBase, Inc. or Integrated DNA Technologies.

RNA profiling of HUVECs or MEFs was performed using the GEArray Q Series Human or Mouse NF-κB Signaling Pathway
Gene Array (SuperArray Bioscience) by following the manufac-
turer’s instructions. Analysis of gene expression array data was
done using the GEArray Expression Analysis Suite software
(SuperArray Bioscience).

**GST Pull-down Assays**—GST fusion constructs containing
various fragments of p65 and Oct-1, and GAPDH
were generated by PCR amplification and cloned into the pGEX-2TA vector (Amer-
sberg Biosciences). DNA sequence analysis verified the authen-
ticity of the fusions. GST forms were expressed in *Escherichia coli* BL21 cells and purified with glutathione-Sepharose beads
(Amersham Biosciences). In preliminary SDS-PAGE studies,
each of the fusion proteins was verified to be appropriate in
molecular weight and expressed at comparable levels (Fig. 3, A
and B). 5 µg of the fusion protein was incubated with 5 µl of
[35S]methionine-labeled protein, generated by *in vitro*
transcription/translation, as described by the manufacturer
(Promega). The proteins were incubated overnight at 4 °C in
binding buffer (20 mM Hepes, pH 7.7, 75 mM potassium chloride, 0.1
mM EDTA, 25 mM magnesium chloride, 0.05% Nonidet P-40, 1
mM dithiothreitol, and 1 mg/ml bovine serum albumin). Bound
proteins were washed three times with binding buffer and
eluted in SDS sample buffer, subjected to SDS-PAGE, and
detected by autoradiography.

**Immunoprecipitation and Western Blot Analysis**—Cells were
washed and collected in ice-cold 1× phosphate-buffered
saline/phosphate inhibitors. Cytoplasmic and nuclear frac-
tions were prepared using the Nuclear Extract Kit (Active
Motif) following the manufacturer’s protocol. Nuclear fraction
samples were centrifuged briefly and precleared with preim-
mune IgG and protein A-agarose beads (Roche Applied Sci-
ence) for 30 min. Precleared extracts were then immunopre-
cipitated overnight at 4 °C with 1–2 µg of each specified
antibody. 50 µl of protein A-agarose beads were added and
incubated with the samples for 2 h at 4 °C. The beads were
washed three times with lysis buffer and then resuspended
in SDS sample buffer and analyzed on a 10% SDS-PAGE. Proteins
were electrophoretically transferred to polyvinylidene difluo-
ride membranes (PerkinElmer Life Sciences), which were then
blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-
HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) and incubated
with anti-Oct-1 IgG for 16 h at 4 °C. Blots were washed three
times with TBST buffer, incubated for 1 h with a secondary
antibody conjugated to horseradish peroxidase, and then
washed three times with TBST. The antigen-antibody interac-
tions were visualized by incubation with SuperSignal West Pico
Chemiluminescent Substrate (Pierce). Blots were exposed to
x-ray film for 5 s to 5 min.

**siRNA Knockdown**—Target sequence for the Oct-1 siRNA
oligonucleotide was designed by using the Dharmacon
siDESIGN Center Web site. The sequence used was 5′-NC-
UCGUGAAGCUUUUAC-3′. The oligonucleotide had been
2′-deprotected, duplexed, and desalted by the manufac-
turer. Sterile RNase-free water was then added to give a final
concentration of 20 µM in 1× universal buffer. siCONTROL
nontargeting siRNA pool obtained from Dharmacon was used
as a control for non-sequence-specific effects. For microinjec-
tion studies, siRNA duplexes were diluted to a final concen-
tration of 50 nM in microinjection buffer (5 mM Na2PO4, pH 7.2,
100 mM KCl). After 24 h, total RNA was isolated from the
microinjected cells, and RT-PCR analysis was performed.

**RESULTS**

Oct-1 Represses E-selectin and VCAM-1 Promoter-Reporter
Gene Expression—Sequence inspection of the E-selectin pro-
moter reveals potential octamer motifs that could bind mem-
ers of the POU domain family of transcription factors. To
determine whether Oct-1, which is ubiquitously expressed, or
Oct-2, a tissue-specific member of the POU family, affects cyto-
kine-induced E-selectin or VCAM-1 expression, we performed
single cell nuclear microinjection assays using either an E-se-
lectin (Fig. 1A) or VCAM-1 promoter/lacZ reporter construct
(data not shown) in Rat-1 fibroblasts. Fibroblasts that were
injected individually with each reporter plasmid showed mini-
mal expression levels without cytokine stimulation. Treatment
of the cells subsequent to injection for 2 h with TNFα
induced expression severalfold. This activation was blocked by co-injec-
tion of an expression plasmid for Oct-1, but parallel injections
with an Oct-2 expression plasmid did not inhibit activation
(Fig. 1A). Similar results were obtained when quiescent cells
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Overexpression of Oct-1 blocks TNFα-induced E-selectin and VCAM-1 gene expression. A, Rat-1 fibroblasts were microinjected with either an E-selectin/lacZ or VCAM-1/lacZ promoter-reporter plasmid. Cells were co-injected with either the Oct-1 or Oct-2 expression construct as indicated. Rhodamine-conjugated dextran was used as a carrier to unambiguously label injected cells. Reporter gene expression was activated, as indicated, by either co-injection of a p65 expression plasmid or by TNFα treatment. Results are reported as the percentage of injected cells (red) that had β-galactosidase activity (blue) as detected by X-gal staining. Representative results are shown in the color panel above the bar graph. B, lacZ reporter constructs containing promoter response elements for the prostate-specific antigen (PSA), the estrogen-responsive gene pS2, or the retinoic acid receptor β were microinjected into Rat-1 fibroblasts. As indicated, cells were co-injected with either the Oct-1 or Oct-2 expression construct. Expression was activated by the addition of dihydrotestosterone (DHT), estradiol (E2), or retinoic acid (RA), and β-galactosidase activity was monitored. Results are shown as the percentage of injected cells that stained blue. C, primary cultures of HUVEC were either left uninjected or injected with the Oct-1 or Oct-2 expression construct. Rhodamine-conjugated dextran was co-injected to unambiguously label successfully injected cells (red). After overnight incubation, the cells were treated with cytokine as indicated. The cells were then fixed and immunostained with anti-E-selectin followed by detection with a fluorescein isothiocyanate-conjugated secondary antibody (green). Nuclei were identified by 4′,6-diamidino-2-phenylindole stain (blue). Representative findings are shown in the top panel. The bottom panel is a bar graph that summarizes the findings. D, small populations of HUVECs (200–500 cells) were spotted on glass coverslips and were left uninjected, or every cell was injected with fluorescent dextran, Oct-1, or Oct-2 expression plasmids. Total RNA was isolated followed by one-step RT-PCR using an equal amount of RNA and gene-specific primers designed to amplify E-selectin, VCAM-1, and GAPDH cDNA.

We also examined the effects of Oct-1 and Oct-2 overexpression on the expression of several NF-κB-independent genes to demonstrate that this repression caused by Oct-1 is specific and not merely an artifact of overexpression. Using microinjection, we introduced the prostate-specific antigen, pS2 (estrogen-responsive protein), or retinoic acid receptor β promoter-reporter genes into fibroblasts and compared their expression levels with or without their respective agonists as well as together with either Oct-1 or Oct-2. As shown in Fig. 1B, co-injection of neither the Oct-1 nor the Oct-2 expression plasmid together with each of the aforementioned reporter genes resulted in any significant decreases in ligand-induced gene expression. Altogether, these findings suggest that Oct-1 selectively represses the transcription of both the E-selectin and VCAM-1 promoter-reporter genes.

Oct-1 Represses Authentic E-selectin and VCAM-1 Gene Expression in Cytokine-activated Endothelial Cells—To determine whether this apparent repression by Oct-1 occurs in vivo on endogenous promoters in a true chromatin environment, we developed a single cell nuclear microinjection assay for monitoring E-selectin expression in primary cultures of HUVECs. Briefly, HUVECs were microinjected with either the Oct-1 or Oct-2 expression vector, and after overnight incubation, the cells were treated with recombinant human TNFα to induce the expression of E-selectin.
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TABLE 1
Selective Oct-1 suppression of genes induced in HUVEC in response to TNFα treatment
The table lists genes that showed a 3-fold or higher increase in expression in response to TNFα when compared to their basal levels and the subset of genes that were significantly down-regulated (>3-fold) in response to transcription factor overexpression in HUVEC.

| Gene symbol | Description | Induction | Oct-1 suppression |
|-------------|-------------|-----------|------------------|
| ATF2        | Activating transcription factor 2 | + | + |
| ELK1        | ETS family member | + | + |
| ELK3        | ETS family member | + | - |
| NFKB1       | Nuclear factor related to κB binding protein | p105 | + | + |
| REL         | c-Rel | + | + |
| RELA        | p65 | + | - |
| SAA1        | Serum amyloid A1 | + | - |
| SELE        | E-selectin | + | + |
| TLR4        | Toll-like receptor 4 | + | + |
| TNFAIP3     | A20 | + | + |

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Repression of NF-κB-dependent Gene Expression by Oct-1 Is Promoter-specific—The observation that Oct-1 specifically represses the expression of two cytokine-inducible NF-κB target genes that belong to two distinct families of adhesion molecules raises the possibility that this octamer-binding transcription factor could have a more general role in the regulation of NF-κB-dependent gene expression. To determine whether Oct-1 generally affects the expression of genes related to NF-κB signaling, we performed gene expression array studies using RNA extracted from a small population of HUVECs that were either un.injected or injected individually with Oct-1 or Oct-2 expression plasmids. As expected, treatment of endothelial cells with TNFα caused certain genes, including E-selectin, to be expressed at levels significantly higher than in quiescent cells (Table 1). More importantly, Oct-1 but not Oct-2 (data not shown), caused a significant down-regulation of some of these activated genes. The fact that only a subset of the genes induced by TNFα were negatively affected by Oct-1 overexpression implies that Oct-1-mediated repression is promoter-specific.

Repression by Oct-1 Is Not Dependent on DNA Binding—Collectively, the results from the previously described experiments demonstrate that Oct-1 inhibits the cytokine-induced expression of E-selectin and VCAM-1 but provide little insight into the mechanism. In an effort to further clarify how Oct-1 decreases the expression of E-selectin and VCAM-1, we examined whether a direct physical interaction between Oct-1 and DNA was required. The role of DNA binding in Oct-1-mediated suppression of NF-κB-dependent gene expression was determined by using a previously characterized mutant of Oct-1 that was incapable of binding DNA (27). Overexpression of the mutant form of Oct-1 by transient transfection decreased p65 expression plasmid or by TNFα treatment (dark gray bars). Results are reported as the percentage of injected cells that stained blue with X-gal.

FIGURE 2. Oct-1-mediated inhibition is not dependent on DNA binding. A, COS-7 cells were transiently transfected with the E-selectin promoter-CAT plasmid, p65 expression plasmid, and increasing amounts of either wild-type Oct-1 or a mutant form of Oct-1 that is incapable of binding DNA (27). 24 h post-transfection, the cells were harvested in lysis buffer, and CAT activity was determined and normalized to β-galactosidase activity. B, reporter constructs containing lacZ under the transcriptional control of either the E-selectin promoter or the VCAM-1 promoter were microinjected together with either wild-type Oct-1 or the Oct-1 DNA binding mutant. Reporter gene expression was stimulated, as indicated, by either co-injection of a p65 expression plasmid or by TNFα treatment (dark gray bars). Results are reported as the percentage of injected cells that stained blue with X-gal.
Oct-1 DNA binding mutant inhibits p65-activated expression of the E-selectin and VCAM-1 promoter/lacZ reporter genes (Fig. 2B) with the same potency as wild-type Oct-1. In a separate set of microinjection experiments, TNFα-induced activation of both lacZ reporter genes was repressed by the Oct-1 DNA binding mutant (Fig. 2B).

Oct-1 Interacts with NF-κB p65—The data presented above suggests that Oct-1 can inhibit the transcriptional activation of NF-κB target genes independent of its ability to bind DNA. To further elucidate the mechanism of this inhibition, we determined whether Oct-1 and either or both of the two NF-κB heterodimer subunits (Fig. 3A) interacted with one another in vitro. Using a GST pull-down assay, we found that full-length Oct-1 was able to directly bind a GST fusion protein of the NF-κB p65 subunit. This interaction was further mapped to amino acids 286–551 of p65, which contains the transcriptional activation domain (Fig. 3C). No interactions were detected with the Rel homology domain of p65 (amino acids 1–321) or GST alone, whereas a weak interaction was detected with p50. Additionally, none of the GST fusion proteins were able to pull down [35S]methionine-labeled luciferase (data not shown). The converse experiment was also performed, in which GST fusion proteins of various Oct-1 fragments (Fig. 3B) were used in interaction studies with intact p65. Interestingly, p65 associated solely with the N-terminal region (amino acids 1–270) of Oct-1 (Fig. 3C). No interactions with the POU homodomain (amino acids 265–444) or C terminus (amino acids 439–744) were evident.

We next investigated the ability of Oct-1 to associate with p65 in vivo using co-immunoprecipitation studies. By examining endogenous nuclear extracts as opposed to overexpressed levels of p65 and Oct-1, we can demonstrate that the interaction between the proteins is physiologically relevant. To this end, we used HUVECs that were rendered either quiescent or activated briefly by TNFα.
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with the in vitro results described above, Oct-1 was found in nuclear extracts precipitated with the p65 antibody but not with preimmune IgG (Fig. 3D). No physical interactions were detected between Oct-1 and p50, and Oct-1 was completely absent in extracts precipitated with an irrelevant antibody (data not shown). In separate control studies, overexpressed Oct-2, unlike Oct-1, does not co-immunoprecipitate with p65 (data not shown), suggesting that p65 and Oct-2 do not interact with one another while at the same time confirming the interaction between p65 and Oct-1.

We then performed microinjection experiments using Oct-1 deletion mutants (Fig. 3E) to test whether Oct-1 is acting through this interaction with NF-κB. As previously observed, microinjection of the wild-type Oct-1 expression construct caused a significant repression of TNFα-induced E-selectin reporter gene expression. More importantly, microinjection of the construct encoding an N-terminal deletion mutant of Oct-1, which lacks the NF-κB interaction domain, did not result in repression. In contrast, the C-terminal deletion mutant of Oct-1, which still has an intact NF-κB interaction domain, repressed to the same extent as wild-type Oct-1. Taken together, these results demonstrate that Oct-1 can directly interact with the p65 subunit of NF-κB both in vitro and in vivo and that this interaction with NF-κB is functionally important, suggesting that Oct-1 could have a direct role in regulating the transactivation function of NF-κB.

**NF-κB-dependent Gene Expression is Up-regulated in Oct-1-deficient MEFs**—To examine the effect of Oct-1 deficiency on the expression of NF-κB-dependent genes, we compared the gene expression profiles of immortalized fibroblast lines derived from wild-type and Oct-1-deficient mouse embryos using a pathway-specific gene expression array system that contains 96 genes related to NF-κB-mediated signal transduction. As predicted, the gene expression patterns of the two cell lines were markedly different (Fig. 4A). Interestingly, several of these NF-κB-related genes were expressed at levels that were at least 3-fold higher in Oct-1-deficient MEFs than in wild-type MEFs, as determined by analysis software provided by the manufacturer (data not shown). To determine whether gene expression in Oct-1-deficient MEFs could be returned to its quiescent levels by exogenous expression of Oct-1, gene expression arrays were performed using Oct-1-deficient MEFs infected with recombinant retroviruses with and without human Oct-1. Reintroduction of human Oct-1 in Oct-1-deficient cells caused a substantial decrease in the expression of several genes to levels that were well below those of quiescent wild-type MEFs (Fig. 4A).

When the gene expression profile of quiescent wild-type MEFs was compared with the profile of wild-type MEFs treated with TNFα, we observed 3-fold or higher increases in the expression of several genes. Interestingly, the expression profiles of Oct-1-deficient MEFs and TNFα-induced wild-type MEFs were very similar (Fig. 4, A and B). Levels of IKKα are increased in Oct-1(−/−) MEFs, which may play a role in increased expression of some NF-κB-dependent genes. Collectively, these results suggest that the presence of endogenous levels of Oct-1 is essential for maintaining at least a basal level of NF-κB-dependent gene expression, and TNFα stimulation leads to an induced state in which endogenous levels of Oct-1 are no longer capable of maintaining quiescence.

Similar findings were observed when single cell nuclear microinjection assays were performed using either the E-selectin/lacZ (Fig. 4C, gray bars) or VCAM-1/lacZ reporter construct (Fig. 4C, black bars) in each of these cell lines. Specifically, the basal expression of both reporter genes increased dramatically when individually introduced into Oct-1-deficient MEFs versus wild-type MEFs. Moreover, exogenous expression of human Oct-1 in the Oct-1-deficient MEFs effectively repressed these increases.

**Oct-1 Expression Is Up-regulated in IL-6-induced Endothelial Cells**—We next looked for mechanisms that might exist that enable the level of Oct-1 to be up-regulated in a given cell type at times during which its repressive function is critical. IL-6 is a pleiotropic cytokine that is expressed by a wide variety of cell types, including macrophages and vascular endothelial cells. Additionally, its expression has been shown to be inducible by inflammatory cytokines, such as TNFα and IL-1 (32, 33), through activation of NF-κB (34, 35). To determine whether Oct-1 expression could be regulated by IL-6 in human endothelial cells, we performed RT-PCR experiments using RNA extracted from HUVECs treated with or without human recombinant IL-6. Indeed, treatment of HUVECs with IL-6 caused a significant increase in Oct-1 mRNA levels compared with basal level, and this increase occurred as early as 1 h after stimulation and was sustained for at least 4 h (Fig. 5A, left). After 24 h, these levels returned to those in resting cells (data not shown). In parallel, we examined Oct-1 protein expression by Western blot analysis using whole cell extracts from HUVECs under similar conditions (Fig. 5A, right) and noted a modest increase by 8 h.

**TNFα-induced E-selectin Reporter Gene Expression Is Repressed by IL-6**—To investigate whether pretreatment of cells with IL-6 could lead to repression of TNFα-induced gene expression, we performed microinjection experiments using a reporter construct. The E-selectin/lacZ reporter plasmid was microinjected into HUVECs followed by no stimulation or stimulation with either TNFα alone, IL-6 alone, or TNFα following IL-6 pretreatment (Fig. 5B, black bars). As expected, TNFα-stimulated cells showed a significant increase (>2-fold) in E-selectin promoter-reporter activity compared with untreated cells, whereas IL-6-stimulated cells showed no appreciable effect. However, pretreatment for 4 h with IL-6 prior to TNFα treatment resulted in greater than 4-fold repression of E-selectin gene expression, suggesting that activation of the IL-6 signaling pathway could have a regulatory role in the TNFα-induced NF-κB activation pathway, possibly through the induction of Oct-1 expression.

To distinguish whether the IL-6-induced expression of Oct-1 causes the repression of TNFα-induced E-selectin reporter gene expression rather than another mechanism unrelated to Oct-1, we performed microinjection experiments similar to those described above. In these experiments, RNA interference was utilized to specifically knock down the expression of Oct-1. HUVECs were co-injected with the E-selectin/lacZ reporter construct and an Oct-1-specific siRNA oligonucleotide followed by incubation for at least 24 h. In control studies, the
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FIGURE 4. Oct-1-deficient MEFs show higher basal NF-κB-dependent gene expression than wild-type MEFs. A, expression profile of NF-κB-related genes in various MEF cell lines. Total RNA was isolated from three different unstimulated MEF cell lines, as indicated, and also from wild-type MEFs treated with TNFα for 2 h. Using a murine pathway-specific gene expression array system, biotin-labeled cDNA probes were synthesized from the isolated RNA, which were then amplified before hybridization to nylon membranes prespotted with cDNA fragments of 96 NF-κB-related genes. Arrays were developed by chemiluminescence followed by exposure to film. The blue boxes on each individual array indicate GAPDH, which was the gene selected as the control. B, clustergram that depicts the relative expression levels of NF-κB-related genes in basal versus TNFα-induced wild-type MEFs, in basal Oct-1-deficient MEFs, and in basal Oct-1-deficient MEFs in which human Oct-1 was reintroduced by a viral vector. Expression levels were normalized across the entire data set using GEArray Expression Analysis Suite. The green color corresponds to the minimal gene expression value, whereas the red color corresponds to the maximum as indicated on the color scale key. C, E-selectin and VCAM-1/lacZ reporter gene expression in different MEF cell lines. Wild-type MEFs, Oct-1-deficient MEFs, and Oct-1-deficient MEFs that had been restored with human Oct-1 were microinjected with either the E-selectin or VCAM-1/lacZ reporter construct (gray and black bars, respectively) and were left untreated or treated for 2 h with TNFα as indicated. Results are reported as the percentage of injected cells that stained blue with X-gal.
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Oct-1 siRNA significantly reduced the Oct-1 transcript (Fig. 5C). The results from these microinjection experiments showed that in the presence of Oct-1-specific siRNA, pretreatment of the cells with IL-6 did not repress TNFα-induced E-selectin reporter gene expression (Fig. 5B, gray bars). In this instance, IL-6 treatment should not lead to increased levels of Oct-1, because newly transcribed Oct-1 mRNA will be selectively destroyed, but IL-6 signaling should otherwise be unaffected. Altogether, these results are consistent with the idea that activation of the IL-6 receptor in response to inflammatory signals, such as TNFα and IL-1, could lead to an increase in Oct-1 expression, which could in turn down-regulate the expression of a specific subset of NF-κB target genes that had been induced by the very same signals.

DISCUSSION

In this paper, we studied the transcriptional regulation of two distinct NF-κB-dependent genes, E-selectin and VCAM-1, both of which are not expressed in quiescent endothelial cells but can be transiently induced by inflammatory mediators, such as TNFα and IL-1. Our results show that the transcriptional activation of both of these genes by the inflammatory cytokine TNFα is selectively inhibited by the ubiquitously expressed transcription factor Oct-1.

Oct-1 and Quiescence in the Vessel Wall—Our findings can be integrated into a model for the repression of TNFα-induced E-selectin and VCAM-1 gene expression in endothelial cells. In response to TNFα, adhesion molecules, such as E-selectin and VCAM-1, are expressed on the surface of endothelial cells. Circulating leukocytes bind to the surface of endothelial cells through interactions with these adhesion molecules. This cellular engagement leads to the activation of leukocytes and the paracrine secretion of the cytokine IL-6 (36, 37). IL-6 then acts on adjacent endothelial cells to up-regulate the expression of Oct-1, which then represses E-selectin and VCAM-1 gene expression back to their normal, quiescent levels.

Several comments should be made in considering this model. First, consistent with our findings with the adhesion molecules, Oct-1 has been shown to repress transcription of the von Willebrand factor gene promoter in endothelial cells (18).

Second, we found that treatment of HUVECs with IL-6 can cause the increased synthesis of Oct-1. Although in agreement with findings described in human embryonal carcinoma cells (38), this observation appears to disagree with previous studies that report the absence of IL-6α transcripts (39, 40) and lack of biological responses to exogenous IL-6 (32, 41) in human endothelial cells. However, a recent study by Waxman et al. (42) clearly demonstrated the expression of IL-6α-chain protein specifically in HUVECs by immunoblotting. Furthermore, Waxman et al. (42) showed that HUVECs exhibit IL-6-induced phosphorylation of STAT3 and p42/44 mitogen-activated protein kinases, which can be blocked by specific antibodies to IL-6. The repression of TNFα-induced E-selectin expression in endothelial cells pretreated with IL-6 and lack of repression in the presence of Oct-1-targeting siRNA oligonucleotides provide further evidence that these cells have fully functional IL-6 receptor complexes that are capable of signaling the increased expression of Oct-1.
Third, although IL-6 has been described as a major mediator of proinflammatory functions, it has also been implicated as having an anti-inflammatory role by down-regulating the level of proinflammatory cytokines (43). Specifically, the levels of TNFα, granulocyte-macrophage colony-stimulating factor, MIP-2, and interferon-γ markedly increased in the circulation of IL-6-deficient mice challenged with lipopolysaccharide. Coincidentally, the genes encoding these cytokines are all specific targets of NF-κB (44–49). IL-6 has also been shown to augment the levels of Oct-1 mRNA and protein in two separate human cell lines (38), suggesting that Oct-1 is a potential target of IL-6 regulation.

Fourth, the Oct-1 repression model may be relevant in other vascular cell types. Although E-selectin expression is largely restricted to endothelial cells, VCAM-1 expression occurs in a variety of cell types in addition to endothelial cells. Our findings demonstrate that selective repression by Oct-1 occurs in both endothelial and nonendothelial cells, indicating that Oct-1 inhibitory function is not cell type-specific. Hence, the transcriptional activation of VCAM-1 in other cell types within the vessel wall or in other tissues may also be regulated by Oct-1-mediated repression.

Consistent with our findings, octamer-binding proteins have been previously shown to play an important role in the biology of smooth muscle cells (SMCs). Although Oct-1 transcript levels were undetectable in SMCs found in the vessel wall, the transcription factor was induced upon disruption of SMC-extracellular matrix interactions (50). Oct-1 was constitutively expressed by cultured SMCs, but its transcription level could be diminished when cells were cultured on specific extracellular matrix proteins. These findings suggest that matrix components can regulate the expression of the transcription factor and implicate Oct-1 in the maintenance of SMC quiescence in the intact vessel wall.

Placing the p65-Oct-1 Interaction in Perspective—In this study, we find that the p65 subunit of NF-κB specifically interacts with the N-terminal region of Oct-1 (amino acids 1–270). Remarkably, this region of the Oct-1 protein is glutamine-rich and is thought to be important for transactivation (27). A previously published report by van Heel et al. (51) also shows that NF-κB p65 interacts with Oct-1. Although both studies demonstrate that p65 interacts with Oct-1, there is disagreement between the studies as to which domain of Oct-1 is important for this interaction. van Heel et al. (51) propose that p65 interacts through the POU homeodomain of Oct-1, but their studies only show that p65 binding to Oct-1 decreases in the absence of the POU homeodomain. They do not specifically show that p65 interacts with this domain alone in the absence of the N-terminal region. Therefore, the importance of the N-terminal region must still be carefully considered.

In contrast to the N terminus of Oct-1, a variety of protein-protein interactions have been mapped to the POU homeodomain region of the octamer protein. Two of the best characterized interactions with octamer factors include the herpes simplex gene product VP16 (52, 53) and the coactivator, designated OBF-1 (or OCA-B or Bob-1) (54, 55). Additionally, the glucocorticoid receptor (GR) binds to both Oct-1 and Oct-2 (56). Binding is mediated through an interface created by the DNA binding domain of the receptor and the POU domain of the octamer proteins. Interestingly, GR-Oct1/2 binding was disrupted by the binding of the receptor to its DNA recognition sequence.

Our data also demonstrate that Oct-1 interacts with the transactivation region of p65, as opposed to the Rel homology domain. It is interesting that this region of p65 can engage in other protein-protein interactions, including interactions with coactivators that are important during transcriptional activation.

Mechanism of Oct-1 Repression—Although the exact mechanism of Oct-1-mediated repression of E-selectin and VCAM-1 is uncertain, we can conclude from our overexpression studies with mutant Oct-1 that repression by Oct-1 is at least in part DNA-independent. Based on the results of our biochemical studies, one potential candidate for Oct-1 repression is the p65 component of NF-κB. In fact, our preliminary chromatin immunoprecipitation analysis shows the presence of Oct-1 simultaneously with p65 on the E-selectin promoter as early as 30 min after TNFα treatment of HUVECs (data not shown). Interestingly, p65 activity has been shown to be controlled by post-translational modifications, such as phosphorylation and acetylation (57, 58). More specifically, it has been proposed that histone deacetylases are involved in the deacetylation of p65, which affects its transactivation function (59, 60). In addition, the recent characterization of the E-selectin promoter in HUVECs shows the presence of two different histone deacetylases simultaneously with p65 at the same 30-min time point following TNFα activation (61). It is conceivable that repression by Oct-1 occurs through some mechanism that involves the recruitment of a specific histone deacetylase-containing corepressor complex, similar to the one recruited by Pit-1 (21), directly to the p65 subunit of NF-κB.

Altogether, the present study provides the first evidence that two distinct cytokine-inducible, NF-κB-dependent genes are actively repressed and can also undergo postinduction repression by the POU domain transcription factor Oct-1. These results suggest that Oct-1 modulates the activity of specific genes involved in immune and inflammatory responses and are consistent with the recent suggestion that Oct-1 is a cellular stress sensor (62).

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REFERENCES
1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
2. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
3. Karin, M., and Delhase, M. (2000) Semin. Immunol. 12, 85–98
4. Israel, A. (2000) Trends Cell Biol. 10, 129–133
5. Karin, M. (1999) J. Biol. Chem. 274, 27339–27342
6. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2927–2932
7. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) Science 275, 523–527
8. Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. (1998) J. Immunol. 161, 4118–4122
9. Sharp, P. A., and Tantin, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9566–9571
10. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
11. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
12. Karin, M., and Delhase, M. (2000) Semin. Immunol. 12, 85–98
13. Israel, A. (2000) Trends Cell Biol. 10, 129–133
14. Karin, M. (1999) J. Biol. Chem. 274, 27339–27342
15. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2927–2932
16. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) Science 275, 523–527
17. Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. (1998) J. Immunol. 161, 4118–4122

