hCTR9, a Component of Paf1 Complex, Participates in the Transcription of Interleukin 6-responsive Genes through Regulation of STAT3-DNA Interactions*[^1][^2][^3]

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PAF, which is composed of Paf1, Cdc73, Ctr9, Leo1, and Rtf1, is a novel complex with multiple functions in transcription-related activities. The PAF complex interacts with histone-modifying enzymes and RNA polymerase II to regulate transcription. With general transcription regulatory potential in yeast, Hyrax/Cdc73 has been reported to associate with β-catenin to control Wnt/Wg signal-specific transcription in Drosophila. Here, we present the first evidence of IL-6 signal-specific transcriptional regulation by SH2BP1/CTR9 in mammals. Upon LPS injection of mice, we observed transient induction of the mammalian PAF complex in the liver. Inhibition of CTR9 specifically abrogated expression of IL-6-responsive genes, but had no effect on genes constitutively expressed or induced by interferon-β, TNFα, or IL-1β. The PAF complex was found in the promoter regions of IL-6-responsive HP and FGG, but not in the promoter region of constitutively active GAPDH. Transcriptional activation by STAT3 was inhibited when CTR9 siRNA was introduced, whereas transcriptional activation was enhanced by mCtr9 overexpression. IL-6-activated Stat3 was found to co-localize and interact with CTR9. In CTR9-depleted cells, decreased STAT3 association with the promoter regions, as well as impaired K4-trimethylation of histone H3 in the coding regions, of target genes was observed. These data suggest that CTR9 participates in the transcription of IL-6-responsive genes through the regulation of DNA association of STAT3 and modification of histone methylation.

Innate immune responses grant the host early protection against infectious agents, resulting in immediate activation of specialized immune cells and production of inflammatory cytokines (1). Upon encountering bacterial lipopolysaccharide (LPS),^4^ immune cells become activated and produce cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 to mediate inflammatory responses in target organs, such as the liver or vascular endothelium (2). In response to inflammatory signals, the innate immune system provokes an extensive transcriptional deviation from homeostasis, referred to as the acute phase response (APR), which includes global adjustments in metabolism and coordinated regulation of genes for innate defense (3).

The yeast PAF complex was originally identified as an RNA polymerase II-associated complex, minimally composed of Paf1p, Cdc73p, Ctr9p, Rtf1p, and Leo1p (4–6). The PAF complex is associated with a number of transcription-related processes, including transcription initiation and elongation, RNA processing, and modification of histone tails. Components of the yeast PAF complex physically interact with the transcription elongation factors Spt4/Spt5 and FACT, and are associated with the promoter and coding regions of genes being actively transcribed (7, 8). In addition, Paf1p also controls the 3’-end formation of non-polyadenylated RNA transcripts, indicating that the PAF complex has regulatory functions in pre-mRNA processing (9–11).

Interactions between the PAF complex and histone modifying enzymes have been strongly established in yeast by genetic assays. The yeast PAF complex is required to recruit the Set-1 containing COMPASS complex and the Dot1 histone methytransferase to RNA polymerase II, which results in histone methylation of H3 at lysine residues 4 and 79 (12). Furthermore, the yeast PAF complex is required for Rad6-Bre1 catalytic activity in mono-ubiquitination of histone H2B at lysine 123, which provides a signal for histone methylation by COMPASS and Dot1 in the promoter region (12–15). In humans, CDC73 (also known as parafibromin) physically associates with a histone methytransferase complex that methylates histone H3 on lysine 4, while inhibition of CTR9 reduces cellular levels of histone H3-K4 mono- or dimethylation, and H3-K77 dimethylation (16, 17).

Beyond its role in general transcriptional regulation, the PAF complex has also been suggested to have target gene specificity.

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[^2]: The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

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[^4]: The abbreviations used are: LPS, lipopolysaccharide; Stat, signal transducer and activator of transcription; TNF, tumor necrosis factor; IL, interleukin; ChiP, chromatin immunoprecipitation assay; DAPI, 4’,6-diamidino-2-phenylindole; APR, acute phase response; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; EST, expressed sequence tag.
The yeast PAF complex controls expression of a subset of genes involved in metabolism and cell cycle regulation (18–21). The Arabidopsis orthologs of the PAF complex, Elf7 and Elf8, specifically regulate gene expression of flowering repressors through the enhancement of histone H3 trimethylation at the lysine 4 residue in flowering locus C (FLC) chromatin (22). In Drosophila, Paf1 and Rtf1 are specifically recruited to active heat shock loci, along with active RNA polymerase II and the elongation factors Spt6 and FACT. Deletion of Paf1 abolishes transcription of the Hsp70 gene and methylation of histone H3 at lysine 4, indicating that Paf1 specifically controls expression of heat shock-inducible genes through the regulation of histone modifications and transcription elongation in the fruit fly (23). Recently, byrax, a Drosophila homolog of yeast Cdc73p, was reported to specifically activate Wnt/Wg target gene transcription mediated by a direct interaction with β-catenin/armadillo (24).

In the work presented here, we show that Sh2bp1/Ctr9, a member of mammalian PAF complex, specifically participates in the transcription of IL-6-inducible inflammatory genes through the regulation of STAT3 association to its target DNA.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—A full-length cDNA of mouse Ctr9 was obtained in the laboratory of S. D., as described (25), and subsequently subcloned into the pSG5-FLAG mammalian expression vector. Amino acids 679–1173 and 1–679 were deleted to generate the truncated mCtr9 constructs, FLAG-mCtr9deltaC and FLAG-mCtr9deltaN, respectively. Full-length cDNAs of mouse Cdc73 and Paf1 were obtained by RT-PCR from livers of LPS-treated mice and cloned into the pENTR/TOPO or pcDNA3.1/myc mammalian expression plasmids. V5-tagged mCtr9, mCdc73, and mPaf1 were generated using the Gateway recombination system (Invitrogen). HA- or FLAG-tagged mCtr9 and mCdc73 were generated by subcloning into the pSG5 mammalian expression vector. Sequences of all cDNAs were verified by automated sequencing. The human haptoglobin promoter (−1553 to +27) was cloned into the upstream region of the luciferase gene derived from pGL3-basic (Promega, Madison, WI).

**Cell Culture and Transfection**—HepG2 cells were maintained in MEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). For cytokine stimulation, cells were treated with rhIL-6 (10 ng/ml) plus IL-6sR (10 ng/ml), IFN-β1a (100 units/ml), IL-1β (10 ng/ml), or TNFα (10 ng/ml) (all from R&D Systems, Minneapolis, MN). Transient transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. An oligonucleotide siRNA targeting CTR9 (5’-AUGCAUCAUCAGUGCUUUG) and a control oligonucleotide siRNA (5’-GAAUUUGGCACGAAACGC) were synthesized by Qiagen-Xeragon.

**LPS Treatment**—C57BL/6j mice (12 to 15 weeks of age) were injected intraperitoneally with 100 µg of LPS (Escherichia coli strain 0111:B4, Difco) in 100 µl of saline, as described (3). At various times after injection, mice were sacrificed by cervical dislocation. Livers were stored in liquid nitrogen until used for RNA extraction.

**RNA Isolation and Analysis**—Total RNA was extracted from livers of LPS-treated mice or HepG2 cells using the TRIzol Reagent (Invitrogen). Northern blot analysis was performed on 20 µg of total RNA using random prime-labeled probes. For conventional or real time PCR, 1 µg of total RNA was reverse-transcribed using an oligo(dT)15 primer and M-MLV reverse transcriptase (Promega). The sequences of the primers for PCR are provided in supplemental Table S1.

**Chromatin Immunoprecipitation (ChIP)**—ChIP assays were performed as described (26). Briefly, HepG2 cells were treated with formaldehyde (1%) for 30 min. Cells were collected in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 µg/ml aprotinin, 2 µg/ml pepstatin, 5 µg/ml leupeptin, and 1 mM benzamidine), and the chromatin was sonicated three times for 40 s each at setting 5.0 using a Branson 250 Sonicator with a microtip. ChIP was performed by incubating extracts with the appropriate antibodies overnight at 4 °C. After reversal of the cross-links and DNA purification, enriched DNA fragments were analyzed by PCR amplification of the promoter and coding regions of target genes. The sequences of the oligomers for PCR are provided in supplemental Table S2. Antibodies against trimethylated histone H3-K4 and dimethylated histone H3-K9 were purchased from Upstate Biotechnology, Inc., and an antibody to STAT3 was purchased from Santa Cruz Biotechnology.

**Reporter Assay**—HepG2, HEK-293, or COS-7 cells in 24-well plates were transfected with m67- or HP-luciferase reporter plasmids (250 ng) together with expression plasmids (250 ng) or siRNA (125 ng). Transfection efficiency was normalized by cotransfection with pRL-TK (2.5 ng). At 36–h post-transfection, cells were treated with IL-6 plus IL-6sR (10 ng/ml each), or OSM (10 ng/ml), for 12 h, and a dual luciferase assay was performed according to the manufacturer’s instructions (Promega).

**Immunoblotting**—HepG2 cells were lysed in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 5 µg/ml aprotinin, 2 µg/ml pepstatin, 5 µg/ml leupeptin, and 1 mM benzamidine). Total cell lysates (40 µg per sample) were resolved using SDS-polyacrylamide gels. Antibodies against the FLAG epitope were purchased from Sigma (clone M2), and antibodies against JAK1 and JAK2 were purchased from Upstate Biotechnology. Antibodies to STAT3 and pTyr-STAT3 were purchased from Santa Cruz Biotechnology. Antibodies against CTR9 have been previously described (25). Antibodies against PAF1, CDC73, and LEO1 were purchased from Bethyl Laboratories ( Montgomery, TX). Immunoreactive signals were detected by enhanced chemiluminescence (Pierce).

**Immunocytochemistry**—HepG2 cells on poly-d-lysine-coated glass coverslips were fixed with ice-cold 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Cells were blocked with 5% horse serum and 2.5% bovine serum albumin for 60 min at room temperature and incubated with rabbit anti-Ctr9 and mouse anti-phospho-STAT3 antibodies overnight at 4 °C. Cells were then incubated with a fluorescein isothiocya-
nate-conjugated anti-rabbit antibody and a rhodamine-conjugated anti-mouse antibody (Jackson Immunoresearch, West Grove, PA). Slides were mounted with mounting medium containing DAPI (Vectashield) and visualized with a Zeiss inverted epifluorescence microscope (Axioplan 2, Zeiss, Oberkochen, Germany).

RESULTS

Transcriptional Induction of the Murine PAF Complex in the Mouse Liver by LPS—To understand the molecular mechanisms of global gene regulation during APR, we specifically searched for putative transcriptional regulators induced by inflammatory signals. In our previously reported microarray data performed in C57BL/6J+129/SvJ mice (3), we found that the EST clone Tspb (GenBank™ accession number AV300608) was significantly induced in the liver by LPS (Fig. 1A). Tspb is the EST of the previously identified mouse gene Sh2bp1, a nuclear phosphoprotein with multiple tetratricopeptide repeat (TPR) domains, which mediate protein-protein interactions (25). Most recently, SH2BP1 was shown to be a human ortholog of yeast Ctr9, a component of the yeast PAF complex (16, 17, 27). Therefore, we examined the expression of the murine PAF complex as a function of time after LPS administration. C57BL/6J mice were intraperitoneally injected with a sublethal dose of LPS (100 μg), and mRNA levels were analyzed by conventional or real-time PCR at various times after injection (Fig. 1B). The transcript levels of murine Ctr9 (mCtr9) were elevated from 3–6 h after LPS stimulation and subsequently declined. The peaks of Ctr9 mRNA accumulation differ between panels A and B, probably due to the genetic differences between C57BL/6 and 129/SvJ strains. We also measured protein levels of CTR9 in LPS-stimulated C57BL/6 mice. In agreement with mRNA expression data, increased levels of Ctr9 protein were detected at 12–24 h after LPS treatment in C57BL/6 mice (Fig. 1C). We next measured the transcript levels of other components of the PAF complex by RT-PCR (Fig. 1B). Murine Cdc73 (mCdc73) and Paf1 (mPaf1) mRNAs also increased in the liver in response to LPS treatment, but their expression patterns differed from that of mCtr9.

SH2BP1/CTR9 Regulates Transcript Levels of IL-6-inducible APP Genes—To investigate the transcriptional regulatory functions of mCtr9 during APR initiated by LPS, we first examined the transcript levels of APPs induced by inflammatory signals using a siRNA specific for CTR9. Transfection with CTR9 siRNA decreased protein levels of endogenous CTR9, and the loss of CTR9 resulted in a reduction of protein levels of other PAF components in HEK293 and HepG2 cells (Fig. 2A).
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Reduced CTR9 expression has been previously reported to alter protein levels of PAF1 and LEO1 in HeLa cells (17). Cells were next stimulated with increasing amounts of LPS, or with the pro-inflammatory cytokine IL-6 plus its soluble receptor (IL-6sR), and the dose- and time-dependent responses of APP gene mRNA levels were analyzed (Fig. 2B). To obtain the highest induction of APP genes in HepG2 cells, we treated cells with IL-6 and IL-6sR as previously reported (28). Because IL-6 plus IL-6sR treatment, but not LPS alone, strongly induced expression of the APP genes fibrinogen (FGGγ) and haptoglobin (HP) in HepG2 cells, we used IL-6 plus IL-6sR as an inflammatory signal for subsequent experiments. We measured transcript levels of the IL-6-inducible APP genes in CTR9-depleted cells (Fig. 2, C and D). Both basal and inducible levels of FGGγ, HP, and SAA were significantly decreased by inhibition of CTR9 expression. Interestingly, transcript levels of the housekeeping genes GAPDH and β-actin remained unchanged, suggesting that CTR9 involves in the regulation of selective subset of gene expressions.

To understand the selectivity of target genes, we examined whether CTR9 controls transcript levels of genes induced by other inflammatory signals. Cells were first stimulated with interferon β and expression of the interferon β-inducible genes, ISG-15 and RIG-I, were determined by RT-PCR in CTR9-depleted cells (Fig. 2E). Interferon β induced expression of ISG-15 and RIG-I to a similar extent in cells transfected with CTR9-specific or control siRNA. In addition, expression patterns of the IL-1β or TNFα-inducible genes ICAM-1 and iNOS were not altered in CTR9-depleted cells (Fig. 2F). To further investigate the signal specific gene regulation by CTR9, we then examined transcript levels of SAA and ICAM-1 genes, whose expressions are induced by both IL-6 and IL-1β. Even though IL-6-inducible SAA expression was down-regulated by inhibition of CTR9 (Fig. 2C), IL-1β-induced expression of SAA was not altered (Fig. 2G). Similarly, transcript levels of ICAM-1 were reduced by CTR9 inhibition, when it was induced by IL-6 (Fig. 2H). These data indicate that CTR9 does not control the transcription of all inflammatory response genes, but specifically participates in transcription of IL-6-responsive genes.

Histone H3 Trimethylation at Lysine 4 in Chromatin Regions Containing IL-6-responsive APP Genes Depends on SH2BP1/CTR9—Because the PAF complex associates with histone-modifying enzymes in various systems, including human (12, 16, 22, 23), we then addressed whether CTR9 is required for methylation of lysine 4 on histone H3 in chromatin regions containing IL-6-responsive APP genes. We performed ChIP on extracts of IL-6-treated HepG2 cells cultured in the presence of control or CTR9-specific siRNAs using an antibody against trimethylated H3-K4 (Fig. 3, A and B). Trimethylation of H3-K4 was increased by IL-6 treatment in chromatin regions containing the HP and FGGγ genes, whereas the GAPDH gene was constitutively trimethylated on H3-K4. Upon inhibition of CTR9 expression, H3-K4 trimethylation was dramatically diminished in the HP and FGGγ genes, while there was little effect on the GAPDH gene. We next examined the methylation of lysine 9 on histone H3 in the chromatin regions of IL-6-responsive APP genes. The coding regions of the HP and FGGγ genes were found to be strongly associated with dimethylated histone H3 on lysine 9 residues (Fig. 3C). However inhibition of CTR9 expression did not substantially alter the dimethylation levels of H3-K9 in any of the tested genes, indicating that CTR9 might control transcription primarily through the regulation of histone H3 methylation on lysine 4 in the chromatin regions of IL-6-responsive APP genes.

Association of SH2BP1/CTR9 in the Chromatin Regions of the IL-6-responsive APP Genes—Drosophila Paf1 is specifically recruited to the chromatin regions of Hsp70 genes upon heat shock activation to mediate histone methylation and gene induction (23). Therefore, we examined occupancy of CTR9 in the chromatin regions of the IL-6-inducible APP genes and GAPDH. We performed ChIP on the promoter and coding regions of the FGG, HP, and GAPDH genes using an anti-CTR9 antibody. Although CTR9 was found to associate with the cod-
ing regions of all genes tested, including GAPDH whose expression was not affected by knockdown of CTR9, its association to the promoter region was observed only in HP and FGGγ genes (Fig. 4, B and E). We also examined occupancy of other PAF members, CDC73 and LEO1, in the promoter and coding regions of HP, FGGγ, and GAPDH. Similar to CTR9, CDC73 and LEO1 were found to associate with the coding regions of all genes tested, but their association was not observed in the promoter region of GAPDH locus (Fig. 4, C–E). These results indicate that the occupancy of CTR9 on the promoter regions of APP gene may explain the specificity of transcriptional control by CTR9.

**SH2BP1/CTR9 Regulates Transcriptional Activity of STAT3 to Control IL-6-responsive Gene Expression**—IL-6 activates the JAK tyrosine kinase and the transcription factor STAT3 to transduce inflammatory signals into the nucleus. Phosphorylated forms of STAT3 translocate into the nucleus and bind to specific DNA elements in the promoter regions of target genes (29–31). To understand how CTR9 regulates transcript levels of IL-6-inducible genes, we monitored STAT3-mediated transcriptional activity in response to IL-6 stimulation in cells with different levels of CTR9 expression. The transcriptional activity of STAT3 was measured with a dual luciferase assay using a reporter under the regulation of a synthetic STAT3-responsive promoter (m67-luc) or the human haptoglobin promoter/enhancer region (HP-luc). Inhibition of CTR9 expression caused an ∼33% decrease in STAT3 activity in cells stimulated with IL-6 (Fig. 5A).

To account for the possibility of nonspecific effects of the siRNA, FLAG-mCtr9 was overexpressed and transcriptional activity of STAT3 was examined using the m67-luc and HP-luc reporters. Increased expression of mCtr9 caused a ∼35% increase in luciferase activity, indicating that CTR9 positively controls the transcriptional activity of STAT3 (Fig. 5B). We also used a siRNA that is specific to human CTR9, but not to mouse Ctr9, to confirm that mCtr9 expression rescued the reduced transcriptional activity by CTR9 siRNA (Fig. 5C). Because the regulatory effect of CTR9 on STAT3 activity was modest, we hypothesized that CTR9 affects the kinetics of STAT3 transcriptional activity rather than its magnitude. To test this hypothesis, we measured transcriptional activity of STAT3 at various times after IL-6 stimulation in FLAG-mCtr9-transfected cells (Fig. 5D). STAT3 transcriptional activity began to increase after IL-6 stimulation and reached a steady state at 2-h poststimulation. In contrast, in CTR9-transfected cells, STAT3 reporter activity reached steady state at ∼3 h after IL-6 stimulation, and the steady state levels were higher than in control cells. These results indicate that increased CTR9 protein levels maintain stronger or longer STAT3-mediated transcriptional activity.

Next, we examined the transcriptional regulatory effect of CTR9 in HEK-293 or COS-7 cells. Cells were transiently transfected with the m67-luc reporter and stimulated with IL-6 or Oncostatin M (OSM), a cytokine which also uses gp130 as a receptor and activates JAK/STAT3 signaling pathways (Fig. 5E). Both IL-6- and OSM-stimulated transcriptional activity of STAT3 was further increased by overexpression of FLAG-mCtr9 in all cell lines tested. These data strongly suggest that CTR9 participates in the STAT3-mediated transcriptional activation of IL-6 or OSM inducible genes. To understand the relationship between CTR9 and the JAK/STAT3 signaling pathway, we examined the effect of CTR9 in cells transfected with the JAK tyrosine kinases JAK1 and JAK2 (Fig. 5F). Overexpressed JAK1 and JAK2 increased both basal and IL-6 inducible levels of STAT3 transcriptional activity, which was significantly reduced by cotransfection with the CTR9-specific siRNA. This result suggests that CTR9 functions downstream of JAK kinase activation.

**SH2BP1/CTR9 contains multiple TPR domains in the N-terminal region, which mediate protein-protein interactions, and serine-rich phosphodomains in the C-terminal region (25).** To identify the region of CTR9 responsible for transcriptional control, we constructed N-terminal (FLAG-mCtr9deltaN) and C-terminal (FLAG-mCtr9deltaC) deletion fragments of mCtr9 (Fig. 5G) and performed luciferase assays using the m67-luc
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FIGURE 5. CTR9 interacts with the JAK/STAT3 signaling pathway to control IL-6-responsive gene expression. A, HepG2 cells were transfected with the m67-luc reporter together with siRNAs and stimulated with IL-6 + IL-6sR (10 ng/ml each). B, cells were transfected with the m67-luc (left) reporter or with the HP-luc (right) reporter containing the haptoglobin promoter together with the FLAG-mCtr9 expression vector. Cells were stimulated with IL-6 + IL-6sR (10 ng/ml each). C, cells were transfected with m67-luc, CTR9 siRNAs specific to the human CTR9, and increasing amounts of the Myc-mCtr9 expression vector. Cells were stimulated with IL-6 + IL-6sR prior to the reporter assay. D, cells were transfected with the m67-luc and the FLAG-mCtr9 expression vector and stimulated with IL-6 + IL-6sR (10 ng/ml each) for the indicated times. E, HEK-293, COS-7, or HepG2 cells were co-transfected with m67-luc and the FLAG-mCtr9 expression vector. Transfected cells were stimulated with IL-6 + IL-6sR (10 ng/ml each), or OSM (10 ng/ml), and were subjected to the luciferase assay. F, HepG2 cells were transfected with m67-luc and the indicated expression vectors and siRNAs, stimulated with IL-6 + IL-6sR (10 ng/ml each), and analyzed using the luciferase assay. Student’s t test, *p < 0.001; **, p < 0.005. G, top, diagram of the full-length and truncated forms of mCtr9. HEK293 cells were transfected with m67-luc and the indicated Ctr9 expression vectors. Cells were stimulated with IL-6 + IL-6sR (10 ng/ml each). Left, luciferase assay, fold induction ratios after IL-6 treatment are marked on top of each bar graph. The results shown are representative of three independent experiments. Right, protein expression of FLAG-tagged mCtr9, mCtr9deltaN, and mCtr9deltaC. Arrows indicate the position of expressed proteins.

reporter in response to IL-6 stimulation. Overexpression of FLAG-Ctr9 further enhanced the transcriptional activity of STAT3, which was not observed in cells transfected with the multiple TPR domain containing N-terminal region of mCtr9 (mCtr9deltaC). In contrast, when cells were transfected with mCtr9deltaN, transcriptional activity of STAT3 was increased to a level comparable to full-length mCtr9. However, the fold-change after IL-6 treatment appears to be the same in the empty or mCtr9deltaN transfected cells, indicating that the serine-rich C-terminal region of mCtr9 may responsible for general transcriptional regulation, but not for IL-6 signal-specific gene regulation. It is noteworthy to mention that the transcriptional regulatory effect of C-terminal region of Ctr9 might be underestimated, due to the poor levels of mCtr9deltaN overexpression. The poor expression was derived from the decreased protein stability of mCtr9deltaN, as its protein level was increased by treatment with 26 S proteasome inhibitor, MG132 (data not shown).

SH2BP1/CTR9 Controls DNA Binding of STAT3 to the Promoter Region of Target Genes—We examined whether CTR9 specifically interacts with nuclear proteins in an IL-6-dependent manner to govern target gene specificity. We first examined the cellular localization of CTR9 and of the phosphorylated forms of STAT3 by immunofluorescent staining in IL-6-stimulated HepG2 cells. The CTR9 protein was observed primarily in the nucleus of HepG2 cells, and its nuclear localization pattern was not significantly changed by treatment with IL-6 (Fig. 6A). However, upon IL-6 stimulation, phosphorylated forms of STAT3 translocated into the nucleus and were predominantly colocalized with CTR9. To examine whether CTR9 interacts with STAT3, we performed coimmunoprecipitation assays using cell lysates obtained from cells transfected with FLAG-mCtr9 or FLAG-mCtr9deltaC mutant. Phosphorylated forms of Stat3 were found to associate with full-length of CTR9 in the presence of IL-6 stimulation, but not with C-terminal domain-deleted form of CTR9, indicating that CTR9 might participate in the regulation of IL-6-inducible gene expression through the interaction with activated form of Stat3 (Fig. 6B). To understand how CTR9 controls the transcriptional activity of STAT3, we then analyzed the kinetics of tyrosine-phosphorylated STAT3 in HepG2 cells stimulated with IL-6 (Fig. 6C). In control and CTR9 siRNA-transfected cells, tyrosine...
phosphorylation of STAT3 was detected by 15 and 30 min after IL-6 stimulation, respectively, with residual activity after 3 h. In addition, the total protein level of STAT3, as well as the JAK1 and JAK2 protein levels, remained unchanged in both CTR9- and control-siRNA-transfected cells, suggesting that the diminished transcriptional activity of STAT3 was not due to the decrease in STAT3 phosphorylation, nor the absolute levels of signaling proteins.

Finally, cells were stimulated with IL-6 and STAT3 binding to promoter regions of target genes was determined by ChIP in cells transfected with control or CTR9-specific siRNA. STAT3 association with the promoter regions of FGGγ was dramatically reduced when CTR9 expression was inhibited (Fig. 6D). To better understand the interaction between CTR9 and STAT3, we compared the dynamics of STAT3 DNA binding in cells transfected with control or CTR9-specific siRNA (Fig. 6E). In cells transfected with control siRNA, STAT3 occupied the promoter region of FGGγ for at least 30 min after IL-6 stimulation. In contrast, when the level of CTR9 expression was reduced, STAT3 dissociated from the promoter region after 15 min of IL-6 stimulation. Taken together, these results indicated that CTR9 participates in the IL-6-inducible gene expression through the regulation of DNA association of STAT3 or accessibility of STAT3 to its target DNA sequences.

DISCUSSION

In the present work, we demonstrated that CTR9 specifically participates in the regulation of the transcript levels of IL-6-responsive genes by interacting with the JAK/STAT3 pathway. Our data suggest that, upon IL-6 stimulation, activated STAT3 binds to its binding sites in the promoter regions of the target genes (HP, FGGγ) and interacts with a CTR9-containing complex. This complex then recruits the histone methyltransferase complex and methylates lysine 4 residues on histone H3 to induce target gene expression. Alternatively, CTR9 may provide specificity at the relevant loci by affecting the accessibility of STAT3 to the DNA in a locus-specific fashion. Although there is relatively low nucleosome occupancy at functional transcription factor binding sites on a genome-wide scale, STAT3 binding sites in the promoter regions of FGGγ and HP (32, 33) are predicted to be occupied by nucleosomes, determined by using the probabilistic nucleosome DNA interaction model (34). Therefore, it is also likely that the chromatin accessibility of STAT3 to the target promoter locus might be affected by the presence of CTR9. Without CTR9, STAT3 binding to its target loci is less stable, which results in failure to recruit the histone methyltransferase complex and impaired gene induction.

CTR9 or members of PAF1 complex has not been reported to have sequence-specific DNA binding ability, yet we observed CTR9 in the promoter regions of selective group of genes. It indicates CTR9 might be recruited to its target genes through direct or indirect physical interactions with sequence-specific transcription factors, as reported in Wnt/Wg target gene expression mediated by Parafibromin (Cdc73) and β-catenin/Armadillo interaction (24). Otherwise, specific chromatin structures or modifications that are differentially distributed throughout the genome might be responsible for controlling the recruitment of CTR9 containing complex. Examine the genome-wide occupancy of CTR9 will be helpful to uncover the full scope of CTR9-mediated transcriptional regulation.

We have shown that stable association of the STAT3 to the promoter region of target gene was strongly affected by the presence of CTR9. In addition, we observed interaction between STAT3 and CTR9 after IL-6 stimulation. Although we do not know yet whether their interaction is direct or requires other nuclear factors, it clearly indicates that CTR9 containing complex participates in the STAT3-mediated transcriptional activation. Further study investigating the nature of CTR9 containing complex in the promoter regions of target gene and their interactions with other transcription factors or histone are necessary to understand the regulatory potential of CTR9 in the transcription.

The effect of protein knockdown of CTR9 on IL-6 target gene expression was appeared to be efficient (Fig. 2), but its effect on STAT3-mediated transcription was somewhat modest (Fig. 4). It suggests that CTR9 might not be the sole regulator of STAT3-mediated gene expression, rather other nuclear or cellular factors are likely involved. It also suggests that other IL-6-mediated signaling pathways that are affected by CTR9 might exist. Because it has been previously reported that IL-6 can transmit its signal through Jak-STAT3 and/or SHP-2 pathways
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(35), it will be interesting to investigate the effect of CTR9 in the IL-6-induced MAPK-mediated signaling pathways.

The yeast Paf1 complex contributes to the post-transcriptional control of transcripts through the regulation of poly(A) site utilization and 3′-end formation (9–11). However, mRNA stability of Fggy and Hp were not significantly altered in the cells with decreased CTR9 expression.5 Furthermore, depletion of CTR9 did not alter the poly(A) tail length significantly, while mutation of yeast Paf1 components results in shortened poly(A) tail length (9). These results suggest that the transcriptional regulation of IL-6-inducible genes by CTR9 might be separable from the control of mRNA stability by the Paf1 complex.6

This is the first report demonstrating that the level of PAF complex can be affected by external stimulus, such as LPS induced inflammation. Intraperitoneal injection of LPS induces systemic inflammation, which involves production of pro- and anti-inflammatory cytokines and activation of immune cells. To identify specific inflammatory cytokine signal that is responsible for the transcriptional regulation of CTR9, we treated cells with various inflammatory cytokines, including IL-6, IL-1β, TNF-α, OSM, IFN-α/β, and IFNγ, for various times up to 24 h. However, none of these individual cytokines significantly changed the transcript or protein levels of CTR9, suggesting that combined signals of various inflammatory cytokines or other mediators of inflammation that are activated by LPS might be necessary to up-regulate transcript of CTR9. Chronic activation of IL-6-mediated signaling and elevated production of APPs are major problems in chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis. Therefore, further studies will be needed to evaluate the therapeutic potential of CTR9.

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