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# Characterization of the PC4 Binding Domain and its Interactions with HNF4α

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**In the presence of oxidative stress, the hepatocellular inflammatory-redox (IR) state upregulates inducible nitric oxide synthase (iNOS) expression as an anti-oxidant function. In IL-1β and peroxide treated hepatocytes, we have identified hepatocyte nuclear factor-4α (HNF4) and the transcriptional co-activator, PC4, to be essential for upregulation of iNOS transcription in this setting. The co-activator, PC4, facilitates activator-dependent transcription via interactions with basal transcriptional machinery that are independent of PC4-DNA binding. The interaction between HNF4 and PC4 has not been previously characterized. In this study utilizing human HepG2 cells, we demonstrate the critical role for p38 MAP kinase mediated HNF4 Ser158 phosphorylation (P-HNF4-S158), binding of PC4 to P-HNF4-S158 and characterize the functional domain of PC4 required for P-HNF4-S158 binding. Our results indicate that the presence of the IR state enhances PC4-HNF4 binding to upregulate transcription of target hepatocyte genes, such as iNOS.**

**Key words: co-activator, inflammation, nitric oxide, nuclear receptor.**

Abbreviations: ChIP, chromatin immunoprecipitation; HNF4, hepatocyte nuclear factor 4-alpha; IL-1, interleukin 1β; iNOS, inducible nitric oxide synthase; IR, inflammatory-redox; MM, mismatch; NO, nitric oxide; PCR, polymerase chain reaction; WT, wild type.

In the presence of oxidative stress, the hepatocellular inflammatory-redox (IR) state upregulates inducible nitric oxide synthase (iNOS) expression as an anti-oxidant function. In IL-1β treated hepatocytes, we have demonstrated that pro-inflammatory cytokine mediated iNOS gene transcription and promoter activity are increased by oxidant stressors, such as peroxide, superoxide or acetaminophen (*1–3*). Subsequently, we identified hepatocyte nuclear factor-4α (HNF4) and the transcriptional co-activator, PC4, to be essential for upregulation of iNOS transcription in these settings (*1*, *2*). HNF4 is a member of the nuclear receptor superfamily of transcription factors and was originally identified in the regulation of liver-specific genes (*4*). Subsequently, >55 distinct target genes involved with lipid, amino acid and glucose metabolism, liver differentiation, cell structure and immune function have been identified for HNF4. HNF4 is highly conserved; amino acid identity between rat and human varies from 89.7% to 100% among the various functional domains of HNF4α. It also binds co-activators in the absence of exogenously added ligand (*4–7*). In the present context, the co-activator, PC4, facilitates activator-dependent transcription via interactions with basal transcriptional machinery that are independent of PC4-DNA binding.

The nature of the interaction between HNF4 and PC4 has not been previously characterized. In this study utilizing HepG2 cells, we demonstrate the critical role for p38 mediated HNF4 Ser158 phosphorylation (P-HNF4-S158), binding of PC4 to P-HNF4-S158 and characterize the functional domain of PC4 required for P-HNF4-S158 binding. Our results indicate that the presence of the IR state enhances PC4-HNF4 binding to upregulate transcription of target hepatocyte genes, such as iNOS.

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## MATERIALS AND METHODS

*Materials*—Phospho-antibody to Ser158 phosphorylated HNF4 was obtained from Sigma.

*Cell Culture*—HepG2 cells (ATCC HB 8065) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% fetal bovine serum (FBS). The medium was changed every 3–4 days, and the cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. In selected instances, the cells were treated with IL-1β (1000 units/ml), $H_2O_2$ (50 μM) or IL-1β + $H_2O_2$ for 8 h. The cells were then washed twice with ice-cold PBS and harvested for further assays.

*Immunoblot Analysis*—Cells were lysed in buffer (0.8% NaCl, 0.02% KCl, 1% SDS, 10% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% $Na_2HPO_4$ and 0.024% $KH_2PO_4$, pH 7.4) and centrifuged at 12,000 × *g* for 10 min at 4°C. Protein concentration was determined by absorbance at 650 nm using protein assay reagent (Bio-Rad, Hercules, CA, USA). Proteins (30 μg) were loaded and run on 4–20% SDS/Polyacrylamide gels, then transferred on to PVDF membranes. Membrane was blocked in 5% (w/v) dried

 

milk in PBST (PBS/0.025% Tween 20). Anti-His antibody (Invitrogen), anti-HNF4 antibody, anti-p38 Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-P-HNF4-S158 polyclonal antibody were diluted 1:800 in 5% dried milk in PBST and incubated with the membrane for 60 min at room temperature (24°C). Three 10 min washes with PBST followed antibody incubation. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:2000 in 5% dried milk in PBST. Reactivity was detected using chemiluminescent reagents (Pierce, Rockford, IL, USA).

*P38 siRNA*—P38 siRNA (si-p38; Santa Cruz Biotechnology, Santa Cruz, CA) was used for transient transfection of HepG2 cells. The cells were transfected with control siRNA (MM-siRNA) or si-p38 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72 h from the initial transfection, cell lysates were collected and analysed.

*Co-immunoprecipitation Assay*—HepG2 cells were transfected with WT-PC4, deletion PC4 or mutant PC4 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), incubated at 37°C for 48 h and then treated with IL-1β (1000 units/ml), $H_2O_2$ (50 μM) or IL-1β + $H_2O_2$ for 8 h. The cells were rinsed twice with PBS and a lysis buffer containing protease and phosphatase inhibitors was added [50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.8 μM aprotinin, 50 μM bestatin, 20 μM leupeptin, 10 μM pepstatin A, 25 μM *p*-bromotetramisole oxalate, 5 μM cantharidin and 5 nM microcystin-LR (microcystin-leucine arginine)]. After a 30 min incubation, the cells were scraped, collected and homogenized. The cell lysates were pre-cleared with normal IgG and protein G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA); immunoprecipitation was performed with primary antibody and protein G plus-agarose for overnight incubation at 4°C. The pellets were collected by centrifugation and washed four times with PBS buffer, followed by SDS/PAGE electrophoresis and transferred on PVDF membranes. The membranes were blocked with 5% (w/v) non-fat dried milk and probed overnight at 4°C with specific primary antibody. After multiple washes with PBST, the membranes were exposed to secondary antibody which was coupled with horseradish peroxidase in 5% non-fat dried milk in PBST for 1 h at room temperature. After extensive washing, the complexes were visualized using the West Pico chemiluminescent kit (Pierce, Rockford, IL, USA).

*Plasmid Constructs and Mutagenesis*—Messenger RNA was isolated from human HepG2 cells using the Qiagen mRNA purification kit. cDNA was synthesized using the purified mRNA and oligo(dT) as a primer using Thermoscript RT–PCR system (Invitrogen, Carlsbad, CA). The product was used to amplify full-length PC4-cDNA. The amplified PC4 cDNA PCR product was inserted into pGEM-T vector (Promega, Madison, WI, USA); positive clones were screened by DNA Miniprep kit (Qiagen, Chatsworth, CA, USA) and fully analysed by automated DNA sequencing. The vector was then cut by the restriction enzyme EcoR I and run in 1% agarose gel. The PC4 cDNA fragment was recovered from the gel and inserted into the EcoRI restriction enzyme site of pcDNA3.1-His expression vector (Invitrogen); the resultant vector, WT-PC4, allows production of a protein with a six His peptide fused at its N-terminus. PC4 deletion constructs, PC4Δ113-127 (CT112 PC4), PC4Δ 96-127 (CT95 PC4), PC4Δ81-127 (CT80 PC4), PC4Δ 53-127 (CT52 PC4) and PC4Δ1-51 (N52 PC4) were polymerase chain reaction (PCR)-amplified using the WT-PC4 vector as templates. Primers have been extended with an EcoR I restriction site at the 5′ ends of the forward primer and reverse primer. PCR products were digested with EcoR I and cloned in the EcoR I site of pcDNA-His vector. PC4 mutants, K34G R85G, R99G, K100G and I102G in which the designated amino acids were mutated to a glycine were constructed. All mutant vectors were generated by using QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and primers as described subsequently.

PC4-K34G-F: 5′-ttgctccagaggtacctgtaaagaaacaaaaga-3
PC4-K34G-R: 5′-ctttacaggtacctctggagcaacttgctttttc-3
PC4-R85G-F: 5′-aattgatattggcgaatattggatggatcctga-3′
PC4-R85G-R: 5′-tccaatattcgccaatatcaattagcactttgc-3′
PC4-R99G-F: 5′-gaaaccaggaggcaaaggtatttctttaaatcc-3
PC4-R99G-R: 5′-aaataccttgcctcctggtttcatttcacctt-3
PC4-K100G-F 5′accaggaagaggcggtatttctttaaatccaga-3
PC4-K100G-R 5′aaagaaataccgcctcttcctggtttcatttcacc-3
PC4-I102G-F 5′ aagaaaaggtggctctttaaatccagaacaatg-3′
PC4-I102G-R 5′ gatttaaagagccacctttttcttcctggtttcatttc

*Chromatin Immunoprecipitation Assay*—HepG2 cells were grown in 10 cm dishes, and DNA–protein cross-linked by addition of formaldehyde at room temperature for 10 min. Assays were performed using the ChIP assay kit (Upstate Biotechnology, Waltham, MA, USA) following the protocol recommended by the manufacturer. Briefly, HepG2 cells were grown in 10 cm dishes, and DNA–protein cross-linked by addition of formaldehyde at room temperature for 10 min. Cell lysates were sonicated to give a DNA size range from 200 to 900 bp. A total of 5 μg goat PC4 polyclonal antibody (Santa Cruz Biotechnology) or P-HNF4-S158 Ab was used for each immunoprecipitation. The DNA was recovered and subjected to analysis by PCR. The primers had the following sequence: 5′-tgaccaattgactggtatgtgtg-3′ (sense strand) and 5′-gctgggctggggagatggctga-3′ (antisense strand) to yield a PCR product of 280 bp. The PCR programme was 94°C for 4 min, followed by 94°C for 45 s, 55°C for 45 s and 72°C for 45 s for a total of 28 cycles, and then 72°C for 7 min. The amplified DNA was visualized by electrophoresis on 1% agarose gel in 1 × TAE (Tris/Acetate/EDTA) buffer after staining with ethidium bromide. Template DNA applied for immunoprecipitation with each antibody was used for PCR as positive input controls.

*Quantitative Real-time PCR*—Each DNA reaction mixture (5-μl aliquot) was used in 50 μl real-time PCR with iQ Supermix PCR 2 × master mix (Bio-Rad, Hercules, CA) supplemented with 0.5 μmol primer. Reactions were subjected to the following amplification conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Amplification, detection and data analysis were performed with an iCycler IQ Real-Time Detection system (Bio-Rad, Hercules, CA). Analyses were done in triplicates. For quantification,

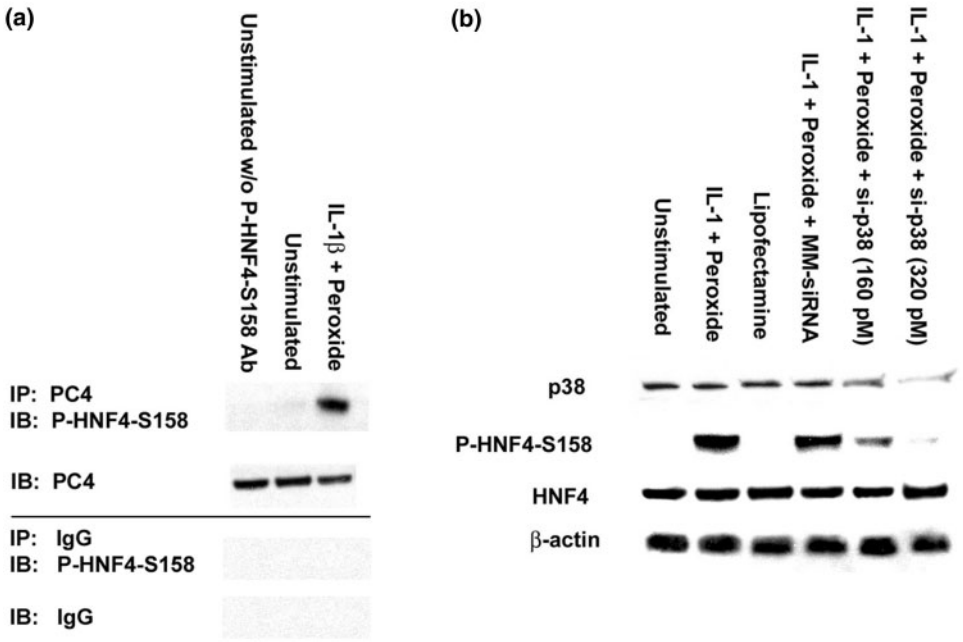


Fig. 1. **P-HNF4-S158 and p38 in HepG2 cells.** (a) Co-IP of PC4 and P-HNF4-S158. HepG2 cells were treated with IL-1β (1000 units/ml), $H_2O_2$ (50 μM) or IL-1β + $H_2O_2$ for 8 h. The cell lysates were pre-cleared, and immunoprecipitation was performed with primary antibody and protein G plus-agarose. Following SDS/PAGE electrophoresis and transfer to PVDF, the membranes were blocked and exposed to secondary antibody. Blot is representative of three experiments. (b) P-HNF4-S158 and p38. HepG2 cells were transfected with control siRNA (MM-siRNA) or p38 siRNA (si-p38) using Lipofectamine. After 72 h from the initial transfection, cells were treated with IL-1β + $H_2O_2$ for 8 h. Cell lysate protein (30 μg) was run on 4–20% SDS/polyacrylamide gels and transferred on to PVDF membranes. Anti-HNF4 antibody or anti-P-HNF4-S158 polyclonal antibody were incubated with the membrane followed by peroxidase-conjugated secondary antibody. Reactivity was detected using chemiluminescent reagents. Blot is representative of three experiments.

a specific standard curve with iNOS promoter DNA serially diluted over 6 logs was used in parallel to the analyses. Negative controls were included in each amplification experiment.

*Statistical Analysis*—Data are expressed as means ± SE. Analysis was performed using Student's *t*-test. *P*-values < 0.05 were considered significant.

## RESULTS

*PC4, p38 and IR-Mediated Formation of P-HNF4-S158*—In HepG2 cells, we have previously demonstrated that an IR-sensitive serine/threonine kinase pathway targets the transcription factor, HNF4, at S158 in the presence of IL-1β + peroxide to enhance binding with the co-activator PC4 and subsequently increase iNOS transcription (*8*). In our model of IR induction in HepG2 cells, co-IP studies demonstrate that PC4 is associated with P-HNF4-S158 in the presence of IL-1β and peroxide only (Fig. 1a). In the absence of stimulation, no interaction is noted. Similarly, no interaction is found in the presence of either IL-1β alone or peroxide alone. (Data not shown.) This data confirm previous observations demonstrating HNF4-PC4 interaction in the presence of IR and indicates specific binding of PC4 to the phosphorylated form of HNF4, P-HNF4-S158. To determine the potential role of the stress activated protein kinase, p38, in the formation of P-HNF4-S158, we inhibited expression of p38 using RNAi. We then performed immunoblot studies for p38, HNF4 and P-HNF4-S158 (Fig. 1b). Total p38 was not altered in the presence or absence of IL-1β and peroxide; in addition, p38 protein was not changed by the presence of MM-siRNA with IL-1β and peroxide. Increasing concentrations of si-p38 (160 pM and 320 pM) in this context significantly decreased p38 levels. In parallel with these findings, P-HNF4-S158 was detected in the presence of IL-1β and peroxide; however, when p38 expression was inhibited, P-HNF4-S158 expression was significantly decreased. Total HNF4 was not altered suggesting that p38 is required for IR mediated P-HNF4-S158 formation.

*P-HNF4-S158 and PC4 Bind to the iNOS Promoter in States of IR*—ChIP assays were then performed to determine *in vivo* binding of PC4 and/or P-HNF4-S158 to the iNOS promoter in our HepG2 model (Fig. 2a). P-HNF4-S158 binds to the IR-sensitive DR1 [direct repeat of RG(G/T)TCA with one base spacing] *cis*-acting activator element (nt –1327 to –1315) in the iNOS (inducible nitric oxide synthase) promoter: AGGTCAGGGGACA (*1*, *8*). Results from the ChIP assay indicate binding of both PC4 and P-HNF4-S158 to this segment of iNOS promoter DNA exclusively in the presence of IL-1β and peroxide. Real-time PCR-ChIP studies suggest equivalent binding of PC4 and P-HNF4-S158 to the iNOS promoter in a 1:1 molar ratio (Fig. 2b).

*C-terminal Pc4 Binds to P-Hnf4-S158*—A series of mutant PC4 expression vectors were then constructed in which successively greater portions of PC4 were

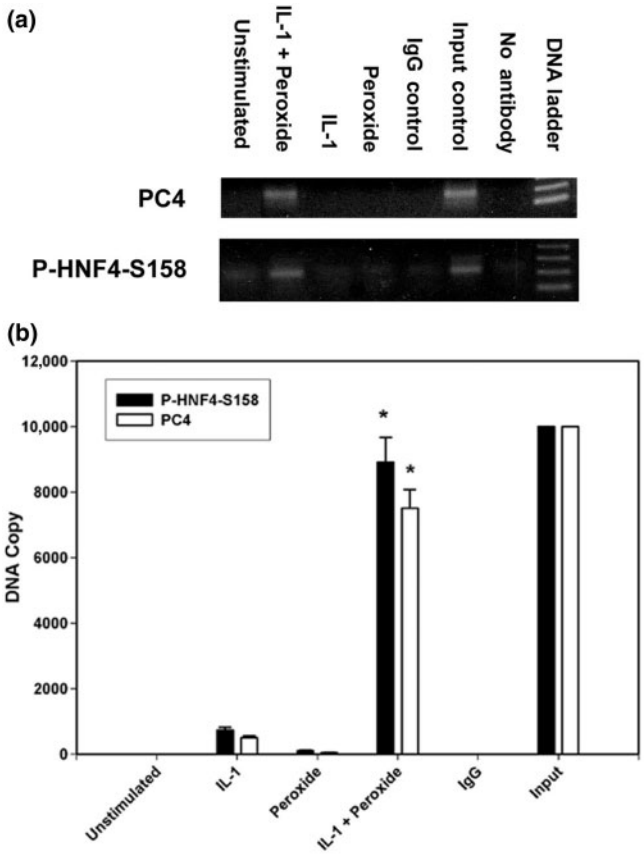


Fig. 2. **PC4 and P-HNF4-S158 binding to the iNOS promoter.** (a) Chromatin immunoprecipitation assay for PC4 and P-HNF4-S158 binding. HepG2 cells were grown in 10 cm dishes, and DNA-protein cross-linked by addition of formaldehyde. Cell lysates were sonicated to give a DNA size range from 200 to 900 bp. A total of 5 μg primary antibody was used for each immunoprecipitation. The DNA was recovered and subjected to analysis by PCR to yield a product of 280 bp. The amplified DNA was visualized by electrophoresis and stained with ethidium bromide. Template DNA applied for immunoprecipitation with each antibody was used for PCR as positive input controls. Gel is representative of four experiments. (b) Quantitative real-time PCR analysis of ChIP assays. Reactions were subjected to the following amplification conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Amplification, detection and data analysis were performed with an iCycler IQ Real-Time Detection system (Bio-Rad). Analyses were done in triplicates. For quantification, a specific standard curve with iNOS promoter DNA serially diluted over 6 logs was used in parallel to the analyses. Negative controls were included in each amplification experiment. Data are expressed as mean ± SEM of three experiments. (*$P < 0.01$ versus IL-1, Peroxide or Unstimulated cells)

truncated from the C-terminal or N-terminal ends (Fig. 3a). A series of co-IP assays were then performed (Fig. 3b). As anticipated, no PC4 interaction with P-HNF-S158 was noted in unstimulated HepG2 cells. When stimulated by IL-1β and peroxide, native PC4 and P-HNF-S158 interactions were found. In the setting of transient transfection with various PC4 mutants, PC4 interaction with P-HNF4-S158 was noted with wild type (full-length) PC4 and CT112 PC4, in which the C-terminal amino acids 113–127 had been deleted. More extended deletions of the PC4 C-terminus did not interact with P-HNF4-S158. When the first 51 amino acids were from the N-terminal of PC4, an interaction was again found with P-HNF-S158. These data suggest that AA 95-112 were critical for PC4 interaction with P-HNF4-S158 in the IR setting. As these residues correspond to the β4 (AA-100 to AA-104) region of PC4, we chose a series of single amino acid mutations to better define the area of interaction (*9*).

To localize the critical interaction site, a series of PC4 expression vectors were then constructed in which a single amino acid were mutated to glycine. These were then tested in the IR model to determine interaction with P-HNF-S158. Co-IP studies were then performed (Fig. 3c). Substitution for R99, K100 or I102 significantly diminished or abolished PC4 interaction with P-HNF4-S158 in the presence of IL-1 and peroxide. Compared with wild type PC4, substitution for K34 did not alter extent of interaction. As expected, no interactions were found in the absence of stimulation. These results indicate a critical region of PC4 at R99-K100-I102 is required for interaction with P-HNF4-S158 in the setting of IR stimulated HepG2 cells.

## DISCUSSION

Metabolic hepatic injury following sepsis or shock remains a poorly characterized clinical problem. The high mortality rates in septic patients with impaired hepatic amino acid uptake and protein synthesis underscore the significance of hepatic dysfunction in this setting (*10–12*). Multiple aetiologies have been proposed, including reactive oxygen species, alteration in microcirculatory blood flow, neutrophil activation and Kupffer cell-induced hepatocyte dysfunction, but the relative roles of these factors remain unknown. Similarly, cytoprotective mechanisms which act to inhibit or reverse sepsis- or shock-induced hepatic injury are also poorly described. In this context, nitric oxide (NO) has received a great deal of attention as a ubiquitous, multifunctional free radical which is produced during shock and sepsis and which may function to eradicate infection and limit tissue injury (*13–15*). The regulatory pathways of hepatic NO production in the milieu of pro-inflammatory cytokines and reactive oxygen species (IR) which characterize sepsis and shock remain poorly understood. In the presence of oxidative stress, the hepatocellular redox state upregulates iNOS expression as an antioxidant function.

We have previously demonstrated that hepatocyte expression of iNOS and synthesis of NO conveys protective antioxidant functions in models of sepsis, shock and reperfusion injury (*2, 16–18*). This hepatocellular redox regulatory system functions independently of both the oxidant species and the specific proinflammatory cytokine. In interleukin (IL)-1β–treated rat hepatocytes, we have demonstrated that iNOS gene transcription and promoter activity are increased by oxidant stress mediated by peroxide, superoxide or acetaminophen. Subsequently, in IL-1β–stimulated rat hepatocytes exposed to superoxide, we identified a redox-sensitive DR1 *cis*-acting activator element

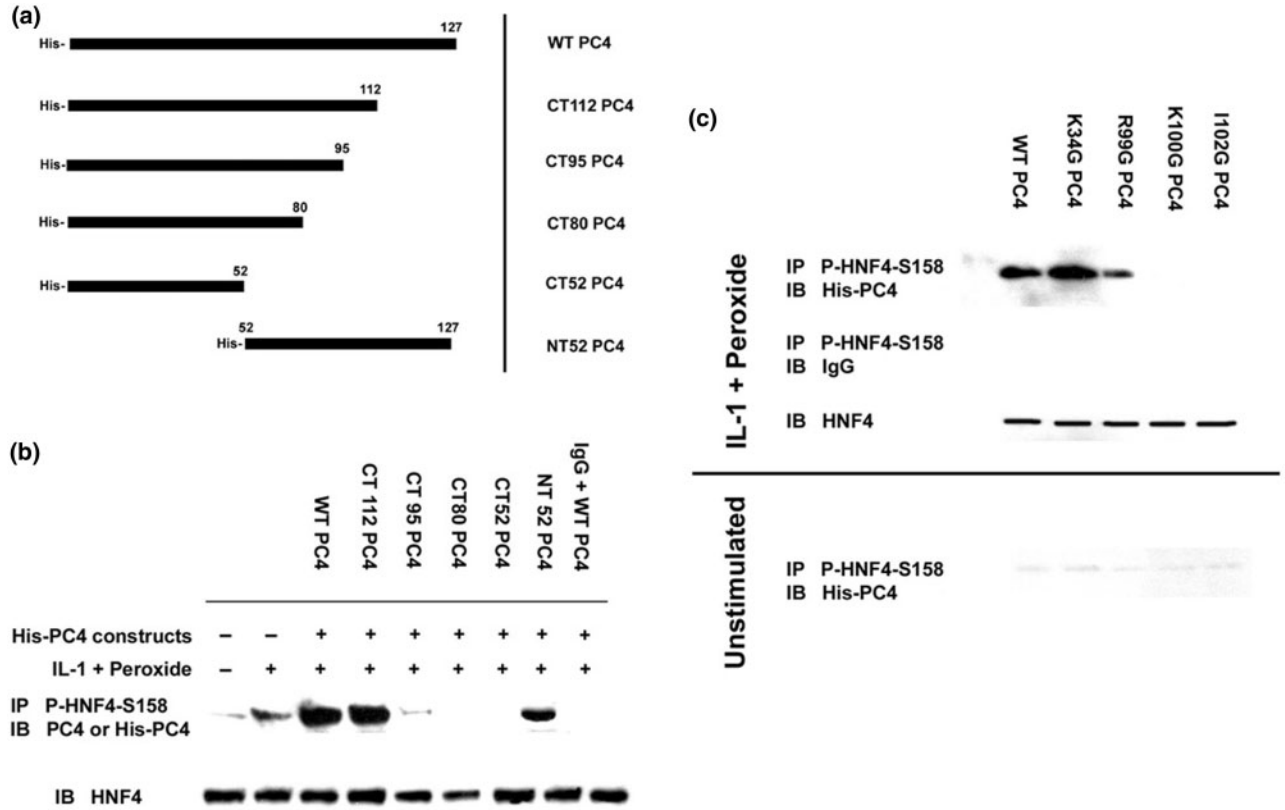


Fig. 3. **Characterization of PC4 interaction with P-HNF4-S158.** (a) Schematic representation of deletion constructs of PC4. (b) Co-IP of PC4 deletion constructs with P-HNF4-S158. HepG2 cells were transfected with His-tagged PC4 deletion constructs using Lipofectamine. After 72 h from the initial transfection, cells were then treated with IL-1β + $H_2O_2$ for 8 h. Cell lysate protein (30 μg) was run on 4–20% SDS/polyacrylamide gels and transferred to PVDF membranes. Anti-HNF4α antibody or anti-P-HNF4-S158 polyclonal antibody were incubated with the membrane followed by peroxidase-conjugated secondary antibody. Reactivity was detected using chemiluminescent reagents. Blot zis representative of three experiments. (c) Co-IP of PC4 single amino acid mutation constructs with P-HNF4-S158.HepG2 cells were transfected with His-tagged PC4 deletion constructs using Lipofectamine. After 72 h from the initial transfection, cells were then treated with IL-1β + $H_2O_2$ for 8 h. Cell lysate protein (30 μg) was run on 4–20% SDS/polyacrylamide gels and transferred to PVDF membranes. Anti-HNF4α antibody or anti-P-HNF4-S158 polyclonal antibody were incubated with the membrane followed by peroxidase-conjugated secondary antibody. Reactivity was detected using chemiluminescent reagents. Blot is representative of three experiments.

(nt −1327 to nt −1315) in the iNOS promoter AGGTCAGGGGACA. The corresponding transcription factor was isolated by DNA affinity chromatography, sequenced, and identified to be hepatocyte nuclear factor-4α (HNF4α). In this setting, transcriptional co-activator PC4 is often isolated with HNF4α in biotinylated DNA-protein pull-down studies. PC4 facilitates activator-dependent transcription via interactions with basal transcriptional machinery that are independent of PC4-DNA binding.

In this study utilizing HepG2 cells exposed to IR stress, we demonstrate that: (i) p38 dependent phosphorylation of HNF4 at S158 results in binding of PC4-P-HNF4-S158 to the iNOS promoter, and (ii) critical residues in the β4 region of PC4 are required for interaction with P-HNF-S158. The nature of the interaction between HNF4 and PC4 has never been examined in this context. PC4 is a 15-kDa polypeptide that serves as a potent co-activator in standard reconstituted *in vitro* transcription systems. It mediates activator-dependent transcription by RNA polymerase II through interactions with the transcriptional activator and basal transcription machinery. It is subject to *in vivo* phosphorylation events that negatively regulate its co-activator functions. The vast majority (95%) of PC4 is phosphorylated and inactive *in vivo*. PC4 is proposed to promote the assembly of the pre-initiation complex (PIC) in activated transcription. However, given that transcription is a multistep process consisting of PIC assembly, promoter opening, initiation, promoter escape, elongation and reinitiation, steps other than PIC assembly are potential targets for regulation as well. Indeed, despite predominant effects of activators—presumably in conjunction with co-activators—on PIC assembly, the effects on the subsequent steps have also been demonstrated in various systems, including promoter opening, promoter escape, elongation and reinitiation (*19–21*). The functional domains of PC4 have not been extensively characterized. However, Jonker and coworkers (*9*) have previously demonstrated an essential role for PC4 C-terminal

domain in binding to the activation domain of VP16. Specifically, the K100G mutation which resides in the β4 domain of PC4 ablated PC4 interaction with VP16 in pull-down assays. In similar studies, Zhong and coworkers (*22*) found that the C-terminal domain was an essential part of PC4 interactions with AP2α. Our findings reinforce the notion that PC4 C-terminal domain is essential for its interaction with a variety of transcription factors, including VP16, AP2α and in our case, HNF4. Within the larger context hepatocyte injury and IR mediated upregulation of iNOS transcription, our studies further define the contribution of HNF4α and PC4 to IR regulation of iNOS transcription.

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