12-2014

Regulation of C-reactive Protein Gene Expression and Function

Avinash N. Thirumalai
East Tennessee State University

Regulation of C-reactive Protein Gene Expression and Function

---

A dissertation presented to

the faculty of the Department of Biomedical Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

---

by

Avinash Thirumalai

December 2014

Dr. Alok Agrawal, Ph.D., Chair

Dr. Antonio E. Rusiñol, Ph.D.

Dr. David A. Johnson, Ph.D.

Dr. Douglas Thewke, Ph.D.

Dr. Gregory A. Ordway, Ph.D.

Dr. Richard M. Kostrzewa, Ph.D.

Keywords: C-reactive protein, OCT-1, Gene Expression, Atherosclerosis, Low-Density Lipoprotein, Site-directed mutagenesis, Reactive Oxygen Species

ABSTRACT

Regulation of C-reactive Protein Gene Expression and Function

by

Avinash Thirumalai

Human C-reactive protein (CRP) is the prototypic acute phase protein whose serum concentration increases rapidly during inflammation. CRP is also associated with atherosclerosis; it is deposited at lesion sites where it may interact with modified lipoproteins. There are 2 major questions regarding CRP: 1. How is the serum concentration of CRP regulated? 2. What are the functions of CRP in atherosclerosis?

Our first aim was to determine the role of the constitutively expressed transcription factor Oct-1 in regulating CRP gene expression. We found that Oct-1 overexpression inhibited (IL-6+IL-1β)-induced CRP gene expression; maximal inhibition required the binding of Oct-1 to an octamer motif at (-59 to -66) on the CRP promoter. Oct-1 overexpression inhibited both (IL-6+IL-1β)-induced and C/EBPβ-induced CRP gene expression even when the Oct-1 site was deleted. These findings suggest that Oct-1 is a repressor of CRP gene expression that acts via binding to its cognate site on the CRP promoter as well as through indirect interactions with other promoter-bound transcription factors.

Our second aim was to investigate the interaction of CRP with oxidized low density lipoprotein (ox-LDL). Acidic pH, a hallmark of atherosclerotic lesions, reversibly alters CRP structure and exposes a hidden binding site that enables CRP to bind ox-LDL. Using site-directed mutagenesis

we constructed a CRP mutant (E42Q) that showed significant binding to ox-LDL at physiological pH. E42Q CRP required a less acidic pH for maximal binding and bound ox-LDL more efficiently than wild type CRP at any pH. We then examined if reactive oxygen species also induced CRP – ox-LDL interaction. H_2O_2-treated CRP bound ox-LDL at physiological pH. Like acidic pH, H_2O_2-treatment induced only a local structural change exposing the ox-LDL binding site. E42Q and H_2O_2-modified CRP are tools to study the function of CRP in animal models of atherosclerosis, which may not have an inflammatory environment sufficient to modify CRP and induce binding to atherogenic ox-LDL.

We conclude that Oct-1 is one of the critical regulators of CRP gene expression, and that CRP can be modified *in vitro* to convert it into an atherogenic LDL-binding molecule.

DEDICATION

This manuscript is dedicated to my mother Mrs. Parimala Vembar. As a single parent, she made many sacrifices to ensure that my brother and I could pursue our education and dreams. What made it more difficult for her has been the fact that I've struggled with depression from the time I was in high school. Despite these issues she allowed me the freedom to choose my own path in life and has been a constant source of support. Her encouragement, faith in me, and patience have never wavered even when I lost confidence in my abilities to work and attain my dreams. I will forever be indebted to her – without her love and support, I would not have been so successful.

ACKNOWLEDGEMENTS

The work presented in this manuscript and also all the progress I've made during my time in graduate school would not have been possible without the influence and help of many wonderful people.

Foremost among them has been Dr. Alok Agrawal, who took a huge gamble on accepting me as his student as I had not been working in research for many years. Dr. Agrawal is primarily responsible for molding my career in science. He taught me not just what questions to ask and how to answer them while doing research but also guided me in the many 'non-science' aspects of doing research. The enthusiasm, dedication, and rigor with which he does science has, and will always, inspire and encourage me. His words of encouragement even when things were not working for me in the lab were always a great source of support. He has been extremely kind, patient, and understanding with me throughout my time here despite my many failings and struggles. Words can do scant justice to all his efforts, and I only hope that I will live up to his expectations of me.

I also thank the members of my thesis advisory committee: Dr. Antonio Rusiñol, Dr. David Johnson, Dr. Richard Kostrzewa, Dr. Gregory Ordway, and Dr. Douglas Thewke for all the advice and encouragement they provided. I appreciate the guidance and support of Dr. Mitchell Robinson and Ms. Beverly Sherwood at the Biomedical Sciences Graduate Program. I am also grateful to all the faculty of the Biomedical Sciences Graduate program who have added to my education.

Dr. Sanjay Singh, David Hammond, and Toh Gang were wonderful people to work with in the lab. They not only taught me a lot about doing science but also provided a

relaxed and friendly environment where I could learn and work to my best capabilities. I owe them an immense debt for making my time in the lab a great experience.

For their help with all administrative issues, my thanks to all the staff at the Biomedical Sciences department, the School of Graduate Studies, and the ETSU International Students' Programs and Services.

I have been fortunate to meet and befriend some wonderful people during my time in school. For most of my time here, I shared an apartment with Justin Beach and I cannot thank him enough for being a great flat-mate and friend. Christopher Daniels, Zhouxi Wu, Christopher McGoldrick, Jessica Crawford, Benjamin Hilton, Suman Dalal, Brigitte Browe, and Natalie Burke have been very good friends to me, and I will treasure the fun times we've had.

Last, but not the least, I would not be here if not for the love, encouragement, and support from my family and friends. I will be eternally gratefully to them.

ABBREVIATIONS

Aβ	Amyloid-beta
ANS	1-anilinonaphthalene-8-sulfonic acid
Ac-LDL	Acetylated low density lipoprotein
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CHO	Chinese hamster ovary
ChIP	Chromatin immune-precipitation
CRP	C-reactive protein
E-LDL	Enzymatically-modified low density lipoprotein
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EU	Endotoxin units
FH	Factor H
h	Hour
HNF	Hepatocyte nuclear factor
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
IL-1	Interleukin-1
IL-6	Interleukin-6
LDL	Low density lipoprotein

Luc	Luciferase
mAb	Monoclonal antibody
Mut	Mutant
NF-κB	Nuclear factor kappaB
OCT	Octamer
OD	Optical density
Oligo	Oligonucleotide
O/N	Overnight
Ova	Ovalbumin
Ova-α-Ova	Ovalbumin – goat anti-ovalbumin immune complex
Ox-LDL	Oxidized low density lipoprotein
PCh	Phosphocholine
PnC	Pneumococcal C-polysaccharide
RLU	Relative luciferase units
SAP	Serum amyloid P component
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline
TGF	Transforming growth factor
TNF	Tumor necrosis factor
WT	Wild-type

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

C-reactive protein (CRP) is an evolutionarily conserved protein that participates in the response to systemic inflammation. CRP was discovered in Oswald Avery's laboratory during their studies of patients infected with *Streptococcus pneumoniae* and is named as such as it precipitated the C-polysaccharide of the pneumococcal cell wall (Abernathy and Avery 1941; Macleod and Avery 1941a; Macleod and Avery 1941b). CRP is a member of the evolutionarily conserved pentraxin family of proteins, which consists of the short pentraxins CRP and Serum Amyloid P component (SAP) and the long pentraxins PTX3, PTX4, and the neuronal pentraxins (reviewed in Agrawal *et al.* 2009).

Structure of CRP

The CRP molecule is a pentamer made up of 5 identical, noncovalently associated ~23 kD subunits arranged symmetrically around a central pore (Fig. 1.1). Each subunit is made up of 206 amino acids folded into 2 antiparallel β sheets with a flattened jelly-roll topology and binds to 2 Ca^{2+} ions. The binding site for phosphocholine (PCh), the classical ligand of CRP, on each subunit is oriented on the same side in the pentamer, thus forming a 'recognition face'. The PCh-binding site is made by the 2 bound Ca^{2+} ions and an adjacent hydrophobic pocket formed by the amino acids Phe^{66}, Thr^{76}, and Glu^{81}. The phosphate group of PCh coordinates directly with the 2 Ca^{2+} atoms, while the choline group lies within the hydrophobic pocket. The 3 methyl groups of choline interact with Phe^{66}, while Glu^{81} interacts with the positively charged nitrogen atom of choline. Thr^{76} is critical for forming an appropriately sized pocket

for PCh (Roux *et al*. 1983; Shrive *et al.* 1996; Thompson *et al*. 1999). This binding site has also been shown to be involved in the binding of CRP to other nonclassical ligands such as phosphoethanolamine, cholesterol, and histones at physiological pH (Szalai *et al*. 1999; Black *et al*. 2003).

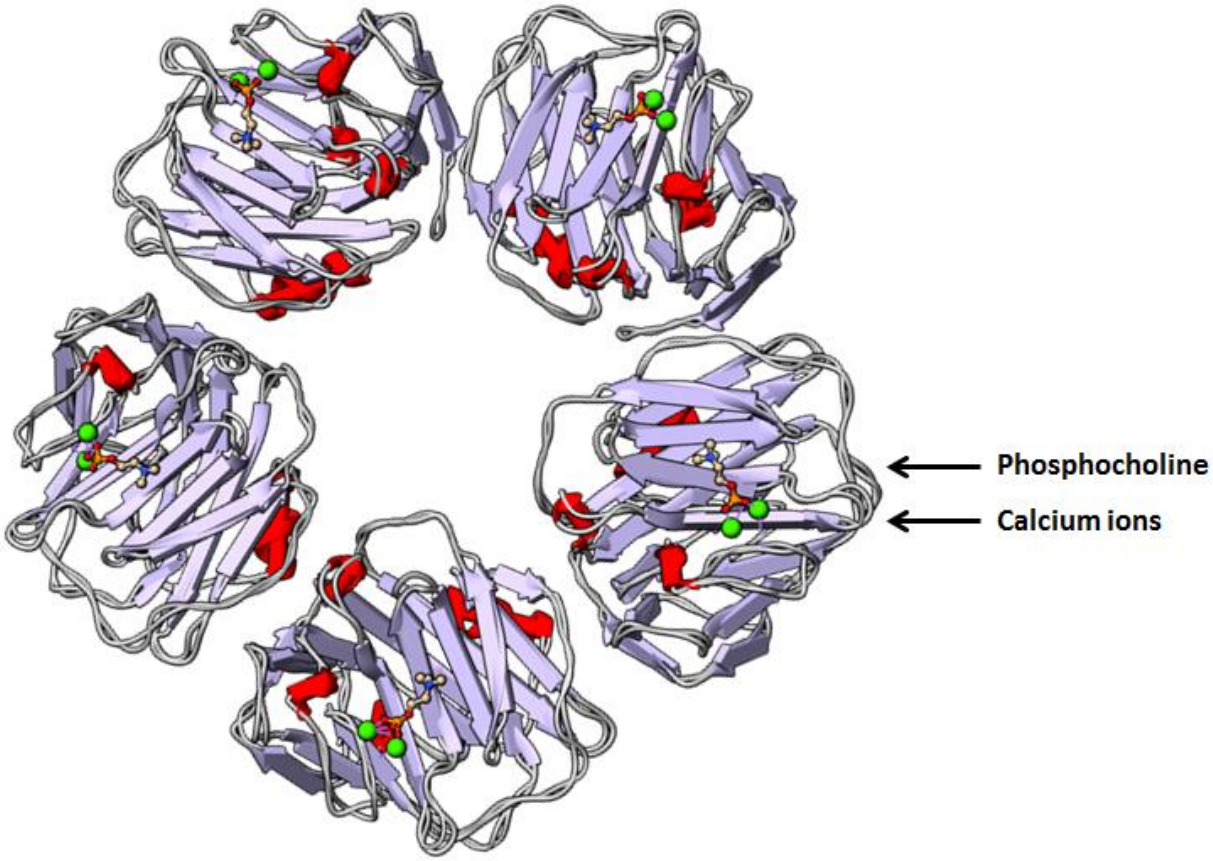

Figure 1.1. Crystal structure of PCh-complexed CRP. The phosphocholine moiety is shown using ball-and-stick representation, while the Ca2+ ions are shown as green spheres (adapted from Thompson *et al*. 1999).

On the opposite 'effector face' of CRP there is a cleft extending from the center of each subunit to the central pore. This cleft is necessary for the interaction of CRP with complement protein C1q and the Fcγ receptors (Agrawal and Volanakis 1994; Agrawal *et al*. 2001; Bang *et al*. 2005). Structural studies have shown that the largely positively charged globular head of

C1q interacts with the largely negatively charged central pore of the CRP pentamer, and that this interaction is critically dependent on several amino acid residues present within the cleft, including the amino acids Asp^{112} and Tyr^{175} (Agrawal and Volanakis 1994; Agrawal *et al*. 2001). Optimal binding of C1q with ligand-bound CRP requires a slight conformational change in the CRP pentamer, and this change seems to depend on the ligand to which CRP is bound (Gaboriaud *et al*. 2003).

Functions of CRP

In humans, CRP has been characterized as a key acute phase protein and mediates the inflammatory response. CRP binds to substances with exposed PCh-groups such as pneumococcal C-polysaccharide on bacterial cell walls, low-density lipoprotein, and apoptotic or damaged cells in a Ca^{2+}-dependent manner (Agrawal *et al*. 1997; Bhakdi *et al*. 1999; Volanakis 2001; Chang *et al*. 2002). The ligand-bound CRP then binds to C1q, the initiating factor of the classical complement pathway (Agrawal and Volanakis 1994; Volanakis 2001). Activation of the classical complement pathway mediates the clearance of pathogens and cellular debris, either by formation of the membrane-attack complex or by opsono-phagocytosis by macrophages. Ligand-bound CRP has also shown to interact directly with Fcγ receptors on macrophages and induce phagocytosis (Bang *et al*. 2005).

Studies using mouse models of pneumococcal infection have shown that CRP protects mice against lethal infection with *S. pneumoniae* by decreasing bacteremia and increasing survival (Mold *et al.* 1981; Yother *et al.* 1982; Szalai *et al.*1995; Simons *et al.* 2014). Passively administered human CRP is protective in mice only when injected 6 h before to 2 h after infection but not when administered 24 h postinfection. The PCh-binding pocket is

necessary for the CRP-mediated initial protection of mice against lethal pneumococcal infection and involves complement activation (Suresh *et al*. 2006; Agrawal *et al*. 2008; Gang *et al*. 2012). Some studies have also shown that CRP can protect mice against lethal pneumococcal infection when the PCh-binding site is mutated, suggesting the involvement of an as yet unknown PCh-binding independent mechanism (Suresh *et al*. 2007). The reasons as to why CRP is not protective when administered during later stages of infection are not known.

Significance of Serum CRP Levels

In healthy individuals the median concentration of CRP in the serum is 0.8μg/ml. During the acute phase response the serum levels of CRP increases to 500 μg/ml or more (Pepys and Hirschfield 2003). The serum levels of CRP have also been known to increase many hundred- to thousand-fold during other acute inflammatory states and also in some noninflammatory conditions such as following stress or cellular injury (Fig. 1.2). Serum CRP levels are therefore used diagnostically as a measure of systemic inflammation. Following the resolution of inflammation, there is an equally rapid reduction of plasma CRP to basal levels (Kushner 1982; Gabay and Kushner 1999).

Minor elevations of serum CRP levels are also found in chronic inflammatory conditions, for example, during atherosclerosis. These have been associated with an increased risk of developing atherosclerosis and subsequent cardiovascular disease (Libby and Ridker 2004). However, it is not clear whether the elevated CRP levels are an independent risk factor and predict the disease or if they are involved in its pathogenesis (Kushner and Elyan 2008; Agrawal *et al*. 2010).

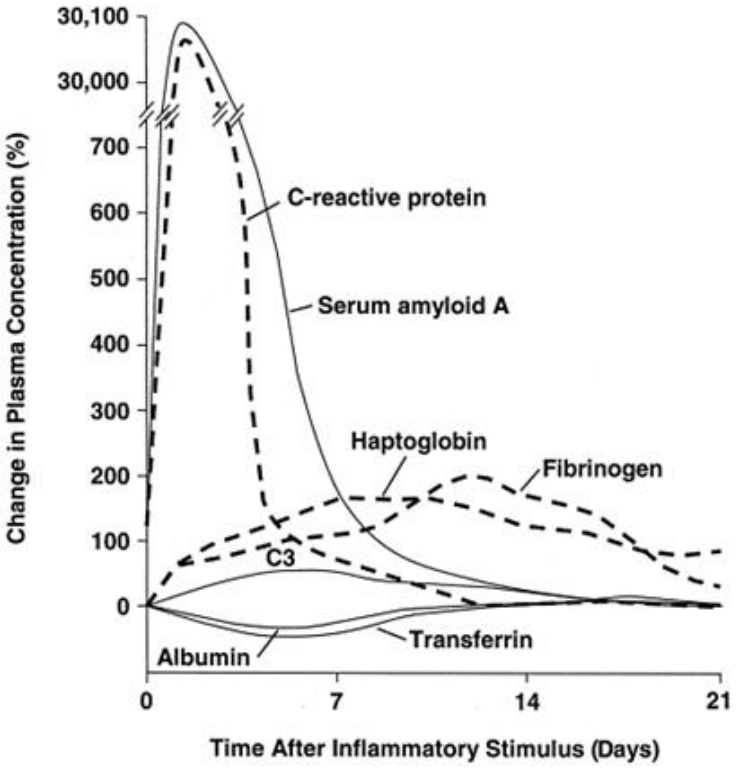

Figure 1.2. Chances in plasma concentrations of proteins during the acute phase response (adapted from Gabay and Kushner 1999).

Regulation of CRP Gene Expression

CRP is primarily produced in the liver, although extra-hepatic synthesis of CRP is known to occur in certain conditions. Hepatic CRP production is the primary determinant of serum CRP levels (Kushner and Kaplan 1961; Hurlimann *et al*. 1966; Kushner and Feldman 1978). Due to the difficulties in obtaining primary human hepatocytes, various human hepatoma cell lines have been used to study transcriptional regulation of the CRP gene. Studies from multiple labs have shown that cytokines like IL-1β, IL-6, IL-17, TGFβ, and TNFα regulate the transcription of the CRP gene in these cell lines (Darlington *et al*. 1986; Ganapathi *et al*. 1988, Poli and Cortese 1989; Castell *et al*. 1990; Yap *et al*. 1991; Zhang *et al*. 1995; Wang *et al*. 1999; Zhang *et al*. 2006; Patel *et al*. 2007). The most commonly used model to study CRP gene expression has been the human hepatoma Hep-3B cell line, and in this cell

line CRP gene expression is primarily regulated by the IL-6 and IL-1β cytokines (Ganter *et al*. 1989).

In human hepatoma Hep-3B cells, IL-6 activates the transcription factors C/EBPβ and STAT3 and induces CRP gene transcription (Poli and Cortese 1989; Ramji *et al*. 1993; Zhang *et al*. 1996; Wang *et al*. 1999, Ochrietor *et al*. 2000; Agrawal *et al*. 2001). IL-1β alone does not induce CRP gene transcription, but synergistically enhances IL-6 induced CRP gene transcription by activating the transcription factor NF-κB (Darlington *et al*. 1986; Ganapathi *et al*. 1988; Agrawal *et al*. 2003; Blashcke *et al*. 2006; Kramer *et al*. 2008). In addition to these cytokine-induced transcription factors, 5 constitutively expressed transcription factors (C/EBPζ, RBP-Jκ, HNF-1, HNF-3, and OCT-1) are known to be involved in CRP gene regulation (Toniatti *et al*. 1990; Li and Goldman 1996; Voleti and Agrawal 2005; Blashcke *et al*. 2006; Nishikawa *et al* 2008; Reiner *et al*. 2008; Grimm *et al*. 2011).

The proximal 157 bp of the CRP promoter (Fig. 1.3) has been shown to be sufficient for this synergistic interaction between IL-6 and IL-1β cytokines (Zhang *et al*. 1995; Li and Goldman 1996; Agrawal *et al.* 2001). The IL-6 induced transcription factor C/EBPβ binds the CRP promoter at 2 sites centered at -52 and -219 (Li and Goldman 1996), while STAT3 binds the promoter at -108 (Zhang *et al*. 1996). The NF-κB p50-p50 homodimer binds to a nonconsensus site at -47, which overlaps the proximal C/EBPβ binding site, and the NF-κB p50-p65 heterodimer binds to a site located at -69 (Agrawal *et al*. 2001; Voleti and Agrawal 2005; Cha-Molstad *et al*. 2007). The constitutively expressed transcription factors C/EBPζ and RBP-Jκ bind to the proximal C/EBPβ and NF-κB p50-p50 sites respectively (Singh *et al*. 2007), while the HNF-1 and HNF-3 binding sites are at -67 and -62 (Li and Goldman 1996). The Oct-1 binding site is centered at -63, and overlaps the HNF-1, HNF-3, and NF-κB p50-

p65 binding sites (Voleti and Agrawal 2005).

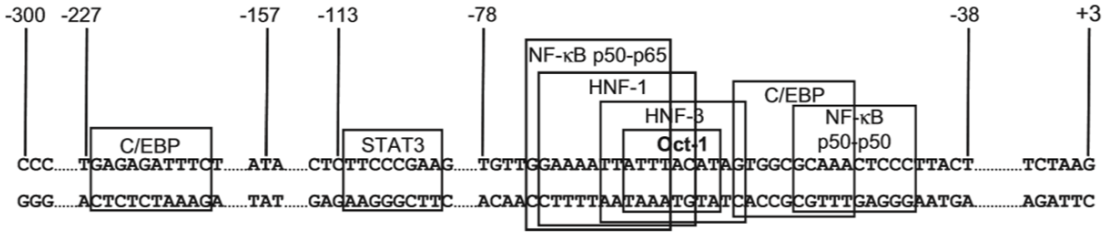

Figure 1.3. -300 to +3 bp of the proximal promoter region of the CRP gene. The binding sites of various transcription factors on the promoter are boxed (adapted from Voleti and Agrawal 2005)

Oct-1 and CRP Gene Expression

Oct-1 is a member of the POU domain transcription factor family that is widely and constitutively expressed (Wang and Jin 2010). Oct-1 functions as both an enhancer, as well as a repressor of gene transcription (Zhou and Yen 1991; Inamoto *et al*. 1997; Dong *et al*. 2009; Shakya *et al*. 2011). Oct-1 binds specifically to an octamer motif (ATGCAAAT) and related sequences on promoters to regulate gene expression (Singh *et al.* 1986; Bhatt *et al*. 1996). In some promoter contexts Oct-1 regulates gene transcription indirectly by interacting with other transcription factors and cofactors that are bound to promoter sequences (Voss *et al.* 1991; Zwilling *et al.* 1992; Nakshatri *et al.* 1995; Ström *et al.* 1996; Prefontaine *et al.* 1999; Shakya *et al.* 2011). In the context of CRP gene expression, the role of Oct-1 was not known at the time of this study. Our lab had previously shown that Oct-1 competed with the NF-κB p50-p65 heterodimer for binding at their overlapping cognate sites (Voleti and Agrawal 2005). In the basal state Oct-1 remains bound to the CRP promoter at its binding site. On cytokine treatment NF-κB activation results in the p50-p65 heterodimer displacing Oct-1 from this site, and thus mediating the synergistic effect of IL-1β. However, the exact role of Oct1-1 in regulating CRP

gene expression and the mechanism involved were not known at the time of this study.

Rationale and Hypothesis

The luciferase reporter assay is a sensitive and accurate assay widely used to measure the role of transcription factors on target gene expression. Reporter plasmids with the luciferase gene cloned downstream of the CRP gene promoter were used to transfect hepatoma Hep-3B cells. The role of Oct-1 in regulating CRP gene expression was studied using reporter plasmids with mutated Oct-1 sites or by cotransfection with Oct-1 overexpressing vectors. Luciferase assays performed with these reagents in both un-induced and cytokine-induced conditions elucidate the role of Oct-1 in regulating CRP gene expression. The mechanism by which Oct-1 exerts its effects on the CRP gene was studied by performing electrophoretic mobility shift assay (EMSA) experiments using nuclear extracts from cytokine-treated and un-treated Hep-3B cells, and with both wild-type and mOct oligos in which the Oct-1 binding site has been mutated.

CRP and Atherosclerosis

Atherosclerosis is an inflammatory disease that is initiated by the retention and subsequent chemical modification of low-density lipoproteins (LDL) in the artery wall. Activation of the endothelium at sites of LDL deposition results in monocyte recruitment into the arterial intima. These monocytes differentiate into macrophages and phagocytize the deposited, modified lipoproteins. Excessive cholesterol and lipid uptake by macrophages transforms them into highly proinflammatory ‘foam cells’ – the key event in the progression of the disease. The failure to resolve initial inflammation slowly leads to the formation of an

atherosclerotic plaque characterized by changes in the extracellular matrix and environment, and in the participation of vascular smooth muscle and immune cells in a chronic inflammatory process (reviewed in Ross 1999; Moore and Tabas 2011).

CRP has been implicated in the pathogenesis of atherosclerosis, although what role it plays is still unclear. Serum CRP levels increase minimally during atherosclerosis, yet CRP is found deposited at sites of atherosclerotic lesions in both humans and in animal models (Reynolds and Vance 1987; Hatanaka *et al.* 1995; Bhakdi *et al.* 1999; Sun *et al.* 2005). The deposition of CRP at lesion sites is independent of its serum level. Native pentameric CRP does not bind unmodified LDL under physiological conditions; however, CRP and LDL can interact with each other if either is immobilized on a solid surface. CRP binds to immobilized enzymatically-modified LDL (E-LDL), an interaction that is Ca^{2+}-dependent and PCh-inhibitable at physiological pH. This binding is dramatically increased in the presence of acidic pH (Singh *et al.* 2008; Singh *et al.* 2009). CRP – E-LDL complex formation also prevents lipid uptake and subsequent foam cell formation (Singh *et al.* 2008). Native CRP does not interact with the major atherogenic form of LDL, oxidized-LDL (ox-LDL) at physiological conditions. However, acidic pH induces a reversible structural modification in the CRP pentamer, and exposes a hidden binding site enabling CRP to bind immobilized ox-LDL. Substitution of one of the amino acids involved in inter-subunit interactions, Pro^{115} with Ala reduced the requirement of acidic pH by 1 unit and also increased ox-LDL binding. This suggested that the CRP molecule was being modified into a 'loosened' pentameric structure (Hammond *et al.* 2010).

To determine the function of CRP in atherosclerosis, human CRP has been used in different animal models of atherosclerosis. Passive administration or transgenic expression of

human CRP was neither atherogenic nor atheroprotective in *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (Hirschfield *et al.* 2005; Torzewski *et al.* 2005; Tennet *et al.* 2008; Ortiz *et al.* 2009). Rabbits transgenically expressing human CRP also did not show any difference in atherosclerotic lesion formation or progression (Koike *et al.* 2009). Studies using the *ApoB*$^{100/100}$*Ldlr*$^{-/-}$ mouse and the *ApoE*$^{-/-}$*CRP*$^{-/-}$ and *Ldlr*$^{-/-}$*CRP*$^{-/-}$ mouse models suggest that CRP might play an atheroprotective role. In the *ApoB*$^{100/100}$*Ldlr*$^{-/-}$ mouse model, administration of human CRP slowed plaque development (Kovacs *et al.* 2007). In the *ApoE*$^{-/-}$*CRP*$^{-/-}$ and *Ldlr*$^{-/-}$*CRP*$^{-/-}$ double knock-out mice the size of atherosclerotic lesions were either equivalent to or increased when compared to the *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, indicating that even mouse CRP, which is present at low levels, may be atheroprotective (Teupser *et al.* 2011).

A hallmark of atherosclerotic plaques is the formation of a highly acidic microenvironment due to macrophage activation, proton and lactate generation, and hypoxic conditions in the artery wall (Silver *et al.* 1988; Leake 1997; Naghavi *et al.* 2002; Haka *et al.* 2009). Acidity is known to induce LDL modification and aggregation, induce binding to extracellular matrix proteins, and increase retention in the artery wall, increasing the uptake of modified LDL by macrophages and monocytes (Sneck *et al.* 2005; Plithari *et al.* 2011; Lähdesmäki *et al.* 2012). The animal models used to study the effect of human CRP in atherosclerosis may not fully develop such a microenvironment at the lesion sites. Human CRP administered to these animals may thus not have undergone the low pH-induced structural modification and, therefore, may not have been able to bind ox-LDL and prevent ox-LDL induced foam cell formation.

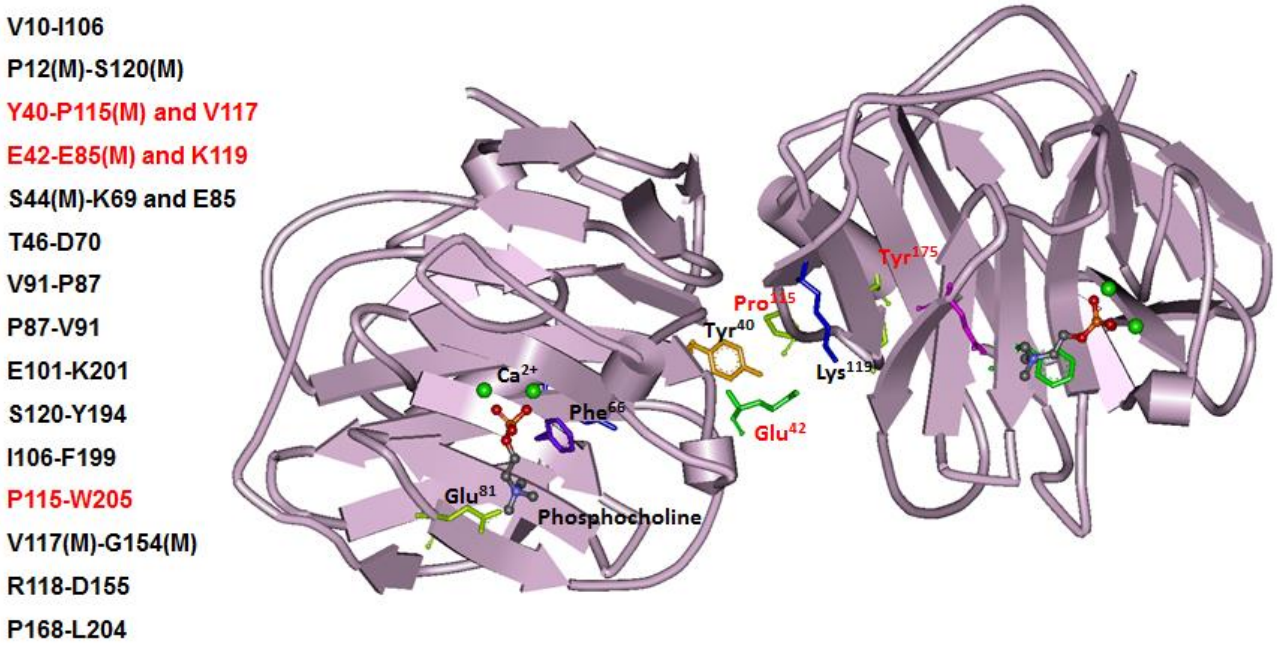

Figure 1.4. Two adjacent subunits of the CRP pentamer, along with amino acids targeted for mutagenesis (red). A list of all amino acids involved in inter-subunit interactions is shown on the left (adapted from (5))

Acidic pH structurally changes the CRP molecule into a 'loosened' pentamer, thus exposing the previously buried ox-LDL binding site. As the pH at lesion sites in animal models of atherosclerosis cannot be modified, it is essential to have a modified form of CRP that can bind to ox-LDL at physiological pH. One approach to achieve this is to use site-directed mutagenesis to make a mutant CRP in which the ox-LDL binding site is no longer buried and permanently exposed. Based on our previous studies using the $Pro^{115} \rightarrow$ Ala (P115A) mutant, we hypothesize that targeting the amino acids involved in inter-subunit interactions will result in a 'loosened' pentamer that can bind to ox-LDL at physiological pH. Figure 1.4 shows the structure of 2 adjacent subunits of the CRP pentamer, with the list of

amino acids involved in inter-subunit interactions on the left. Highlighted in red are the amino acids that were targeted by site-directed mutagenesis. We choose to make a $Glu^{42} \rightarrow$ Gln substitution to generate the E42Q mutant. The binding of the E42Q and P115A mutants to ox-LDL at physiological pH was then tested and compared with that of WT CRP. The Y175A mutant ($Tyr^{175} \rightarrow$ Ala), in which an amino acid not involved in inter-subunit interactions has been substituted was used as a negative control.

In addition to acidic pH endothelial dysfunction and macrophage activation results in the generation of reactive oxygen species (ROS) like O_2^- and OH^- ions and H_2O_2 at atherosclerotic lesions. ROS-induced deregulation of the redox environment is known to enhance the deposition and modification of lipoproteins, activate inflammatory signaling, and increase foam cell formation (Patel *et al.* 2000; Papaharalambus and Griendling 2007; Ottaviano *et al.* 2008; Kondo *et al.* 2009; Hulsmans and Holvoet 2010). Studies from other labs have suggested that ROS-mediated modifications of CRP may influence its function (Miyazawa *et al.* 1988; Wang *et al.* 2011). We hypothesized that ROS may structurally alter the CRP pentamer, similar to that induced by acidic pH, and enable it to bind to immobilized ox-LDL. To this purpose, we choose the oxidizing agent H_2O_2 as an example to investigate if ROS modify CRP and enable it to bind immobilized ox-LDL.

Specific Aims

1. To determine the role of Oct-1 in the regulation of CRP gene expression
    a. To determine whether Oct-1 acts as an activator or repressor of CRP gene expression in Hep-3B cells
    b. To determine the requirement of the Oct-1-binding site on the CRP promoter for

Oct-1-mediated regulation of CRP gene expression

- We published the findings, and these are reproduced in Chapter 2

2. To generate modified CRP which can bind to ox-LDL at physiological pH
    a. To use site-directed mutagenesis and construct a CRP mutant that binds to ox-LDL at physiological pH
        - We published the findings, and these are reproduced in Chapter 3
    b. To determine whether the molecules characteristic of the sites of inflammation can react with CRP and generate modified CRP that binds to ox-LDL at physiological pH
        - Results of preliminary experiments carried out are presented in Chapter 4

3. To determine whether modified CRP (from aim 2) can prevent formation of ox-LDL-induced macrophage foam cells
        - Results are not presented as we were yet to establish a technique to measure ox-LDL-induced foam cell formation

CHAPTER 2

Oct-1 acts as a transcriptional repressor on the C-reactive protein promoter

Bhavya Voleti[1, 2], David J. Hammond Jr.[1], Avinash Thirumalai[1], and Alok Agrawal[*]

Department of Biomedical Sciences, Quillen College of Medicine, P. O. Box 70577, East Tennessee State University, Johnson City, TN 37614, USA.

Keywords: C-reactive protein; Oct-1; C/EBPβ; Hep3B cells; κB site

[1]These authors contributed equally to this work.
[2]Present address: Departments of Psychiatry and Neurobiology, Yale University School of Medicine, New Haven, CT 06508, USA

[*]Address correspondence and reprint requests to: Dr. Alok Agrawal, Department of Biomedical Sciences, P. O. Box 70577, Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
Email address: agrawal@etsu.edu Tel.: +1 423 439 6336; Fax: +1 423 439 8044

Reference: Voleti B, Hammond DJ Jr, Thirumalai A, Agrawal A. 2012. Mol. Immunol; 52(3-4): 242–248. doi:10.1016/j.molimm.2012.06.005.)

Abstract

C-reactive protein (CRP), a plasma protein of the innate immune system, is produced by hepatocytes. A critical regulatory region (−42 to −57) on the CRP promoter contains binding site for the IL-6-activated transcription factor C/EBPβ. The IL-1β-activated transcription factor NF-κB binds to a κB site located nearby (−63 to −74). The κB site overlaps an octamer motif (−59 to −66) which is the binding site for the constitutively active transcription factor Oct-1. Oct-1 is known to function both as a transcriptional repressor and as an activator depending upon the promoter context. Also, Oct-1 can regulate gene expression either by binding directly to the promoter or by interacting with other transcription factors bound to the promoter. The aim of this study was to investigate the functions of Oct-1 in regulating CRP expression. In luciferase transactivation assays, overexpressed Oct-1 inhibited (IL-6+IL-1β)-induced CRP expression in Hep3B cells. Deletion of the Oct-1 site from the promoter drastically reduced the cytokine response because the κB site was altered as a consequence of deleting the Oct-1 site. Surprisingly, overexpressed Oct-1 inhibited the residual (IL-6+IL-1β)-induced CRP expression through the promoter lacking the Oct-1 site. Similarly, deletion of the Oct-1 site reduced the induction of CRP expression in response to overexpressed C/EBPβ, and overexpressed Oct-1 inhibited C/EBPβ-induced CRP expression through the promoter lacking the Oct-1 site. We conclude that Oct-1 acts as a transcriptional repressor of CRP expression and it does so by occupying its cognate site on the promoter and also via other transcription factors by an as yet undefined mechanism.

Introduction

C-reactive protein (CRP) is defined as an acute phase protein whose serum concentration increases in inflammatory conditions, such as rheumatoid arthritis, and in some non-inflammatory conditions, such as stress and cellular injury (Agrawal et al., 2009; Kushner et al., 2006). CRP is primarily produced by hepatocytes in response to IL-6 and IL-1β and its synthesis is regulated at the transcriptional level (Castell et al., 1990; Eklund, 2005; Goldberger et al., 1987; Toniatti et al., 1990a; Voleti and Agrawal, 2006; Yoshida et al., 2006; Zhang et al., 1995). In human hepatoma Hep3B cells, IL-6 induces CRP expression by activating transcription factors C/EBPβand STAT3 (Ochrietor et al., 2000; Poli and Cortese, 1989; Ramji et al., 1993; Turkson et al., 1998; Wang et al., 1999; Young et al., 2008; Zhang et al., 1996). IL-1β, which alone does not induce CRP expression in Hep3B cells, synergistically enhances the effects of IL-6 by activating transcription factor NF-κB (Agrawal et al., 2003a; Darlington et al., 1986; Ganapathi et al., 1988; Kramer et al., 2008; Zhang et al., 1995). The proximal 157 bp of the CRP promoter are sufficient for the synergy between IL-6 and IL-1β (Zhang et al., 1995). Transcription factor c-Rel participates in regulating CRP expression, without binding to the promoter, via interactions with C/EBPβ (Agrawal et al., 2003b; Cha-Molstad et al., 2007). Five constitutively active transcription factors (C/EBPζ, RBP-Jκ, HNF-1, HNF-3, and Oct-1) are also involved in regulating CRP expression (Blaschke et al., 2006; Grimm et al., 2011; Nishikawa et al., 2008; Reiner et al., 2008; Toniatti et al., 1990b; Voleti and Agrawal, 2005).

The binding sites of various transcription factors on the CRP promoter are shown in Fig. 2.1A. C/EBPβbinds to two sites centered at positions −52 and −219 (Li and Goldman, 1996). The STAT3-binding site is located at −108 (Zhang et al., 1996). There are five NF-κB proteins, p50, p52, p65, c-Rel and Rel B, which form homodimers or heterodimers and bind to κB sites on

the promoters to regulate gene expression (Ghosh and Hayden, 2012). On the CRP promoter, NF-κB p50-p50 binds to a non-consensus κB site located at −47, overlapping the proximal C/EBP site, and NF-κB p50-p65 binds to a κB site located at −69 (Agrawal et al., 2001; Cha-Molstad et al., 2000; Voleti and Agrawal, 2005). C/EBPζ binds to the same proximal C/EBP site where C/EBPβbinds (Singh et al., 2007). RBP-Jκ binds to the same site where NF-κB p50-p50 binds (Singh et al., 2007). The binding sites for HNF-1 and HNF-3 are located at positions −67 and −62, respectively (Li and Goldman, 1996). Oct-1 binds to a site centered at position −63 (Arcone et al., 1988; Li and Goldman, 1996).

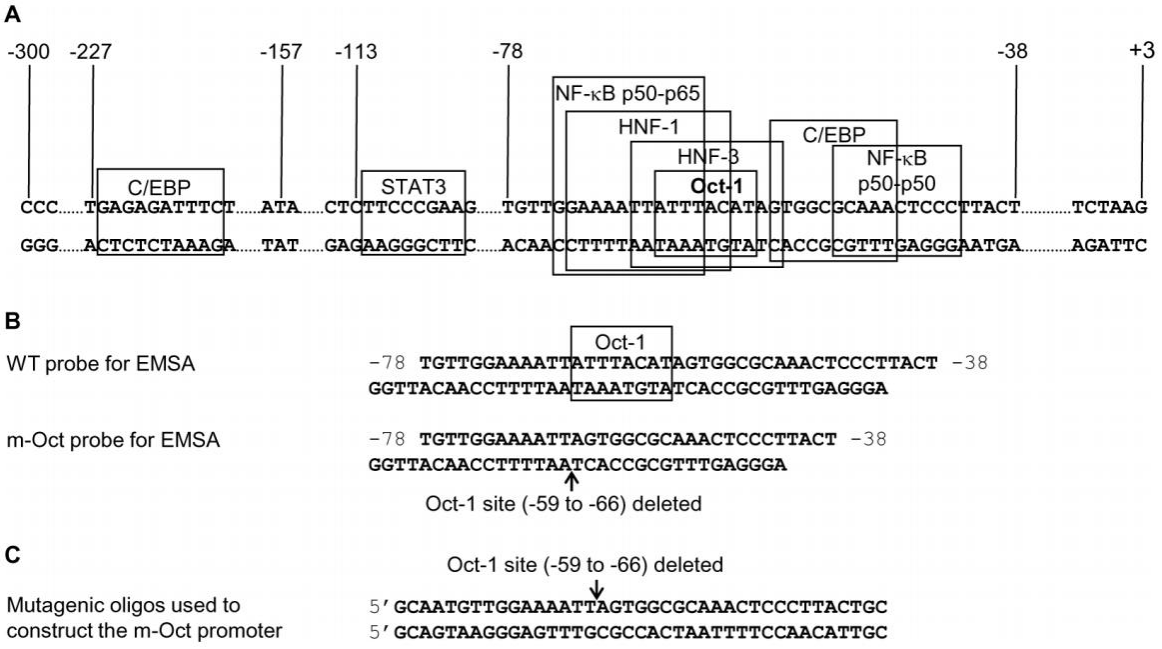

Figure 2.1: CRP promoter and oligos used to study the role of Oct-1 in CRP gene regulation. (A) The −300 to +3 region of the CRP gene is shown. The binding sites of various transcription factors on the promoter are boxed. (B) Sequences of the oligos derived from the CRP promoter and used as probes in EMSA. (C) Sequences of the mutagenic oligos used for mutagenesis of the CRP promoter to construct the m-Oct promoter.

Oct-1 is a ubiquitous and constitutively active transcription factor (Singh et al., 1986; Sturm et al., 1988). Generally, Oct-1 functions as an activator of transcription (Dong et al., 2009;

Inamoto et al., 1997; Shakya et al., 2011; Zhou and Yen, 1991). However, Oct-1 also represses transcription of some genes (Bhat et al., 1996; Shakya et al., 2011). Oct-1 binds specifically to an octamer motif (ATGCAAAT) and related sequences on promoters to regulate gene expression (Bhat et al., 1996; Singh et al., 1986). Oct-1 also functions by associating with basal transcription factors and other tissue-specific transcription factors and cofactors (Nakshatri et al., 1995; Préfontaine et al., 1999; Shakya et al., 2011; Ström et al., 1996; Voss et al., 1991; Wysocka and Herr, 2003; Zwilling et al., 1994).

It is not known how Oct-1 acts on the CRP promoter to regulate CRP gene expression. Previously, we reported that Oct-1 competes with NF-κB for binding to its cognate site which overlaps the κB site on the CRP promoter (Voleti and Agrawal, 2005). The aim of this study was to determine whether Oct-1 acts as a repressor or as an activator of CRP expression. Another aim of this study was to determine the requirement of the Oct-1-binding site on the CRP promoter for Oct-1 to regulate CRP expression.

Materials and Methods

Electrophoretic mobility shift assays (EMSA)

Hep3B cells were cultured in 100 mm dish. Cells were subjected to serum starvation overnight and then treated with IL-1βfor 15 min, as described previously (Voleti and Agrawal, 2005). The confluency of cells was approximately 60% at the time of IL-1β-treatment. IL-β(R&D) was used at a concentration of 200 U/ml. Nuclear extracts were prepared by using NE-PER nuclear and cytoplasmic kit (Pierce), as described previously (Singh et al., 2007). The sequences of the oligonucleotides (oligo) used in EMSA are shown in Fig. 1B. Oligos were obtained from Integrated DNA Technologies. To prepare the probes, complementary oligos were annealed and labeled with [γ-32 P] ATP. The probe-nuclear extract reaction buffer contained 16 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 2.5 mM DTT, 0.15% Nonidet P-40, 8% Histopaque, and 1 μ-g of poly dI-dC. In super shift experiments, antibodies (2 μg) were added to the reaction mixture and incubated on ice for 15 min before addition of the probe. In oligo competition experiments, 150 ng of unlabeled oligos was added to the reaction mixture before addition of the antibody and probe. The antibodies to p50 (H119), p65 (H286), HNF-1 (H205), HNF-3 (C20) and Oct-1 (C20) were purchased from Santa Cruz Biotechnologies. DNA-protein complexes were resolved in native 5% polyacrylamide gels containing 2.5% glycerol and visualized in a phosphorimager using Image-Quant software (GE Healthcare).

Construction of CRP promoter-luciferase (Luc) reporter vectors

The construction of wild-type (WT) CRP promoter constructs, Luc-157 WT (−157/+3 of CRP gene) and Luc-300 WT (−300/−1 of CRP promoter), has been reported previously (Agrawal et al., 2001; Kleemann et al., 2003; Voleti and Agrawal, 2005). These two WT

constructs were used as templates for mutagenesis (deletion of all 8 bp of the Oct-1 site) of the CRP promoter by using QuickChange site-directed mutagenesis kit (Stratagene). The mutagenic primers are shown in Fig. 1C. Mutations were verified by DNA sequencing. Plasmids were purified using maxi prep plasmid isolation kit (Eppendorf).

Luciferase transactivation assays (Luc assays)

Hep3B cells were cultured in 6-well plates. Transfections were performed using TransIT-LT1 (Mirus) according to manufacturer's instructions. CRP promoter-Luc reporter constructs were used at 1 μg plasmid per well and the transcription factor expression vectors were used as mentioned in the figure legends. The confluency of cells was approximately 60% at the time of transfection. After transfection, cells were left in serum-free medium. After 16 h of transfection and serum starvation, the transfected cells were either treated with IL-6 and IL-1βfor 24 h or left untreated. IL-6 (R&D) was used at a concentration of 1100 U/ml and IL-1βwas used at a concentration of 200 U/ml. After 40 h of transfection, Luc assays were performed following the protocol supplied by the manufacturer (Promega). Luc activity was measured in a luminometer (Molecular Devices), as described previously (Singh et al., 2007).

Results

Oct-1 binds to its site on the CRP promoter and competes with the binding of NF-κB to the overlapping κB site

To demonstrate that Oct-1 binds to its cognate site on the CRP promoter, we performed EMSA using a 45 bp oligo (WT oligo; Fig. 2.1B) which contained the overlapping Oct-1 and κB sites and the adjacent C/EBP site (Fig. 2.2). We used nuclear extract from IL-1β-treated Hep3B cells as the source of NF-κB. Four specific complexes were formed on the probe (lanes 1 and 2). The fastest migrating complex was NF-κB (p50–p65) because the antibodies to p50 and p65 reduced the intensity of the complex (lanes 3 and 4). By using antibodies to HNF-1 and Oct-1, the top two complexes were found to contain HNF-1 and Oct-1 (lanes 5 and 6). Another specific complex contained HNF-3 (confirmation using antibodies to HNF-3 is shown in Fig. 2.3). When the Oct-1-containing complex was abolished (and, in part, super shifted) by treatment of the nuclear extract with anti-Oct-1 antibodies, the intensity of the NF-κB complex increased (compare lanes 1 and 6). These results suggest that Oct-1 binds to its site on the CRP promoter and competes with the binding of NF-κB to the overlapping κB site.

Oct-1 does not bind to CRP promoter in the absence of the Oct-1 site

The purpose of the next EMSA was to characterize the m-Oct promoter (promoter with the 8 bp Oct-1 site deleted; Fig. 2.1B) that we used in the Luc assays. We performed EMSA using WT oligo and m-Oct oligo as probes and nuclear extracts from untreated Hep3B cells (Fig. 2.3). Three specific complexes containing HNF-1, Oct-1 and HNF-3 were formed on the WT

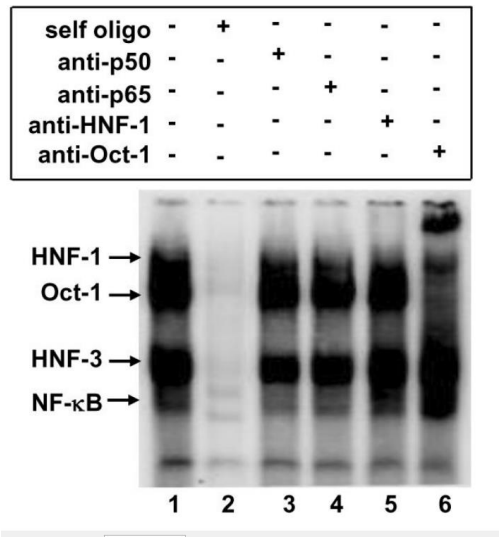

Figure 2.2. Oct-1 binds to its site on the CRP promoter and competes with the binding of NF-κB to the overlapping κB site. A representative of three EMSA is shown. Radiolabelled WT oligo (−38 to −78) was used as the probe and nuclear extract from IL-β-treated Hep3B cells was used as the source of NF-βB. Self oligo competitor (unlabeled WT oligo) and antibodies were added to nuclear extract before the addition of the probe. DNA probe-protein complexes were visualized by using a phosphorimager. Arrows point to the complexes formed on the probe. The mobility of the free probe is not shown.

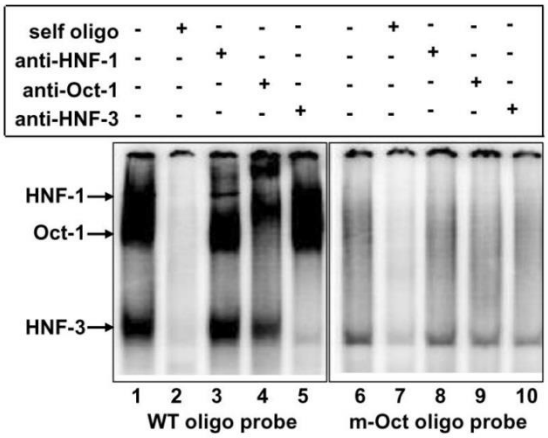

Figure 2.3. Oct-1 does not bind to CRP promoter in the absence of the Oct-1 site. A representative of two EMSA is shown. Radiolabelled WT oligo (lanes 1–5) and m-Oct oligo (8 bp Oct-1 site deleted, lanes 6–10) were used as probes. Nuclear extract from untreated Hep3B cells was used as the source of constitutively active transcription factors. Self oligo competitors (unlabeled WT and m-Oct oligos) and antibodies were added to nuclear extract before the addition of the probe. DNA probe-protein complexes were visualized by using a phosphorimager. Arrows point to the complexes formed on the WT probe. The mobility of the free probe is not shown.

probe (lanes 1–5), whereas deletion of the Oct-1 site abolished the binding of not only Oct-1 but also HNF-1 and HNF-3 to the probe (lanes 6–10). These results suggested that Oct-1 would not bind directly to the m-Oct CRP promoter that we used in the Luc assays.

Oct-1 inhibits (IL-6+IL-1β)-induced CRP expression

To investigate the role of Oct-1 in regulating CRP expression, we conducted Luc assays using Luc 157-WT and Luc 300-WT promoter constructs and determined the effects of overexpressed Oct-1 on (IL-6+IL-1β)-induced CRP promoter-driven Luc activity. As shown in Fig. 2.4, Oct-1 inhibited (IL-6+IL-1β)-induced CRP promoter-driven Luc activity in a dose dependent manner, irrespective of the size of the CRP promoter, that is, irrespective of the presence of one or both of the C/EBP sites (located at positions −52 and −219). From these results we conclude that Oct-1 acts as a transcriptional repressor of CRP expression.

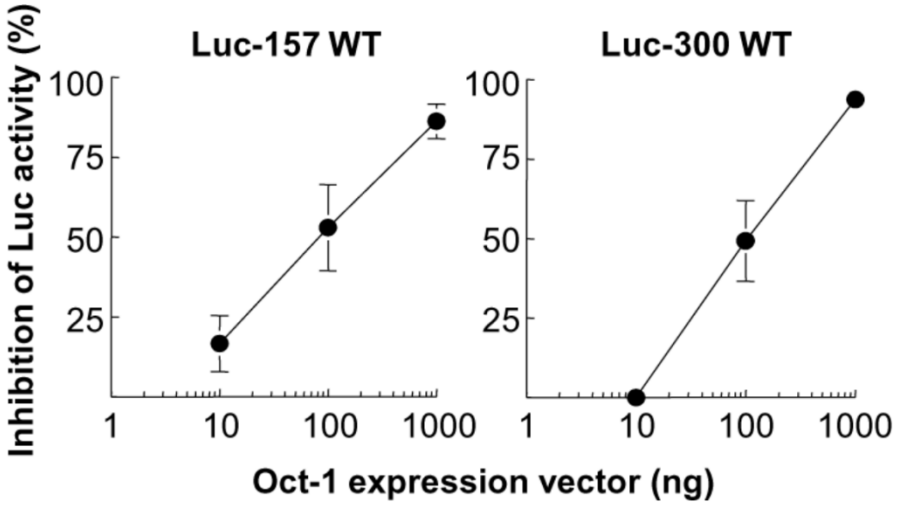

Figure 2.4. Oct-1 inhibits (IL-6+IL-1β)-induced CRP promoter-driven Luc activity. Luc-157 WT and Luc-300 WT CRP promoter constructs were transfected into Hep3B cells along with increasing amounts of the expression vector encoding Oct-1. After 16 h, cells were treated with IL-6 and IL-1β for 24 h. CRP expression was measured as Luc activity. Percent inhibition of Luc activity is plotted on the y-axis. Average ± S.E.M. of three experiments are shown. For some data points, error bars are not visible because the S.E.M. was low.

Effects of deleting the Oct-1 site on basal and (IL-6+IL-1β)-induced CRP expression

To explore the mechanism of Oct-1-mediated repression of CRP expression, our approach was to delete the Oct-1-site (−59 to −66) from the promoter and use the m-Oct promoter construct, which does not bind Oct-1, in the Luc assays. We determined the contribution of the Oct-1 site, which overlaps the HNF-1, HNF-3 and κB sites, to the basal and (IL-6+IL-1β)-induced CRP expression. Deletion of the Oct-1 site from the promoter did not affect basal CRP expression (Fig. 2.5A). However, (IL-6+IL-1β)-induced CRP expression was drastically reduced when the Oct-1 site was deleted, irrespective of the size of the CRP promoter used in the assay (Fig. 2.5B). CRP expression was reduced by about 87% (from 14-fold to 2-fold) when Luc-157 m-Oct promoter was used and by about 91% (from 113-fold to 11-fold) when Luc-300 m-Oct promoter was used, compared to their respective WT constructs. These results suggest that the −59 to −66 region of the CRP promoter is required for full (IL-6+IL-1β)-induced CRP expression.

Oct-1 inhibits (IL-6+IL-1β)-induced CRP expression even if the Oct-1 site is deleted from the promoter

Because some residual (IL-6+IL-1β)-induced CRP transcription activity was present even if the Oct-1 site was deleted, we next determined whether overexpressed Oct-1 would have an effect on the residual (IL-6+IL-1β)-induced CRP expression. Surprisingly, overexpressed Oct-1 inhibited the (IL-6+IL-1β)-induced CRP expression through the m-Oct promoters of both size; the inhibition was about 55%, 71%, 83% and 64% on Luc-157 WT, Luc-157 mOct, Luc-300 WT and Luc-300 m-Oct promoters, respectively (Fig. 2.6). The Oct-1-mediated inhibition of CRP expression on the m-Oct promoters was not significantly different from that on the WT

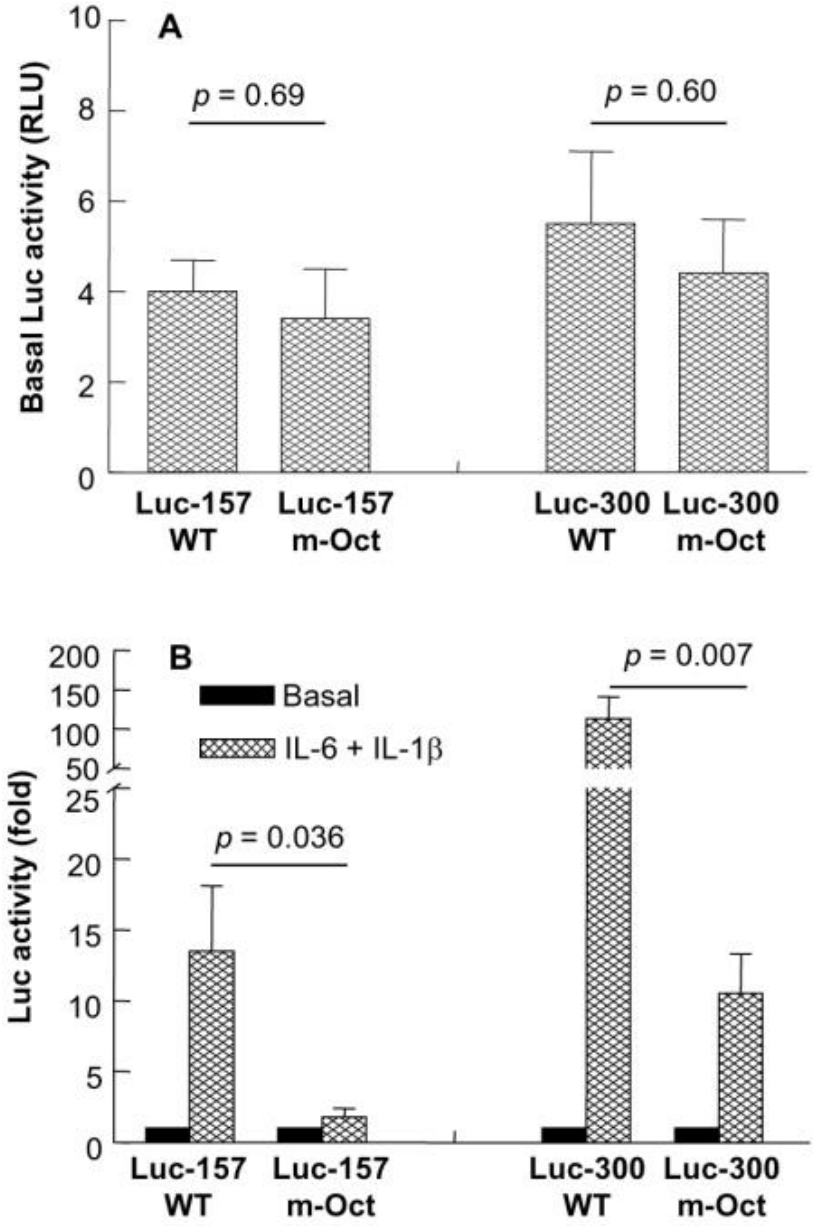

Figure 2.5. Effects of deleting the Oct-1 site on basal and (IL-6+IL-1β)-induced CRP promoter-driven luciferase activity. (A) Hep3B cells were transfected with Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct CRP promoter constructs. After 40 h, CRP transcription was measured as Luc activity and plotted on the y-axis. Average ± S.E.M. of four experiments are shown. Unpaired two-tailed Students *t*-test was used to calculate *p* values. (B) Hep3B cells were transfected with Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct CRP promoter constructs. After 16 h, cells were either treated with IL-6 and IL-1β or left untreated for 24 h. CRP expression was measured as Luc activity. Basal Luc activity was taken as 1 and (IL-6+IL-1β)-induced Luc activity was plotted on the y-axis as fold over basal Luc activity. Average ± S.E.M. of five experiments are shown. Unpaired two-tailed Students *t*-test was used to calculate *p* values.

promoters. These results suggest that the inhibitory effect of Oct-1 on CRP expression is not mediated solely through the Oct-1 site and that Oct-1 represses CRP expression even when Oct-1 is not bound directly to the promoter.

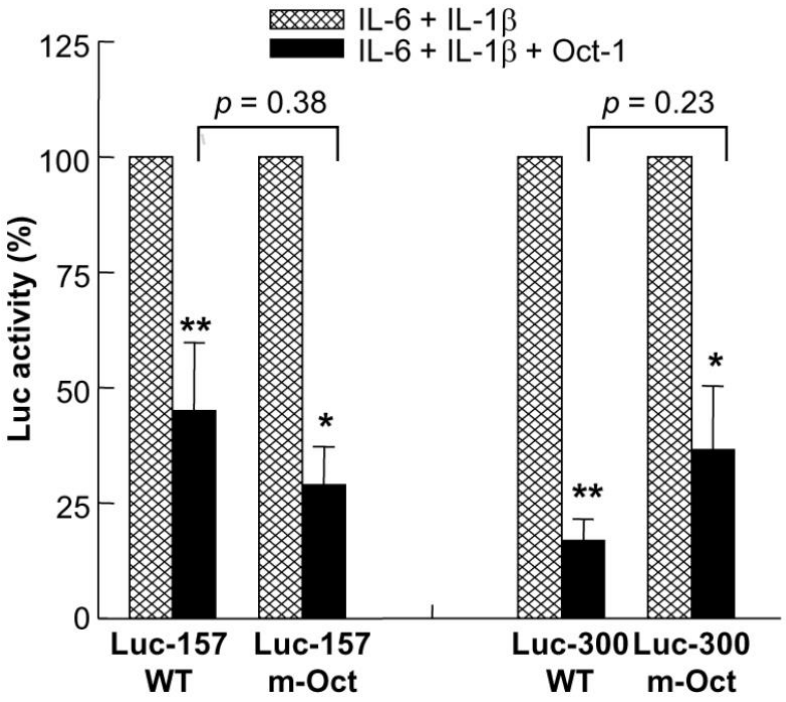

Figure 2.6. Oct-1 inhibits (IL-6+IL-1β)-induced CRP expression even if the Oct-1 site is deleted from the promoter. Hep3B cells were transfected with Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct CRP promoter constructs. One set of cells were co-transfected with the expression vector encoding Oct-1 (1 μg). After 16 h, cells were either treated with IL-6 and IL-1β or left untreated for 24 h. CRP expression was measured as Luc activity. Luc activity in the absence of Oct-1 was taken as 100%. Average ± S.E.M. of four experiments are shown. The *p* values for the difference between (IL-6+IL-1β) and (IL-6+IL-1β+Oct-1) groups were calculated by using paired two-tailed Students *t*-test (*p< 0.05, **p< 0.005). The *p* values for the difference between WT and corresponding m-Oct groups were calculated by using unpaired two-tailed Students *t*-test.

Oct-1 inhibits C/EBPβ-induced CRP expression regardless of the Oct-1 site

To further explore the mechanism of repressive action of Oct-1 on the CRP promoter, we investigated the effects of overexpressed Oct-1 on overexpressed C/EBPβ-induced CRP expression, in the presence and absence of the Oct-1 site. We first determined the effects of

deleting the Oct-1 site on C/EBPβ-induced CRP expression. C/EBPβ-induced CRP expression was reduced when the Oct-1 site was deleted, irrespective of the size of the CRP promoter used in the assay (Fig. 2.7A). CRP expression was reduced by about 53% (from 40-fold to 19-fold) when Luc-157 m-Oct promoter was used and by about 38% (from 59-fold to 37-fold) when Luc-300 m-Oct promoter was used, compared to their respective WT constructs. These results suggest that the −59 to −66 region of the CRP promoter is also required for full C/EBPβ-induced CRP expression. We next investigated whether overexpressed Oct-1 would have an effect on C/EBPβ-induced CRP expression. Surprisingly, overexpressed Oct-1 inhibited C/EBPβ-induced CRP expression through the m-Oct promoters of both size; the inhibition was about 71%, 78%, 57% and 79% on Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct promoters, respectively (Fig. 2.7B). These results further suggest that the inhibitory effect of Oct-1 on CRP expression is also mediated via interactions with other transcription factors bound to the CRP promoter.

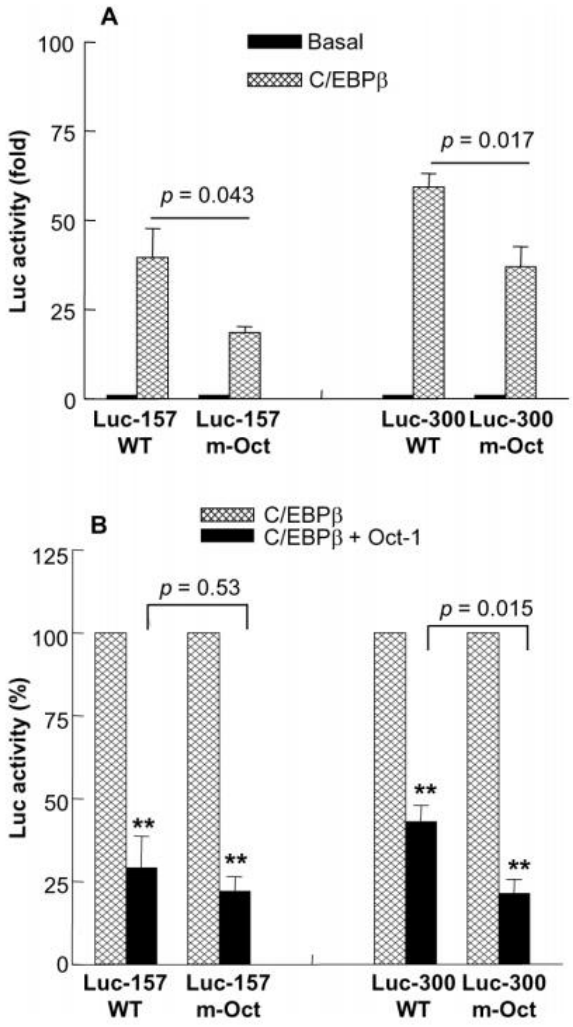

Figure 2.7. Oct-1 inhibits C/EBPβ-induced CRP expression regardless of the Oct-1 site. (A) Hep3B cells were transfected with Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct CRP promoter constructs. One set of cells were co-transfected with the expression vector encoding C/EBPβ (50 ng). After 40 h, CRP expression was measured as luciferase activity. Basal Luc activity was taken as 1 and C/EBPβ-induced Luc activity was plotted on the y-axis as fold over basal Luc activity. Average ± S.E.M. of four experiments are shown. Unpaired two-tailed Students *t*-test was used to calculate *p* values. (B) Hep3B cells were transfected with Luc-157 WT, Luc-157 m-Oct, Luc-300 WT and Luc-300 m-Oct CRP promoter constructs. One set of cells were co-transfected with the expression vector encoding C/EBPβ (50 ng). Another set of cells were co-transfected with the expression vectors encoding C/EBPβ (50 ng) and Oct-1 (1 μg). After 40 h, CRP expression was measured as Luc activity. Luc activity in the absence of Oct-1 was taken as 100%. Average ± S.E.M. of four experiments are shown. The *p* values for the difference between C/EBPβ and (C/EBPβ+ Oct-1) groups were calculated by using paired two-tailed Students *t*-test. (**p< 0.005). The *p* values for the difference between WT and corresponding m-Oct groups were calculated by using unpaired two-tailed Students *t*-test. was reduced when the Oct-1 site was deleted, irrespective of the size of the CRP promoter used.

Discussion

The aim of this study was to determine whether Oct-1 acts as a transcriptional repressor or as an activator of CRP expression. Our approach included overexpressing Oct-1 in (IL-6+IL-1β)-treated Hep3B cells transfected with CRP promoter linked to Luc reporter. We used a 157 bp promoter which contained only one C/EBP-site and a 300 bp promoter which contained both C/EBP-sites. Our major findings were: 1. Overexpressed Oct-1 inhibited (IL-6+IL-1β)-induced CRP promoter-driven Luc expression. 2. The inhibition of 157 bp CRP promoter-driven Luc expression was not different from that of 300 bp CRP promoter driven Luc expression. These findings suggest that Oct-1 acts as a transcriptional repressor of CRP expression and that the proximal 157 bp of the CRP promoter is sufficient for the repressive action of Oct-1.

Because Oct-1 is also known to regulate expression of certain genes by associating with other transcription factors and cofactors (Nakshatri et al., 1995; Préfontaine et al., 1999; Shakya et al., 2011; Ström et al., 1996; Voss et al., 1991; Wysocka and Herr, 2003; Zwilling et al., 1994), we determined whether Oct-1 can regulate CRP expression without binding to its cognate site on the CRP promoter. Our approach included overexpressing Oct-1 in (IL-6+IL-1β)-treated Hep3B cells transfected with either WT CRP promoter or m-Oct CRP promoter linked to Luc reporter. Another approach was to investigate the effects of overexpressed Oct-1 on overexpressed C/EBPβ-induced CRP expression in Hep3B cells transfected with either WT CRP promoter or m-Oct CRP promoter linked to Luc reporter. Our major findings were: 1. (IL-6+IL-1β)-induced CRP expression was drastically reduced when the Oct-1 site was deleted, indicating that the −59 to −66 region of the CRP promoter was required for full (IL-6+IL-1β)-induced CRP expression. This finding was expected because the −59 to −66 region overlaps the binding site for the IL-1β-activated transcription factor NF-κB. 2. Overexpressed Oct-1 inhibited the residual

(IL-6+IL-1β)-induced CRP expression through the m-Oct promoter, indicating that the repressive effect of Oct-1 on CRP expression was not mediated solely via the binding of Oct-1 to the Oct-1 site. 3. C/EBPβ-induced CRP expression was reduced when the Oct-1 site was deleted, indicating that the −59 to −66 region of the CRP promoter was also required for full C/EBPβ-induced CRP expression. This finding was unexpected because the −59 to −66 region does not overlap the C/EBP-site and, therefore, the deletion of this region should not have affected C/EBPβ-induced CRP expression. This finding therefore suggests that C/EBPβcooperates with one or more transcription factors bound to the −59 to −66 region. 4. Overexpressed Oct-1 inhibited the residual C/EBPβ-induced CRP expression through the m-Oct promoter, further indicating that the repressive effect of Oct-1 on CRP expression was not mediated solely via the binding of Oct-1 to the Oct-1 site.

We have previously shown that Oct-1 binds to a 25 bp oligo containing the overlapping Oct-1 and κB sites derived from the CRP promoter and that Oct-1 and NF-κB compete with each other for binding to their overlapping binding sites (Voleti and Agrawal, 2005). In this study, we found similar results using the 45 bp oligo derived from the CRP promoter which contained the overlapping Oct-1 and κB sites and the adjacent C/EBP-site. Deleting the Oct-1 site from the oligo abolished the binding of not only Oct-1 to the oligo but also abolished the binding of HNF-1 and HNF-3 and perhaps also of NF-κB. This result was expected because the binding sites for these four transcription factors overlap. Our attempts to specifically abolish the binding of only Oct-1 to the CRP promoter, without disrupting the binding of HNF-1, HNF-3 and NF-κB to their binding sites on the promoter, have been unsuccessful; mutating either the last 2 bp or 4 bp of the Oct-1 site did not abolish the binding of Oct-1 to its site (data not shown).

It has been reported previously that the −42 to −57 region of the CRP promoter, where

NF-κB p50-p50, C/EBPβ, C/EBPξ, RBP-Jκ, and c-Rel form complexes with the promoter, is a critical regulatory region participating in CRP expression (Agrawal et al., 2001, 2003a, 2003b; Cha-Molstad et al., 2000, 2007; Singh et al., 2007; Voleti and Agrawal, 2005; Young et al., 2008). Our current data suggest that the −57 to −74 region, where Oct-1, HNF-1, HNF-3, and NF-κB form complexes with the promoter, is also a critical regulatory region on the CRP promoter, that Oct-1 works through both regulatory regions, and that the two regulatory regions on the CRP promoter regulate CRP expression cooperatively.

Combined data suggest that Oct-1 acts as a transcriptional repressor of CRP gene expression and that it does so through two mechanisms. The first mechanism involves the binding of Oct-1 to its site; the competition between Oct-1 and NF-κB for binding to their overlapping sites contributes to the regulation of CRP expression, as we have proposed previously (Voleti and Agrawal, 2005). Competition between Oct-1 and NF-κB, between Oct-1 and C/EBPβ, and between Oct-1 and NF-Y, and subsequent repression of the promoter activity, has been shown for several other genes (dela Paz et al., 2007; Osborne et al., 2004; Wu et al., 1997). It is possible that when Oct-1 binds to an overlapping site of a transcriptional activator, then Oct-1 usually acts as a transcriptional repressor. The second mechanism of Oct-1-mediated repression of CRP expression, as yet undefined, involves possible interactions with other transcription factors bound to the CRP promoter.

Acknowledgements

We are grateful to Dr. Peter F. Johnson for the gift of expression vector encoding C/EBPβand Dr. Winship Herr for the gift of expression vector encoding Oct-1.

This work was supported in part by the National Institutes of Health (R01HL071233; A.A.)

References

Agrawal A, Cha-Molstad H, Samols D, Kushner I. Transactivation of C-reactive protein by IL-6 requires synergistic interaction of CCAAT/enhancer binding protein β (C/EBPβ) and Rel p50. J. Immunol. 2001; 166:2378–2384.

Agrawal A, Cha-Molstad H, Samols D, Kushner I. Overexpressed nuclear factor-κB can participate in endogenous C-reactive protein induction, and enhances the effects of C/EBPβ and Signal Transducer and Activator of Transcription-3. Immunology. 2003a; 108:539–547.

Agrawal A, Samols D, Kushner I. Transcription factor c-Rel enhances C-reactive protein expression by facilitating the binding of C/EBPβ to the promoter. Mol. Immunol. 2003b; 40:373–380.

Agrawal A, Singh PP, Bottazzi B, Garlanda C, Mantovani A. Pattern recognition by pentraxins. Adv. Exp. Med. Biol. 2009; 653:98–116.

Arcone R, Gualandi G, Ciliberto G. Identification of sequences responsible for acute-phase induction of human C-reactive protein. Nucl. Acids Res. 1988; 16:3195–3207.

Bhat R, Weaver JA, Sterling KM, Bresnick E. Nuclear transcription factor Oct-1 binds to the 5'-upstream region of CYP1A1 and negatively regulates its expression. Int. J. Biochem. Cell Biol. 1996; 28:217–227.

Blaschke F, Takata Y, Caglayan E, Collins A, Tontonoz P, Hsueh WA, Tangirala RK. A nuclear receptor corepressor-dependent pathway mediates suppression of cytokine-induced C-reactive protein gene expression by liver X receptor. Circ. Res. 2006; 99:e88–e99.

Castell JV, Gomez-Lechon MJ, David M, Fabra R, Trullenque R, Heinrich PC. Acute-phase response of human hepatocytes: Regulation of acute-phase protein synthesis by interleukin-6. Hepatology.1990; 12:1179–1186.

Cha-Molstad H, Agrawal A, Zhang D, Samols D, Kushner I. The Rel family member p50 mediates cytokine-induced C-reactive protein expression by a novel mechanism. J. Immunol. 2000; 165:4592–4597.

Cha-Molstad H, Young DP, Kushner I, Samols D. The interaction of c-Rel with C/EBP-beta enhances C/EBP-beta binding to the C-reactive protein gene promoter. Mol. Immunol. 2007; 44:2933–2942.

Darlington GJ, Wilson DR, Lachman LB. Monocyte-conditioned medium, interleukin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. J. Cell Biol.1986; 103:787–93.

Dela Paz NG, Simeonidis S, Leo C, Rose DW, Collins T. Regulation of NF-κB-dependent gene expression by the POU domain transcription factor Oct-1. J. Biol. Chem. 2007; 282:8424–8434.

Dong B, Huang C, Li D, Zhao FQ. Oct-1 functions as a transactivator in the hormonal induction of beta-casein gene expression. Mol. Cell. Biochem. 2009; 328:93–99.

Eklund CM. Proinflammatory cytokines in CRP baseline regulation. Adv. Clin. Chem. 2009; 48:111–136.

Ganapathi MK, Schultz D, Mackiewicz A, Samols D, Hu S-I, Brabenec A, Macintyre SS, Kushner I. Heterogeneous nature of the acute phase response: Differential regulation of human serum amyloid A, C-reactive protein, and other acute phase proteins by cytokines in Hep3B cells. J. Immunol. 1988; 141:564–569.

Ghosh S, Hayden MS. Celebrating 25 years of NF-κB research. Immunol. Rev. 2012; 246:5–13.

Goldberger G, Bing DH, Sipe JD, Rits M, Colten HR. Transcriptional regulation of genes encoding the acute-phase proteins CRP, SAA, and C3. J. Immunol. 1987; 138:3967–3971.

Grimm AA, Brace CS, Wang T, Stormo GD, Imai S-I. A nutrient-sensitive interaction between Sirt1 and HNF-1α regulates CRP expression. Aging Cell. 2011; 10:305–317.

Inamoto S, Segil N, Pan Z-Q, Kimura M, Roeder RG. The cyclin-dependent kinase-activating kinase (CAK) assembly factor, MAT1, targets and enhances CAK activity on the POU domains of octamer transcription factors. J. Biol. Chem. 1997; 272:29852–29858.

Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HMG, Kooistra T. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFκB-C/EBP-β complex formation. Blood. 2003; 101:545–551.

Kramer F, Torzewski J, Kamenz J, Veit K, Hombach V, Dedio J, Ivashchenko Y. Interleukin-1β stimulates acute phase response and C-reactive protein synthesis by inducing an NF-κB- and C/EBPβ-dependent autocrine interleukin-6 loop. Mol. Immunol. 2008; 45:2678–2689.

Kushner I, Rzewnicki D, Samols D. What does minor elevation of C-reactive protein signify? Am. J. Med. 2006; 119:166.e17–166.e28.

Li S-P, Goldman ND. Regulation of human C-reactive protein gene expression by two synergistic IL-6 responsive elements. Biochemistry. 1996; 35:9060–9068.

Nakshatri H, Nakshatri P, Currie RA. Interaction of Oct-1 with TFIIB: Implications for a novel response elicited through the proximal octamer site of the lipoprotein lipase promoter. J. Biol. Chem. 1995; 270:19613–19623.

Nishikawa T, Hagihara K, Serada S, Isobe T, Matsumura A, Song J, Tanaka T, Kawase I, Naka T, Yoshizaki K. Transcriptional complex formation of c-Fos, STAT3, and hepatocyte NF-1α is essential for cytokine-driven C-reactive protein gene expression. J. Immunol. 2008; 180:3492–3501.

Ochrietor JD, Harrison KA, Zahedi K, Mortensen RF. Role of STAT3 and C/EBP in cytokine dependent expression of the mouse serum amyloid P-component (SAP) and C-reactive protein (CRP) genes. Cytokine. 2000; 12:888–899.

Osborne AR, Zhang H, Fejer G, Palubin KM, Niesen MI, Blanck G. Oct-1 maintains an intermediate, stable state of HLA-DRA promoter repression in Rb-defective cells: An Oct-1-containing repressosome that prevents NF-Y binding to the HLA-DRA promoter. J. Biol. Chem. 2004; 279:28911–28919.

Préfontaine GG, Walther R, Giffin W, Lemieux ME, Pope L, Hache RJG. Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. J. Biol. Chem. 1999; 274:26713–26719.

Poli V, Cortese R. Interleukin 6 induces a liver-specific nuclear protein that binds to the promoter of acute-phase genes. Proc. Natl. Acad. Sci. USA. 1989; 86:8202–8206.

Ramji DP, Vitelli A, Tronche F, Cortese R, Ciliberto G. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBPδ/NF-IL6β, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. Nucl. Acids Res. 1993; 21:289–294.

Reiner AP, Barber MJ, Guan Y, Ridker PM, Lange LA, Chasman DI, Walston JD, Cooper GM, Jenny NS, Rieder MJ, Durda JP, Smith JD, Novembre J, Tracy RP, Rotter JI, Stephens M, Nickerson DA, Krauss RM. Polymorphisms of the HNF1A gene encoding hepatocyte nuclear

factor-1α are associated with C-reactive protein. Am. J. Human Genet. 2008; 82:1193–1201.

Shakya A, Kang J, Chumley J, Williams MA, Tantin D. Oct1 is a switchable, bipotential stabilizer of repressed and inducible transcriptional states. J. Biol. Chem. 2011; 286:450–459.

Singh H, Sen R, Baltimore D, Sharp PA. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature. 1986; 319:154–158.

Singh PP, Voleti B, Agrawal A. A novel RBP-Jκ-dependent switch from C/EBPβ to C/EBPζ at the C/EBP binding site on the C-reactive protein promoter. J. Immunol. 2007; 178:7302–7309.

Ström A-C, Forsberg M, Lillhager P, Westin G. The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. Nucl. Acids Res. 1996; 24:1981–1986.

Sturm RA, Das G, Herr W. The ubiquitous octamer-binding protein Oct-1contains a POU domain with a homeobox subdomain. Genes Dev. 1988; 2:1582–1599.

Toniatti C, Arcone R, Majello B, Ganter U, Arpaia G, Ciliberto G. Regulation of the human C-reactive protein gene, a major marker of inflammation and cancer. Mol. Biol. Med. 1990a; 7:199–212.

Toniatti C, Demartis A, Monaci P, Nicosia A, Ciliberto G. Synergistic trans-activation of the

human C-reactive promoter by transcription factor HNF-1 binding at two distinct sites. EMBO J. 1990b; 9:4467–4475.

Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. Mol. Cell. Biol. 1998; 18:2545–2552.

Voleti B, Agrawal A. Regulation of basal and induced expression of C-reactive protein through an overlapping element for OCT-1 and NF-κB on the proximal promoter. J. Immunol. 2005; 175:3386–3390.

Voleti B, Agrawal A. Statins and nitric oxide reduce C-reactive protein production while inflammatory conditions persist. Mol. Immunol. 2006; 43:891–896.

Voss JW, Wilson L, Rosenfeld MG. POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. Genes Dev. 1991; 5:1309–1320.

Wang Y, Ripperger J, Fey GH, Samols D, Kordula T, Wetzler M, Van Etten RA, Baumann H. Modulation of hepatic acute phase gene expression by epidermal growth factor and Src protein tyrosine kinases in murine and human hepatic cells. Hepatology. 1999; 30:682–697.

Wu GD, Lai EJ, Huang N, Wen X. Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to

overlapping elements within the interleukin-8 promoter: The role of Oct-1 as a transcriptional repressor. J. Biol. Chem. 1997; 272:2396–2403.

Wysocka J, Herr W. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem. Sci. 2003; 28:294–304.

Yoshida T, Yamagishi S, Nakamura K, Matsui T, Imaizumi T, Inoue H, Ueno T, Sata M. Pigment epithelium-derived factor (PEDF) blocks the interleukin-6 signaling to C-reactive protein expression in Hep3B cells by suppressing Rac-1 activation. Life Sci. 2006; 79:1981–1987.

Young DP, Kushner I, Samols D. Binding of C/EBPβ to the C-reactive protein (CRP) promoter in Hep3B cells is associated with transcription of CRP mRNA. J. Immunol. 2008; 181:2420–2427.

Zhang D, Jiang S-L, Rzewnicki D, Samols D, Kushner I. The effect of interleukin-1 on C-reactive protein expression in Hep3B cells is exerted at the transcriptional level. Biochem. J. 1995; 310:143–148.

Zhang D, Sun M, Samols D, Kushner I. STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. J. Biol. Chem. 1996; 271:9503–9509.

Zhou D-X, Yen TSB. The ubiquitous transcription factor Oct-1 and the liver-specific factor

HNF-1 are both required to activate transcription of a hepatitis B virus promoter. Mol. Cell. Biol. 1991; 11:1353–1359.

Zwilling S, Annweiler A, Wirth T. The POU domains of the Oct1 and Oct2 transcription factors mediate specific interaction with TBP. Nucl. Acids Res. 1994; 22:1655–1662.

CHAPTER 3

Exposing a Hidden Functional Site of C-reactive protein by Site-directed Mutagenesis

Sanjay K. Singh[‡, 1], Avinash Thirumalai[‡, 1], David J. Hammond, Jr.[‡, 1], Michael K. Pangburn[§], Vinod K. Mishra[¶], David A. Johnson[‖], Antonio E. Rusiñol[‖], and Alok Agrawal[‡, 2]

[‡]Department of Pharmacology and [‖]Department of Biochemistry and Molecular Biology, Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614,

[§]Department of Biochemistry, Center for Biomedical Research, University of Texas Health Science Center, Tyler, Texas 75708,

[¶] Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

[1]These authors contributed equally to this work.

[2]Address correspondence and reprint requests to: Dr. Alok Agrawal, Department of Biomedical Sciences, P. O. Box 70577, Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
Email address: agrawal@etsu.edu Tel.: +1 423 439 6336; Fax: +1 423 439 8044

Reference: Singh SK, Thirumalai A, Hammond DJ Jr, Pangburn MK, Mishra VK, Johnson DA, Rusiñol AE, Agrawal A. J Biol Chem. 2012 Jan 27;287(5):3550-8.
(doi: 10.1074/jbc.M111.310011.)

Abstract

C-reactive protein (CRP) is a cyclic pentameric protein whose major binding specificity, at physiological pH, is for substances bearing exposed phosphocholine moieties. Another pentameric form of CRP, which exists at acidic pH, displays binding activity for oxidized LDL (ox-LDL). The ox-LDL-binding site in CRP, which is hidden at physiological pH, is exposed by acidic pH-induced structural changes in pentameric CRP. The aim of this study was to expose the hidden ox-LDL-binding site of CRP by site-directed mutagenesis and to generate a CRP mutant that can bind to ox-LDL without the requirement of acidic pH. Mutation of Glu^{42}, an amino acid that participates in inter-subunit interactions in the CRP pentamer and is buried, to Gln resulted in a CRP mutant (E42Q) that showed significant binding activity for ox-LDL at physiological pH. For maximal binding to ox-LDL, E42Q CRP required a pH much less acidic than that required by wild-type CRP. At any given pH, E42Q CRP was more efficient than wild-type CRP in binding to ox-LDL. Like wild-type CRP, E42Q CRP remained pentameric at acidic pH. Also, E42Q CRP was more efficient than wild-type CRP in binding to several other deposited, conformationally altered proteins. The E42Q CRP mutant provides a tool to investigate the functions of CRP in defined animal models of inflammatory diseases including atherosclerosis because wild-type CRP requires acidic pH to bind to deposited, conformationally altered proteins, including ox-LDL, and available animal models may not have sufficient acidosis or other possible modifiers of the pentameric structure of CRP at the sites of inflammation.

Introduction

C-reactive protein (CRP) is a pentameric protein comprised of five identical, non-covalently joined subunits (reviewed in Ref.1). Each CRP subunit binds two calcium ions (2). At physiological pH, CRP binds to molecules and cells bearing exposed phosphocholine (PCh) moieties, such as pneumococcal C-polysaccharide (PnC), in a Ca^{2+}-dependent manner (3, 4). We have shown previously that pH regulates the ligand-binding function of CRP. Acidic pH transforms native pentameric CRP into another pentameric configuration that recognizes immobilized ligands made up of conformationally altered proteins (5,6).

CRP is a plasma protein that is also found in the extracellular matrix at the sites of inflammation, such as atherosclerotic lesions (7–10, reviewed in Ref. 11). Atherosclerosis is an inflammatory disease (12), and generation of an acidic extracellular milieu is a hallmark of inflammatory processes. It has been suggested that at the sites of inflammation, the pH may become acidic because of activated macrophages, hypoxia, lactate generation, and proton generation (13–20, reviewed in Ref. 21). Naghavi and co-workers (20) measured the pH of both human and rabbit atherosclerotic plaques and found the plaques to contain areas in which the pH was decreased to 5.5. The functions of CRP in the development of atherosclerosis are not known.

The binding of CRP to two atherogenic forms of LDL, oxidized LDL (ox-LDL) and enzymatically modified LDL (E-LDL), has been investigated previously (8, 9, 22–26). We found that CRP did not bind to ox-LDL at physiological pH but gained the ability to bind to ox-LDL at acidic pH (6). Similarly, CRP bound to E-LDL at physiological pH, but the binding was dramatically enhanced at acidic pH (5). In addition, using E-LDL, we showed that CRP-bound E-LDL did not cause formation of macrophage foam cells (27). These *in vitro* data suggested that CRP should be able to bind to atherogenic LDL *in vivo* because of acidosis at the sites of

inflammation and prevent foam cell formation.

However, CRP is neither proatherogenic nor atheroprotective in murine and rabbit models of atherosclerosis (28 –35). The reason for the unresponsiveness of human CRP in animal models of atherosclerosis is not clear. Because atherogenic LDL can be efficiently bound by CRP only when CRP is present in its acidic pH-modified structural form and because the animal models of atherosclerosis may lack an inflammatory environment (12, 36), a CRP mutant that can bind to atherogenic LDL at physiological pH is needed to investigate the possible atheroprotective role of CRP using available animal models.

The aim of this study was to generate a CRP mutant that can bind to ox-LDL without the requirement of acidic pH. Because the ox-LDL-binding site in pentameric CRP is formed at acidic pH, our choice of amino acids in CRP for mutagenesis was on the basis of the hypothesis that acidic pH loosens the CRP pentamer to expose amino acids that are otherwise hidden at physiological pH, and it may be possible to mimic the effect of acidic pH on CRP by mutating certain amino acids. Accordingly, we focused on the amino acids participating in the inter-subunit

interactions in the CRP pentamer. The following amino acid pairs have been implicated in inter-subunit interactions (37–39): Val^{10}-Ile^{104}, that is, Val^{10} of one subunit interacts with Ile^{104} of adjacent subunit; Pro^{12}-Ser^{118}; Tyr^{40}-Pro^{115}; Tyr^{40}-Val^{117}; Glu^{42}-Glu^{85}; Ser^{46}-Glu^{85}; Glu^{42}-Lys^{119}; Ser^{44}-Lys^{69}; Val^{90}-Pro^{87}; Gly^{101}-Lys^{201}; Ser^{120}-Tyr^{197}; Pro^{115}-Trp^{205}; and Arg^{118}-Asp^{155}. In this study, we mutated two amino acids,Glu^{42}(to Gln) and Pro^{115}(to Ala), from the inter-subunit contact region, and one amino acid, Tyr^{175}(to Ala), which is not a part of the inter-subunit contact region (Fig. 3.1), and evaluated the effect of these mutations on the binding of CRP to several immobilized proteins, including ox-LDL.

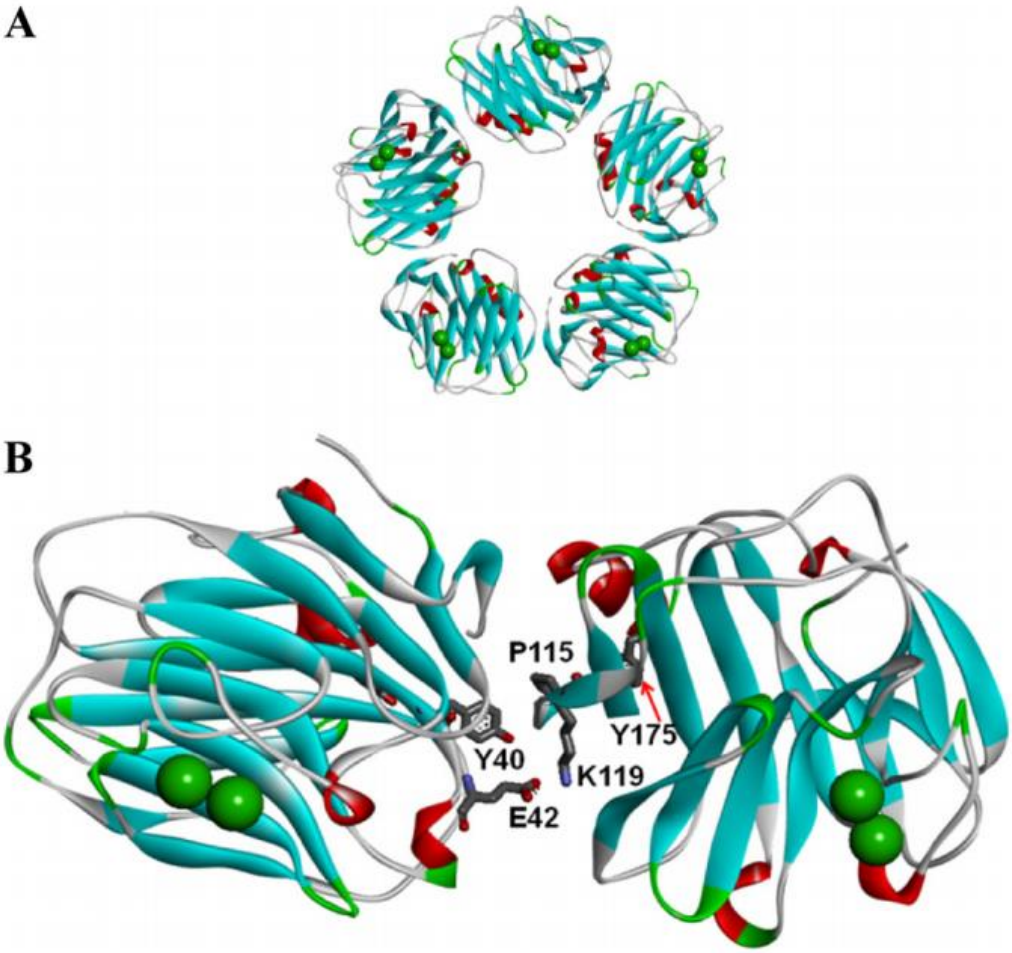

Figure 3.1. The structure of CRP. *A*, Discovery Studio Visualizer 3.0 software (Accelrys Software, Inc.) was used to generate a ribbon diagram of the crystal structure of pentameric CRP obtained from RCSB Protein Data Bank (PDB ID 1B09). *B*, two of the five subunits are shown. The side chains of Tyr^{40}, Glu^{42},Pro^{115}, Lys^{119}, and Tyr^{175}, relevant for this work, are highlighted. Calcium ions are shown as *green balls*.

Experimental Procedures

Construction and expression of CRP mutants

The construction of the CRP mutants E42Q (substitution of Glu^{42} with Gln), P115A substitution of Pro^{115} with Ala), and Y175A (substitution of Tyr^{175} with Ala) has been reported previously (6, 40, 41). All three mutants were stably expressed in CHO cells as described previously (41). CHO cell lines expressing each CRP mutant were isolated by a series of subcloning steps.

Purification of CRP

Native WT CRP was purified from discarded human pleural fluid as described previously (6) and stored frozen. Purification of CRP mutants from the CHO cell culture supernatants involved two steps: a Ca^{2+}-dependent affinity chromatography on a PCh-Sepharose column (Pierce) followed by gel filtration on a Superose12 column (GE Healthcare) as described previously (6) and stored frozen. On the day of the experiments, CRP was repurified by gel filtration on a Superose12 column to remove any form of modified CRP that might have been generated because of storage of CRP. Repurified CRP was stored in 10 mM TBS (pH 7.2), containing 2 mM $CaCl_2$ at 4 °C, and was used within a week.

Preparation of Ox-LDL

Native LDL was isolated from human plasma by sequential ultracentrifugation (1.019<d>1.063 g/ml), as described previously (42). Ox-LDL was prepared by treating LDL with 20μM $CuCl_2$ in PBS for 12 h at 37 °C (43). The Cu^{2+}-mediated oxidation was terminated by adding EDTA to the reaction at a final concentration of 0.5 mM. Following dialysis against PBS,

ox-LDL was passed through chelex-100 resin (Bio-Rad) to remove any traces of Cu^{2+}, filter sterilized, and stored in the dark at 4 °C. The degree of oxidation of LDL was evaluated by agarose gel electrophoresis. Ox-LDL had 3.2- to 3.5-fold higher R_F values than native LDL. The degree of oxidation was also determined by TBARS assay (Cayman Chemical Co.), a colorimetric assay using malondialdehyde as a standard. Ox-LDL used in this study resulted in 30.5 nmoles malondialdehyde/mg of protein. Protein concentrations of the ox-LDL preparations were measured using the microBCA protein assay kit (Pierce).

Ox-LDL-binding assay

Microtiter wells were coated with 10 µg/ml ox-LDL diluted in TBS (350 µl/well) overnight at 4 °C. Purified CRP (WT and mutants), diluted in TBS (pH 7.2), containing 0.1% gelatin, 0.02% Tween 20, and 2 mM $CaCl_2$ (TBS-Ca), was added in duplicate wells (100 µl/well) and incubated for 2 h at37°C, unless otherwise mentioned in the figure legends. After the CRP incubation step, the wells were washed with TBS-Ca. Rabbit anti-CRP antibody (Sigma), diluted 1/1000 in TBS-Ca, was used (100 µl/well, 1 h at37°C) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (GE Healthcare), diluted in TBS-Ca, was used (100 µl/well, 1 h at 37 °C) as the secondary antibody. Color was developed, and the absorbance was read at 405 nm in a microtiter plate reader (Molecular Devices).

PnC-binding assay

Binding activity of CRP for PCh was evaluated by using PnC (Statens Serum Institut) as the ligand. Microtiter wells were coated (350 µl/well) with 10 µg/ml PnC in TBS overnight at 4 °C. CRP diluted in TBS-Ca (various pH, various Ca^{2+} concentrations) was added (100 µl/well) in

duplicate wells. After incubating the plates for 2 h at 37°C, the wells were washed with appropriate TBS-Ca. The anti-CRP monoclonal antibody HD2.4 (0.5 µg/ml), diluted in TBS-Ca, was used (100 µl/well, 1 h at 37°C) to detect bound CRP. HRP-conjugated goat anti-mouse IgG, diluted in TBS-Ca, were used (100 µl/well, 1 h at 37°C) as the secondary antibody. Color was developed, and the absorbance was read at 405 nm in a microtiter plate reader.

Protein ligand-binding assay

This assay was used to determine the binding of CRP to proteins other than ox-LDL and was performed as described for the ox-LDL-binding assay, except that the microtiter wells were coated with 10 µg/ml complement factor H (purified from normal human plasma) (44), amyloid β (fragment 1–38, catalog no. A0189, Sigma-Aldrich), BSA (catalog no. A0281, Sigma-Aldrich), or gelatin (catalog no. G9382, Sigma-Aldrich) diluted in TBS (350 µl/well) overnight at 4°C.

Molecular modeling of CRP

Molecular modeling was on the basis of the x-ray crystal structure of the WT CRP-PCh complex (39). The PDB file 1BO9.pdb was imported into SwissPdbViewer (also known as DeepView) (45) and used to substitute Glu^{42} with Gln. The *in silico* mutated structure was saved as a PDB file and opened in the free version of Discovery Studio Visualizer 3.0 (Accelrys) for measuring distances and for creating figures. On-screen images were captured with Snagit 10 (TechSmith) and saved as portable network graphic files. Salt bridges were analyzed by inputting the atomic coordinates for the α and β subunits from each PDB file using web-based software (46, 47).

CD spectroscopy

The CD spectra of CRP (100 µg/ml) were recorded at 25°C using a J-815 CD spectrometer equipped with a Peltier-type temperature control system (JASCO model PTC-423S/15) and interfaced to a personal computer. The instrument was calibrated with (1S)-(+)-10-camphorsulfonic acid. The CD spectra were measured from 200 nm to 250 nm every 0.5 nm with 4-s averaging per point and a 2-nm bandwidth. A 0.1-cm path length cell was used for obtaining the spectra. The CD spectra were signal-averaged by adding four scans and base line-corrected.

Gel filtration

Gel filtration analysis of CRP at pH 5.6 was carried out on a Superose12 column. The column was equilibrated and eluted with TBS (pH 5.6) containing 2 mM$CaCl_2$ at a flow rate of 0.3 ml/min. Fractions (60 fractions, 0.25 ml each) were collected, and absorbance at 280 nm was measured to locate the elution volume of CRP from the column.

1-Anilinonaphthalene-8-sulfonic acid (ANS)-binding fluorescence assay

The hydrophobic fluorescent probe ANS was purchased from AnaSpec, Inc. The ANS-binding fluorescence assays were performed as described previously (6) to investigate the structural changes in CRP at pH 5.6. CRP (50 µg/ml) in TBS containing 2 mM $CaCl_2$, at various pH levels, was mixed with ANS at a final concentration of 100 µM. The fluorescence intensity of the binding of ANS to CRP was measured by using the excitation and emission wavelengths of 390 nm and 460 nm respectively, in a spectrofluorometer (Fluostar Galaxy, BMG Lab Technologies).

Results

E42Q CRP binds to ox-LDL more efficiently than WT CRP

The construction, expression, purification and some characterization of E42Q, P115A, and Y175A CRP mutants have been reported previously, and their overall structure was found not to be different from WT native and WT recombinant CRP (6, 40, 41). The elution profile of each CRP mutant from the gel filtration column was identical to that of WT CRP, suggesting that all CRP mutants were pentameric (Fig. 3.2A). The purity of CRP preparations was confirmed by denaturing SDS-PAGE (Fig. 3.2B). The PCh-binding activity of all CRP mutants was also identical to that of WT CRP (Fig. 3.2, C and D).

The binding of CRP mutants to ox-LDL was first determined at pH 7.0, a pH at which WT CRP does not bind to ox-LDL (6). To show maximum possible binding of CRP to ox-LDL that can be measured by this assay, pH 5.0 was used, a pH at which WT CRP binds efficiently to ox-LDL (6). At pH 7.0, the binding of E42Q CRP to ox-LDL was significantly higher than the negligible binding of either WT or P115A and Y175A CRP mutants to ox-LDL (Fig. 3.3A). The minimal binding of WT and mutants P115A and Y175A to ox-LDL seen at pH 7.0 may be due to the exposure of a few PCh groups in some ox-LDL molecules (22). As expected, at pH 5.0, the binding of the CRP mutants to ox-LDL was not different from that of WT CRP. These data indicated that E42Q CRP gained the ability to bind to ox-LDL at physiological pH. However, further structural change was required for maximal binding. Therefore, the binding of each CRP species to ox-LDL was evaluated from pH 7.0 down to pH 5.0, where maximal binding for all species occurred (Fig. 3.3B). The binding of mutants P115A and Y175A to ox-LDL, as a function of pH, was not different from that of WT CRP. However, the binding of E42Q CRP to ox-LDL was enhanced between pH 6.5 and 5.5, as demonstrated by the shift in the binding curve

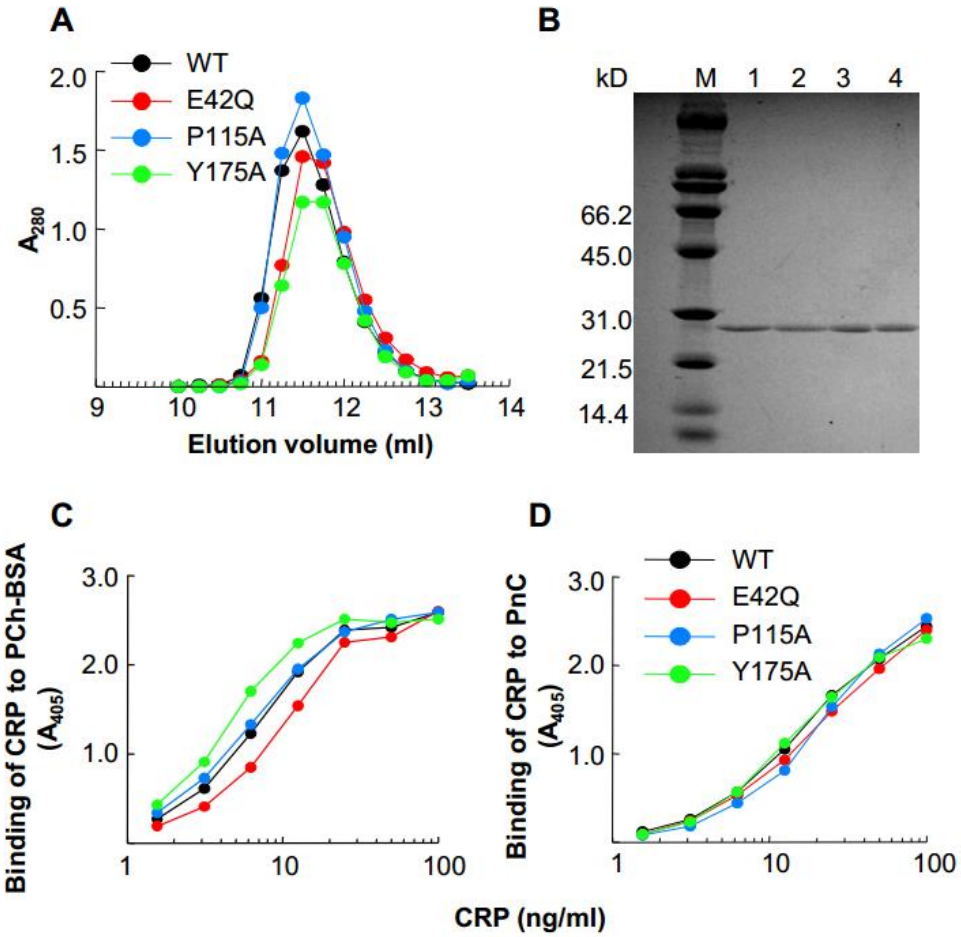

Figure 3.2. E42Q, P115A and Y175A mutant CRPs retain their pentameric structure and phosphocholine binding ability *A*, Elution profiles of WT and mutant CRP from the Superose12 gel filtration column. CRP in TBS, pH 7.2, containing 2 mM $CaCl_2$ (TBS-Ca) was applied to the column equilibrated with TBS-Ca, as follows: WT CRP (1.6 mg), E42Q CRP (1.0 mg), P115A CRP (1.7 mg) and Y175A CRP (0.9 mg). CRP was eluted with TBS-Ca at the flow rate of 0.3 ml/min. Sixty fractions (0.25 ml) were collected and protein measured to locate the elution volume of CRP from the column. *B*, Denaturing SDS-PAGE (4%-20% gel) of CRP. 5 µg of each CRP was applied to the gel; the gel was stained with Coomassie brilliant blue. M, BioRad's broad range molecular weight marker; Lane 1, WT CRP; Lane 2, E42Q CRP; Lane 3, P115A CRP, and Lane 4, Y175A CRP. *C*, Binding of WT and mutant CRP to PCh-substituted BSA (PCh-BSA). *D*, Binding of WT and mutant CRP to PnC. Binding activity of CRP for PCh-containing ligands was evaluated by two assays using PCh-BSA and PnC in the solid phase. Wells were coated with either PCh-BSA or PnC at 10 µg/ml in TBS (350 µl/well), overnight at 4 ºC. CRP diluted in TBS-Ca was added (100 µl/well) in duplicate wells. After incubating the plates for 2 h at 37 ºC, the wells were washed with TBS-Ca. The anti-CRP mAb HD2.4 (0.5 µg/ml), diluted in TBS-Ca, was used (100 µl/well; 1 h at 37 ºC) to detect bound CRP. HRP-conjugated goat anti-mouse IgG, diluted in TBS-Ca, were used (100 µl/well; 1 h at 37 ºC) as the secondary antibody. Color was developed and the absorbance was read at 405 nm in a microtiter plate reader.

of the E42Q mutant relative to the other forms. Consequently, pH 5.6 was chosen for performing a CRP dose response assay to compare the efficiency of binding of various CRP species to ox-LDL (Fig. 3.3C). The resulting curves indicate that E42Q CRP was ~4-fold more efficient than WT CRP in binding to ox-LDL. These data indicate that E42Q CRP is more potent than WT CRP. E42Q CRP binds to ox-LDL at physiological pH (Fig. 3.3A), E42Q CRP requires less acidic pH for maximal binding to ox-LDL (Fig. 3.3B), and more E42Q CRP binds to ox-LDL at any pH (Fig. 3.3C).

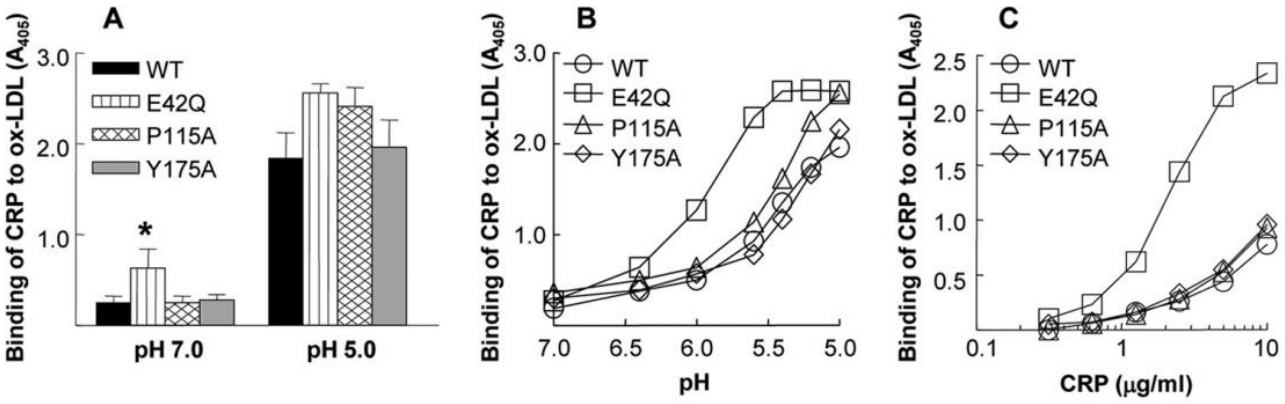

Figure 3.3. Binding of CRP to ox-LDL.*A,* CRP (10 μg/ml), diluted in TBS-Ca (pH 7.0 and 5.0) containing 2 mM$CaCl_2$, was added to ox-LDL-coated wells. Bound CRP was detected using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm. Results are shown as mean±S.D. of five assays. *,$p<0.005$ between WT and E42Q CRP.*B*, CRP (10 μg/ml), diluted in TBS-Ca (pH 7.0 to 5.0), containing 2 mM $CaCl_2$, was added to ox-LDL-coated wells. Bound CRP was detected as in A. *C*, Increasing concentrations of CRP, diluted in TBS-Ca (pH 5.6) containing 2 mM $CaCl_2$, was added to ox-LDL-coated wells. Bound CRP was detected as in A. A representative of four experiments is shown for B and C.

E42Q CRP Is neither monomerized nor aggregated at any pH

Gel filtration, ox-LDL-binding reversibility assays, ANS binding fluorescence assays, and CD spectra were used to investigate the possibility that the mutation and acidic pH might have caused monomerization and/or aggregation of CRP and that the observed binding of CRP to

ox-LDL might be due to monomerized and aggregated forms of CRP.

Gel filtration was used to determine whether E42Q CRP remained pentameric at pH 5.6 after incubation for 2 h at 37°C, the conditions used in the ox-LDL-binding assays. The elution profiles of E42Q CRP at pH 7.2 and 5.6 were identical (Fig. 3.4A). These data indicated that E42Q CRP remained pentameric at pH 5.6. It was neither monomerized nor aggregated.

Next, the effect of pH neutralization on the ox-LDL-binding activity of CRP was evaluated (Fig. 3.4B). Only E42Q CRP showed significant binding to ox-LDL at pH 7.0. At pH 5.6, each CRP species efficiently bound to ox-LDL. When a CRP solution, which had been incubated at pH 5.6 for 2 h at 37°C, was neutralized and the binding assay was performed at pH 7.0, no CRP bound to ox-LDL. Because it is known that monomerized CRP binds to ox-LDL at pH 7.0 (25) and that monomers of CRP cannot form pentamers *in vitro*, these data suggested that CRP was not monomerized at acidic pH and that any structural change in CRP at pH 5.6 was reversible at pH 7.0.

ANS-binding fluorescence assays were used to evaluate hydrophobic changes in the structure of CRP at pH 5.6 (Fig.3.4C). Incubation of ANS with all forms for CRP at pH 7.0 resulted in a negligible increase in fluorescence. In contrast, ANS binding to CRP at pH 5.6 resulted in significantly increased fluorescence compared with that at pH 7.0. Neutralization of the pH 5.6 CRP solutions, which had been incubated for 2 h at 37°C to pH 7.0, followed by the addition of ANS resulted in no increase in fluorescence consistent with no binding of ANS. These data suggested that the hydrophobicity of CRP was increased at acidic pH but that the change was reversible. The reversibility of the structural changes in the various forms of CRP indicated that they were neither monomeric nor aggregated at acidic pH.

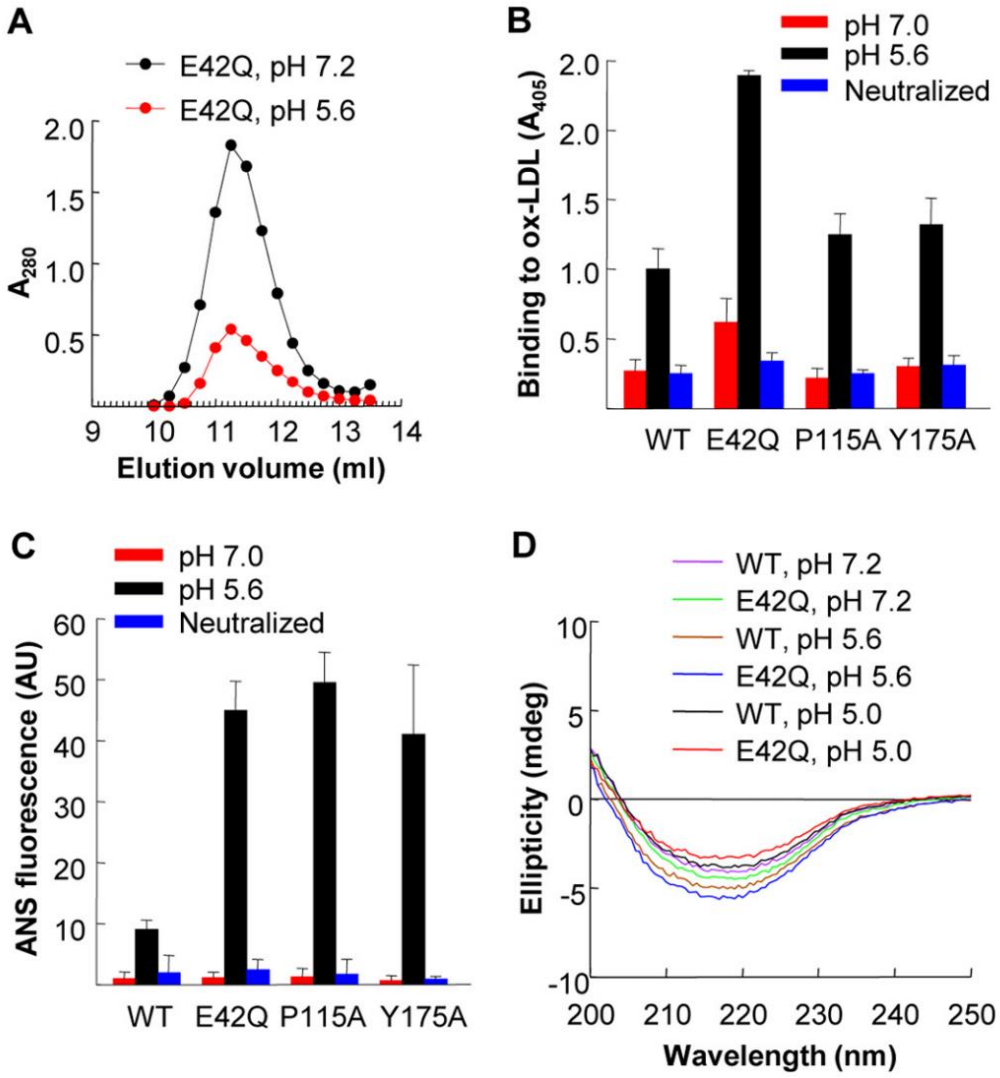

Figure 3.4. CRP is neither monomerized nor aggregated at acidic pH. *A*, Elution profiles of E42Q CRP from the Superose12 gel filtration column. CRP (250 µl of 5.6 mg/ml) in TBS (pH 7.2) containing 2 mM $CaCl_2$ was applied to the column equilibrated with the same buffer (black). CRP (250 µl of 2.2 mg/ml), after incubation for 2 h at 37°C in TBS (pH5.6) containing 2 mM $CaCl_2$ was applied to the column equilibrated with the same buffer (red). CRP was eluted with the respective buffers. A representative of two experiments is shown. *B*, Reversibility of the ox-LDL-binding activity of CRP. CRP at pH 5.6 was first incubated for 2 h at 37°C.After removing an aliquot, the pH was neutralized. These CRP (10 µg/ml) samples were then added to ox-LDL-coated microtiter wells. Bound CRP was detected as in Fig. 3.3. Red bars, CRP in TBS-Ca (pH 7.2); black bars, CRP in TBS-Ca (pH 5.6), and incubated at 37 °C for 2 h before adding to the wells; blue bars, as in black, except that the pH was neutralized before adding CRP to the wells. Results are shown as mean±S.D. of five independent assays. *C*, ANS-binding fluorescence of CRP samples used in B. Red bars, CRP at pH 7.2; black bars, CRP at pH 5.6 pre-incubated at 37 °C for 2 h; blue bars, after incubating CRP at pH 5.6 at 37 °C for 2 h, the pH was neutralized. Results are shown as mean±S.D. of five experiments. *D*, Far UV CD spectra of WT and E42Q CRP in TBS-Ca at various pH are shown.

Finally, we used CD spectroscopy to determine global changes in the secondary structure of CRP because of mutation of Glu42 and acidic pH (Fig. 3.4D). The comparison of the far UV CD spectra of WT and E42Q CRP at pH 5.0, 5.6, and 7.2 showed minimal changes in the secondary structures of both CRP because of acidic pH, and the changes in the secondary structure of E42Q was not different from the changes in WT CRP. These data indicated that the overall secondary structure of WT CRP was maintained in E42Q CRP at any pH.

Combined data suggested that, at acidic pH, CRP was not monomerized, CRP was not completely denatured, CRP was not aggregated, the pentameric structure of CRP was modified although there were no global changes in the secondary structure, and the modifications were reversible.

Acidic pH causes localized structural changes in CRP

Because E42Q CRP required a buffer less acidic than that required by WT CRP for comparable binding to ox-LDL and because there were no global changes at acidic pH in any CRP, we hypothesized that the structural changes in CRP in response to acidic pH and mutations were only subtle. Localized conformational changes in CRP were investigated by determining the effects of acidic pH on the Ca^{2+}-binding site of CRP by measuring the Ca^{2+} requirement of CRP to bind to one of its Ca^{2+}-dependent ligands, PnC. A change in the Ca^{2+} requirement for comparable PnC-binding activity at different pH would reflect a change in the Ca^{2+} affinity of CRP. At pH 7.2, WT CRP bound efficiently, even at 0.06 mM Ca^{2+}, to PnC (Fig. 3.5A). However, at pH 5.0, 2 mM Ca^{2+} was required for efficient binding of CRP to PnC. The increase in the Ca^{2+} requirement was directly related to the decrease in pH. These results suggested that acidic pH modified the Ca^{2+}-binding site of CRP and reduced the Ca^{2+} affinity of CRP and that 2

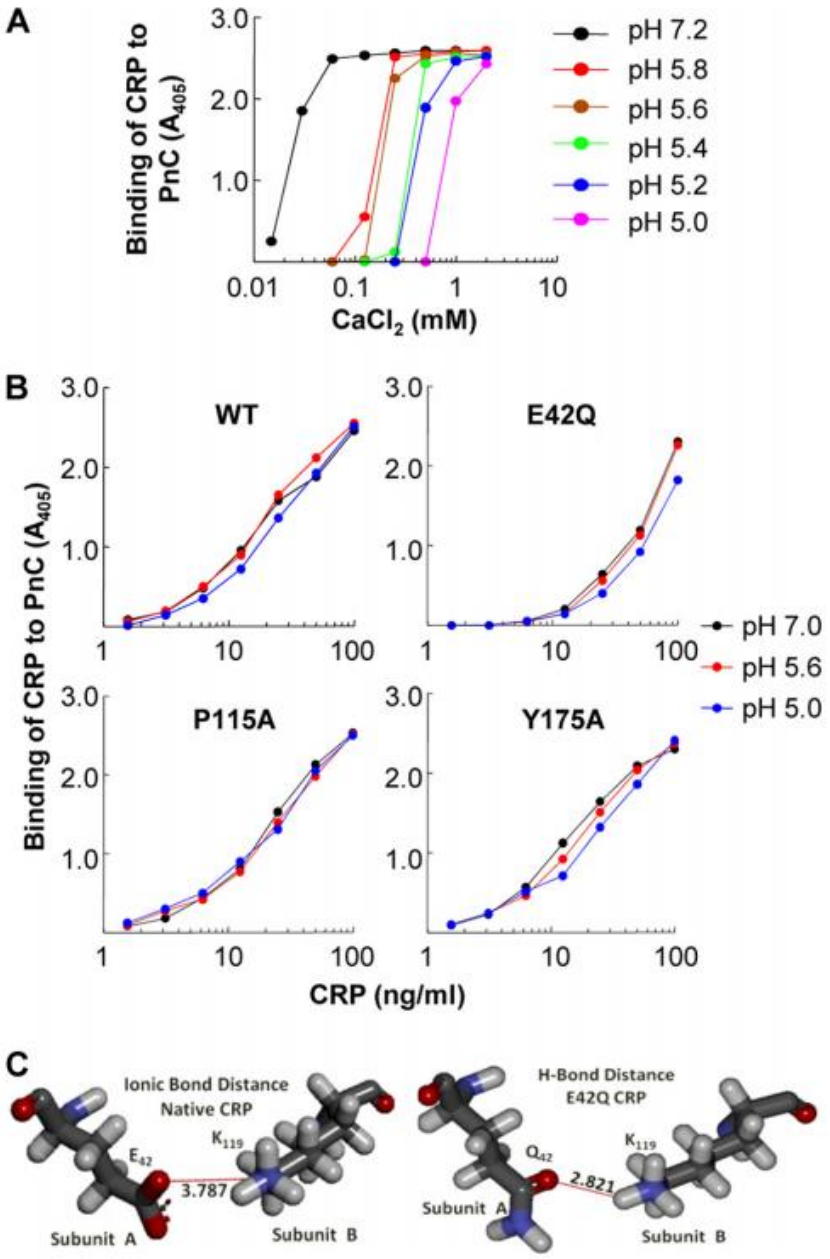

Figure 3.5. Determination of local structural changes in CRP. *A*, PnC-binding activity of WT CRP as a function of pH and Ca^{2+} concentration. WT CRP (50 ng/ml), diluted in TBS-Ca (various pH, various concentrations of Ca^{2+}) was added to PnC-coated wells. A representative of three experiments is shown. *B*, PnC-binding activity of CRP mutants as a function of pH and Ca^{2+} concentration. Increasing concentrations of CRP diluted in TBS-Ca (various pH) containing 2 mM $CaCl_2$ was added to PnC-coated wells. In both A and B, bound CRP was detected by using a mouse anti-CRP antibody and HRP-conjugated goat anti-mouse IgG. The absorbance of the color was read at 405 nm. A representative of three experiments is shown. *C*, molecular modeling of CRP on the basis of the x-ray crystal structure of the WT CRP-PCh complex (1B09.pdb) is shown.

mM Ca^{2+} should be used to perform ligand-binding assays at acidic pH. As shown in Fig. 3.5B, all CRP species were similar in binding to PnC at any pH in 2 mM Ca^{2+}, suggesting that the mutation of CRP did not reduce the Ca^{2+} affinity of CRP.

Next, we evaluated the structural change in CRP caused by the substitution of Glu^{42} to Gln by using molecular modeling (Fig. 3.5C). In WT CRP, there is an ionic bond between Glu^{42} and Lys^{119} at a distance of 3.787 Å. In E42Q CRP, this ionic bond is lost. Instead, there is the possibility of a weak H-bond between Gln^{42} and Lys^{119}. The shortest distance between the H on the Lys^{119} ϵ-amino group and the O on the side chain of Gln^{42} is 2.821 Å, whereas H-bonds are normally about 2.0 Å. The modeling of WT and E42Q CRP further suggested that there might be a localized structural change in CRP caused by the substitution of Glu^{42} to Gln.

Reexamination of our previously reported findings

Recently we reported that, at pH 4.6 and in 0.1 mM Ca^{2+}, CRP did not bind to PnC, that a temperature of 37 °C was required for the binding of CRP to ox-LDL, and that the P115A CRP mutant was more efficient than WT CRP in binding to ox-LDL (6). Our current finding (Fig. 3.5A) that the affinity of CRP for Ca^{2+} was drastically reduced at pH 5.0 prompted us to reexamine our previously reported findings.

As shown in Fig. 3.6, at pH 4.6, CRP did not bind to PnC, even in 2mM Ca^{2+}. Just for this reason we did not include pH 4.6 in this investigation. As shown in Fig. 3.7, at acidic pH and in 2 mM Ca^{2+}, 37 °C was not necessary for the binding of E42Q CRP to ox-LDL, suggesting that the efficiency of binding of CRP to ox-LDL depends upon all three factors: pH, temperature, and the extent of the structural change in CRP. As shown in Fig. 3.8, P115A CRP bound to ox-LDL more efficiently than WT CRP in 0.1 mM Ca2+ but not in 2 mM Ca2+, whereas E42Q

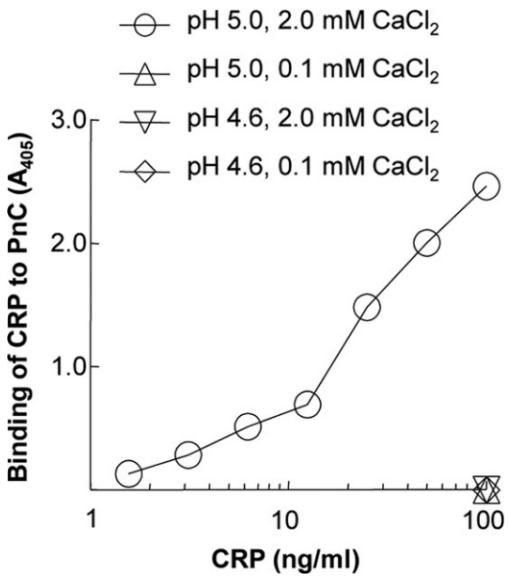

Figure 3.6. Acidic pH reduces the affinity of CRP for Ca^{2+}. As in Fig. 3.5A, PnC-binding activity of WT CRP as a function of pH and Ca^{2+} concentration is shown. CRP diluted in TBS-Ca (pH 5.0 and 4.6) containing 0.1 mM or 2 mM $CaCl_2$ was added to PnC-coated wells. After 2 h at 37°C, the wells were washed once with respective TBS-Ca and then with TBS-Ca (pH 7.2) containing 2 mM $CaCl_2$. Bound CRP was detected using a mouse anti-CRP antibody and HRP-conjugated goat anti-mouse IgG. The absorbance of the color was read at 405 nm. A representative of three experiments is shown.

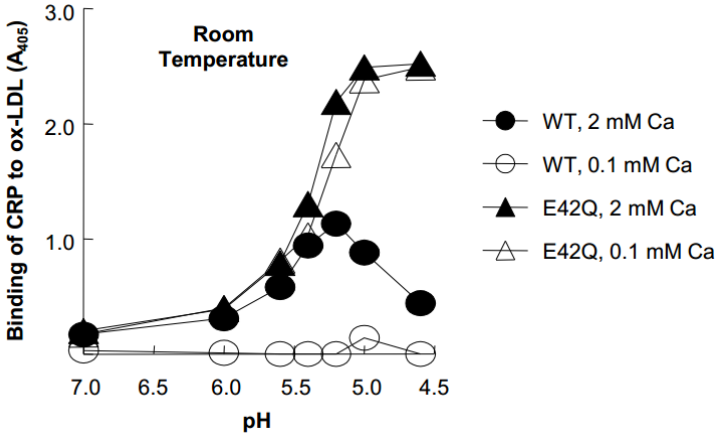

Figure 3.7. Effect of temperature on the binding of WT and mutant CRP to ox-LDL. Microtiter wells were coated with 10 µg/ml of ox-LDL diluted in TBS (350 µl/well). CRP (10 µg/ml), diluted in TBS-Ca (2 mM or 0.1 mM $CaCl_2$), pH 7.0 to 4.6, was added to the wells and incubated at room temperature (23 ºC) for 2 h. Bound CRP was detected by using a rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the developed color was read at 405 nm. A representative of two experiments is shown.

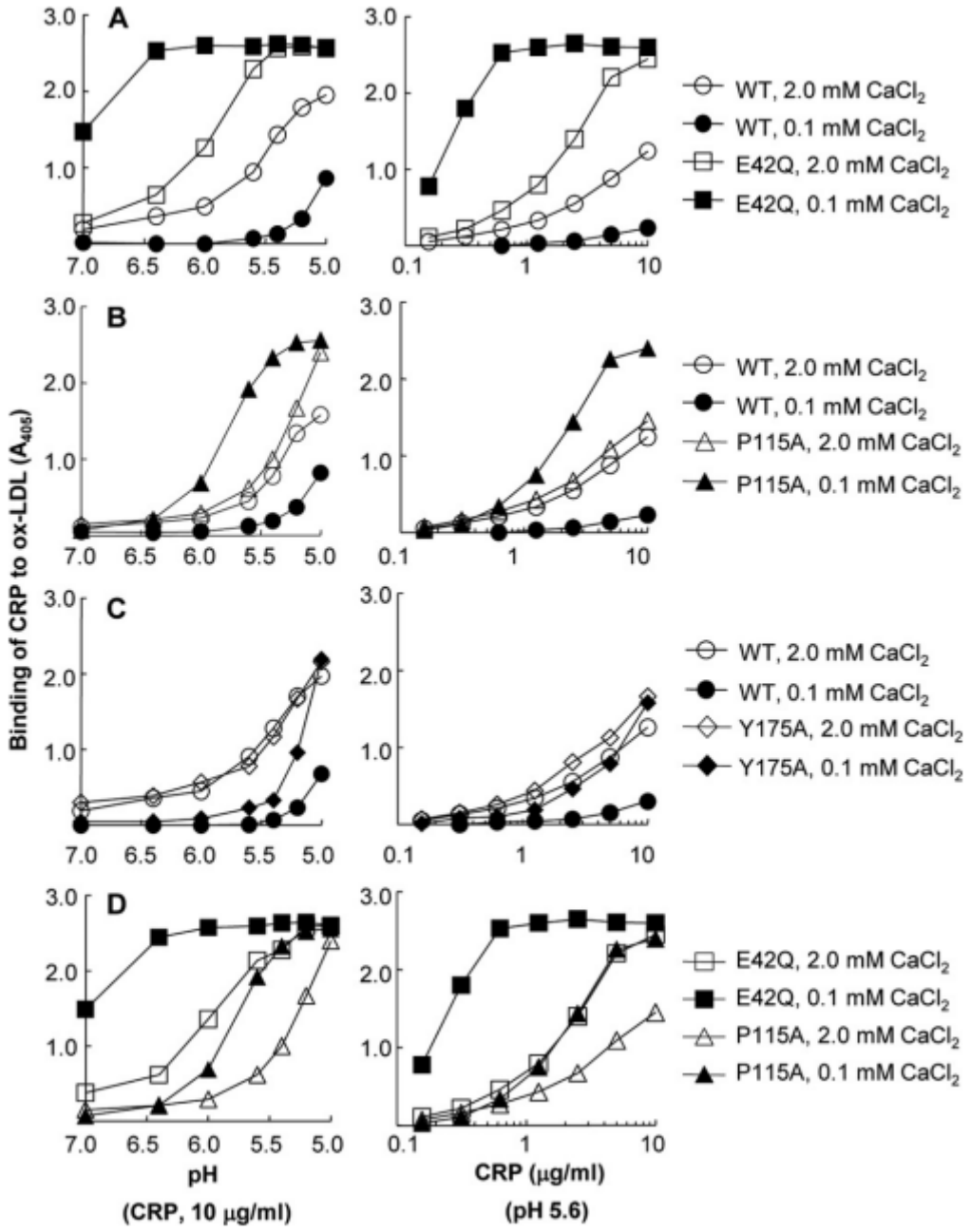

Figure 3.8. Effect of Ca^{2+} on the binding of WT and mutant CRP to ox-LDL. Left panel, CRP (10 μg/ml), diluted in TBS-Ca (2 mM or 0.1 mM $CaCl_2$) (pH 7.0 to 5.0) was added to ox-LDL-coated wells. Right panel, increasing concentrations of CRP, diluted in TBS-Ca (2 mM or 0.1 mM $CaCl_2$) (pH 5.6) was added to ox-LDL-coated wells. Bound CRP was detected by using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm. *A*, comparison of WT and E42Q CRP.*B*, comparison of WT and P115A CRP.*C*, comparison of WT and Y175A CRP.*D*, comparison of E42Q and P115A CRP. A representative of three experiments is shown.

CRP bound to ox-LDL more efficiently than WT CRP at 2 mM Ca2+ also. Thus, a decrease in the concentration of Ca2+ is not necessary for E42Q CRP to bind to ox-LDL.

E42Q CRP has higher avidity than WT CRP for binding to a variety of other immobilized proteins

We reported previously that the binding of CRP to ox-LDL at acidic pH was not due to its specificity for binding to ox-LDL but due to its specificity for binding to immobilized, modified, and conformationally altered proteins, irrespective of the identity of the protein (5, 6). Therefore, we investigated the binding activity of E42Q CRP at acidic pH for a few immobilized proteins other than ox-LDL. Binding curves of WT CRP and E42Q CRP to the immobilized proteins, factor H, amyloid β, BSA, and gelatin, over a pH range from 7.0 down to 5.0, are shown in Fig. 3.9. In every case, more E42Q CRP was bound to the tested proteins, and this binding required less acidic pH compared with that required by WT CRP. Interestingly, E42Q CRP also bound to gelatin at acidic pH. However, the presence of 0.1% gelatin in the binding buffer used in the assays did not inhibit the binding of E42Q CRP to immobilized gelatin (data not shown). These results indicated that the substitution of Glu^{42} to Gln exposed a hidden ligand-binding site in CRP for deposited and conformationally altered proteins present in an acidic pH environment. These findings also suggested that the protein component of ox-LDL was the CRP ligand.

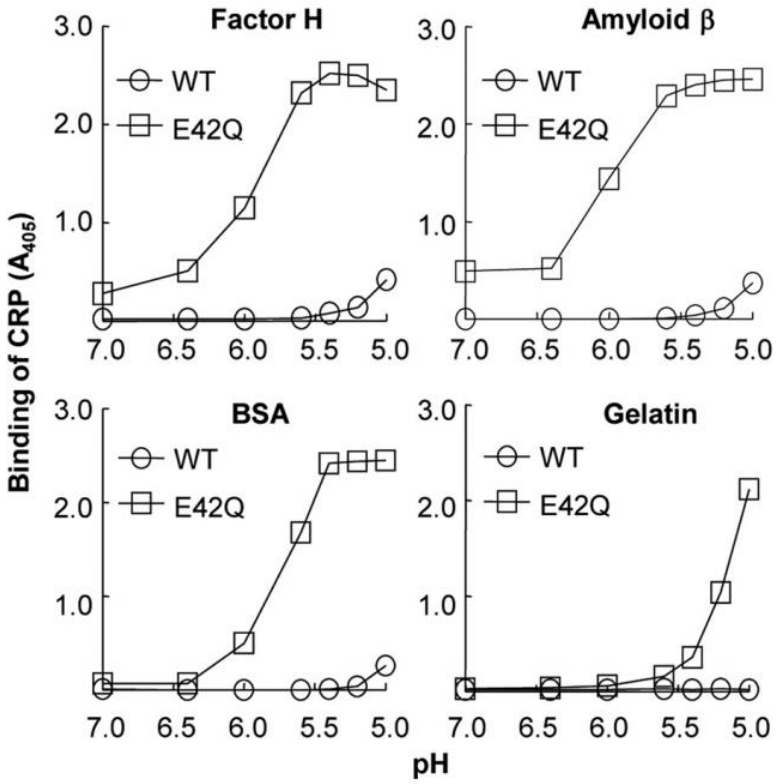

Figure 3.9. Binding of CRP to immobilized proteins at acidic pH. Results of a protein ligand-binding assay are shown. Microtiter wells were coated with complement factor H, amyloidβ, BSA, and gelatin. CRP (10 µg/ml), diluted in TBS-Ca (pH 7.0 to 5.0), was then added to the wells. Bound CRP was detected by using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the developed color was read at 405 nm. A representative of three experiments is shown

Discussion

The goal of this investigation was to use site-directed mutagenesis to generate a CRP mutant capable of binding to ox-LDL without the requirement of acidic pH. Our major findings were: 1) Mutation of Glu^{42}, an amino acid that participates in inter-subunit interactions in the CRP pentamer, to Gln resulted in a CRP mutant (E42Q) that showed significant binding to ox-LDL at physiological pH. For maximal binding to ox-LDL, the E42Q CRP required a pH less acidic than that required by WT CRP. Also, at any given pH, the binding of E42Q CRP to ox-LDL was more efficient than the binding of WT CRP to ox-LDL. 2) The acidic pH did not measurably change the secondary structures of WT or E42Q CRP and did not monomerize or aggregate CRP. However, the acidic pH changed the hydrophobicity of CRP and reduced affinity for Ca^{2+}. These acidic pH-induced changes were reversible at physiological pH. 3) E42Q CRP had a higher avidity than WT CRP for binding to not only ox-LDL but also to other immobilized, and therefore conformationally altered, proteins when both CRP and immobilized proteins were exposed to acidic pH.

Our data suggest that CRP undergoes a pH-dependent reversible transition between two conformational forms without a significant disruption of its secondary structure, providing new insight into the functions of CRP in inflammatory diseases. The data also suggest that the binding of CRP to ox-LDL at acidic pH is due to the acidic pH-dependent changes in CRP, perhaps because of the loosening of the CRP pentamer. In CRP, the side chain of Glu^{42} in one subunit ionically interacts with the main chain of Glu^{85} (the distance between the Glu^{42} and the α amino of Glu^{85} is 6.242 Å away, which is beyond a salt bridge distance of usually only 4 Å) and with the side chain of Lys^{119} of the adjacent subunit. The increased binding of E42Q CRP at pH values closer to physiological conditions are consistent with the loss of an ionic interaction

between Glu^{42} and Lys^{119} and suggestive of a loosening of the pentameric structure. Partial exposure of the hidden ox-LDL-binding site of CRP by mutagenesis raises the possibility that conditions other than acidic pH may also be able to switch the structure of CRP to a more active configuration.

Acidic pH is not the only characteristic of inflammatory sites. Free radicals and other oxidants may also be present at the sites of inflammation in arteries and may oxidize CRP in addition to oxidizing LDL to facilitate the binding of CRP to ox-LDL. Although acidic pH modifies the structure of CRP (6, 48) and is just one possible *in vivo* modifier of the structure of CRP, other modifiers of the structure of CRP have also been reported (49,50). CRP modified with active oxygen species has been shown to modulate the stimulus-dependent activation of platelets (49). A redox switch in CRP involving the reduction of its single disulfide bond has been shown to modulate the activation of endothelial cells by CRP (50). Also, CRP is not the only host defense protein that is activated by a conformational change. Other host defense proteins of the immune system, for example, β-defensin 1 and NPR1, have been shown recently to be activated by conformational changes in response to redox conditions (51, 52) and nitrosylation (52). We are currently investigating the effects of several other inflammation-related protein modifiers, including redox conditions and nitrosylation, on the binding of CRP to deposited, conformationally altered, and amyloidogenic proteins.

In animal models of atherosclerosis, WT CRP is neither atheroprotective nor proatherosclerotic (28 –35). We hypothesize that the lack of an effect of CRP in animal models is due to the absence of an inflammatory environment that is needed for appropriately altering the structure of CRP so it can capture atherogenic LDL. Indeed, atherosclerosis is not naturally developed in these animal models, and in humans it is developed over a period of several years

or decades (12, 36). The E42Q CRP might be useful in investigating the functions of CRP in defined animal models of atherosclerosis. E42Q CRP may provide a better molecule for testing the hypothesis that CRP requires a structural change to bind to ox-LDL *in vivo*. Our finding that the binding of E42Q CRP to ox-LDL was dramatically increased at physiological pH when the concentration of Ca^{2+} was 20-fold lower than normal (0.1 mM compared with 2 mM) is also significant because it suggests that E42Q CRP will remain active even at low Ca^{2+} concentrations at the sites of inflammation. Therefore, we propose that E42Q CRP will bind to atherogenic LDL *in vivo* even if the acidic pH component of inflammation is missing or mild in the arterial walls of the animal models of atherosclerosis to prevent foam cell formation and thus reduce the development of atherosclerosis.

The reversibility of the structural changes in the CRP pentamer and of its ligand-binding activities at physiological pH indicates that the functions of CRP in circulation are different from those at the localized sites of inflammation, where both CRP and the ligands of CRP are exposed to an inflammatory environment. Interestingly, E42Q CRP efficiently bound not only to ox-LDL but to a variety of immobilized proteins, which includes proteins that might be deposited or bound to structures in the body. For example, deposition of factor H has been implicated in age-related macular degeneration and pneumococcal infection (53–58). It is of interest that chaperone proteins, the family of proteins recognizing misfolded proteins, are also, like CRP, ancient proteins (59). Overall, our findings suggest that E42Q CRP may serve as a tool to investigate the functions of CRP in each and every inflammatory disease involving deposition of proteins, such as autoimmune diseases, and in which CRP has been implicated (60 –63). Investigations using E42Q CRP in animal models of inflammatory diseases may also establish the therapeutic potential of E42Q CRP. It may also be possible to design small-molecule compounds that can

target and change the structure of endogenous native CRP to mimic the structures of acidic pH-treated CRP or E42Q CRP.

Acknowledgements

This work was supported, in whole or in part, by National Institutes of Health Grants R01HL071233 (to A. A.), R01DK35081 (to M. K. P.), R15HL091770 (to D. A. J.), and 1R15HL091502 (to A. R.).

References

1. Black, S., Kushner, I., and Samols, D. (2004) C-reactive Protein. J. Biol. Chem.279,48487–48490
2. Mullenix, M. C., and Mortensen, R. F. (1994) Calcium ion binding regions in C-reactive protein. Location and regulation of conformational changes. Mol. Immunol.31,615–622.
3. Volanakis, J. E., and Kaplan, M. H. (1971) Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc. Soc. Exp. Biol. Med.136,612–614
4. Dong, A., Caughey, W. S., and Du Clos, T. W. (1994) Effects of calcium, magnesium, and phosphorylcholine on secondary structures of human C-reactive protein and serum amyloid P component observed by infrared spectroscopy. J. Biol. Chem.269,6424–6430
5. Singh, S. K., Hammond, D. J., Jr., Beeler, B. W., and Agrawal, A. (2009) The binding of C-reactive protein, in the presence of phosphoethanolamine, to low-density lipoproteins is due to phosphoethanolamine-generated acidic pH. Clin. Chim. Acta 409,143–144.
6. Hammond, D. J., Jr., Singh, S. K., Thompson, J. A., Beeler, B. W., Rusiñol, E., Pangburn, M. K., Potempa, L. A., and Agrawal, A. (2010) Identification of acidic pH-dependent ligands of pentameric C-reactive protein. J. Biol. Chem.285,36235–36244
7. Kushner, I., and Kaplan, M. H. (1961) Studies of acute phase protein. I. An immunohistochemical method for the localization of Cx-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. J. Exp. Med.114,961–974
8. Bhakdi, S., Torzewski, M., Klouche, M., and Hemmes, M. (1999) Complement and atherogenesis. Binding of CRP to degraded, nonoxidized LDL enhances complement activation. Arterioscler. Thromb. Vasc. Biol.19,2348–2354

9. Torzewski, M., Rist, C., Mortensen, R. F., Zwaka, T. P., Bienek, M., Waltenberger, J., Koenig, W., Schmitz, G., Hombach, V., and Torzewski, J. (2000) C-reactive protein in the arterial intima. Role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. Arterioscler. Thromb. Vasc. Biol.20,2094–2099
10. Kushner, I., Samols, D., and Magrey, M. (2010) A unifying biologic explanation for "high-sensitivity" C-reactive protein and "low-grade" inflammation. Arthritis Care Res.62,442–446
11. Agrawal, A., Hammond, D. J., Jr., and Singh, S. K. (2010) Atherosclerosis related functions of C-reactive protein. Cardiovasc. Haematol. Disord. Drug Targets10,235–240
12. Libby, P., Ridker, P. M., and Hansson, G. K. (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473,317–325
13. Silver, I. A., Murrills, R. J., and Etherington, D. J. (1988) Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp. Cell Res.175,266–276
14. Leake, D. S. (1997) Does an acidic pH explain why low density lipoprotein is oxidized in atherosclerotic lesions? Atherosclerosis 129,149–157
15. Björnheden, T., Levin, M., Evaldsson, M., and Wiklund, O. (1999) Evidence of hypoxic areas within the arterial wall in vivo. Arterioscler. Thromb. Vasc. Biol.19, 870–876
16. Sneck, M., Kovanen, P. T., and Oörni, K. (2005) Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans. J. Biol. Chem.280,37449–37454
17. Hayashi, M., Fujimoto, K., Urushibata, K., Takamizawa, A., Kinoshita, O., and Kubo, K. (2006) Hypoxia-sensitive molecules may modulate the development of atherosclerosis in

sleep apnea syndrome. Respirology 11, 24–31

18. Gallagher, F. A., Kettunen, M. I., Day, S. E., Hu, D. E., Ardenkjaer-Larsen, J. H., Zandt, R., Jensen, P. R., Karlsson, M., Golman, K., Lerche, M. H., and Brindle, K. M. (2008) Magnetic resonance imaging of pH in vivo using hyperpolarized 13C-labelled bicarbonate. Nature 453,940–944
19. Haka, A. S., Grosheva, I., Chiang, E., Buxbaum, A. R., Baird, B. A., Pierini, L. M., and Maxfield, F. R. (2009) Macrophages create an acidic extracellular hydrolytic compartment to digest aggregated lipoproteins. Mol. Biol. Cell 20,4932–4940
20. Naghavi, M., John, R., Naguib, S., Siadaty, M. S., Grasu, R., Kurian, K. C.,
21. van Winkle, W. B., Soller, B., Litovsky, S., Madjid, M., Willerson, J. T., and
22. Casscells, W. (2002) pH heterogeneity of human and rabbit atherosclerotic plaques. A new insight into detection of vulnerable plaque. Atherosclerosis 164,27–35
23. Oörni, K., and Kovanen, P. T. (2006) Enhanced extracellular lipid accumulation in acidic environments. Curr. Opin. Lipidol.17,534–540
24. Chang, M. K., Binder, C. J., Torzewski, M., and Witztum, J. L. (2002) C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand. Phosphorylcholine of oxidized phospholipids. Proc. Natl. Acad. Sci. U.S.A.99,13043–13048
25. Taskinen, S., Kovanen, P. T., Jarva, H., Meri, S., and Pentikäinen, M. O. (2002) Binding of C-reactive protein to modified low-density-lipoprotein particles. Identification of cholesterol as a novel ligand for C-reactive protein. Biochem. J.367,403–412
26. van Tits, L., de Graaf, J., Toenhake, H., van Heerde, W., and Stalenhoef, A. (2005) C-reactive protein and annexin A5 bind to distinct sites of negatively charged phospholipids

present in oxidized low-density lipoprotein. Arterioscler. Thromb. Vasc. Biol.25,717–722

27. Ji, S. R., Wu, Y., Potempa, L. A., Qiu, Q., and Zhao, J. (2006) Interactions of C-reactive protein with low-density lipoproteins. Implications for an active role of modified C-reactive protein in atherosclerosis. Int. J. Biochem. Cell Biol.38,648–661
28. Singh, U., Dasu, M. R., Yancey, P. G., Afify, A., Devaraj, S., and Jialal, I.(2008) Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats. J. Lipid Res.49,1015–1023
29. Singh, S. K., Suresh, M. V., Prayther, D. C., Moorman, J. P., Rusiñol, A. E., and Agrawal, A. (2008) C-reactive protein-bound enzymatically modified low-density lipoprotein does not transform macrophages into foam cells.. J. Immunol.180,4316–4322
30. Hirschfield, G. M., Gallimore, J. R., Kahan, M. C., Hutchinson, W. L., Sabin, C. A., Benson, G. M., Dhillon, A. P., Tennent, G. A., and Pepys, M. B.(2005) Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 102, 8309–8314
31. Reifenberg, K., Lehr, H. A., Baskal, D., Wiese, E., Schaefer, S. C., Black, S., Samols, D., Torzewski, M., Lackner, K. J., Husmann, M., Blettner, M., and Bhakdi, S. (2005) Role of C-reactive protein in atherogenesis. Can the apolipoprotein E knockout mouse provide the answer? Arterioscler. Thromb. Vasc. Biol.25,1641–1646
32. Trion, A., de Maat, M. P., Jukema, J. W., van der Laarse, A., Maas, M. C., Offerman, E. H., Havekes, L. M., Szalai, A. J., Princen, H. M., and Emeis, J. J. (2005) No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-leiden/human C-reactive protein transgenic mice. Arterioscler. Thromb. Vasc. Biol.25,1635–1640

33. Torzewski, M., Reifenberg, K., Cheng, F., Wiese, E., Küpper, I., Crain, J., Lackner, K. J., and Bhakdi, S. (2008) No effect of C-reactive protein on early atherosclerosis in LDLR-/-/human C-reactive protein transgenic mice. Thromb. Haemost.99,196–201

34. Ortiz, M. A., Campana, G. L., Woods, J. R., Boguslawski, G., Sosa, M. J., Walker, C. L., and Labarrere, C. A. (2009) Continously-infused human C-reactive protein is neither proatherosclerotic nor proinflammatory in apolipoprotein E-deficient mice. Exp. Biol. Med.234,624–631

35. Teupser, D., Weber, O., Rao, T. N., Sass, K., Thiery, J., and Fehling, H. J. (2011) No reduction of atherosclerosis in C-reactive protein (CRP)-deficient mice. J. Biol. Chem.286,6272–6279

36. Kovacs, A., Tornvall, P., Nilsson, R., Tegnér, J., Hamsten, A., and Björkegren, J. (2007) Human C-reactive protein slows atherosclerosis development in a mouse model with human-like hypercholesterolemia. Proc. Natl. Acad. Sci. U.S.A.104,13768–13773

37. Koike, T., Kitajima, S., Yu, Y., Nishijima, K., Zhang, J., Ozaki, Y., Morimoto, M., Watanabe, T., Bhakdi, S., Asada, Y., Chen, Y. E., and Fan, J.(2009) Human C-reactive protein does not promote atherosclerosis in transgenic rabbits.Circulation120,2088–2094

38. Singh, V., Tiwari, R. L., Dikshit, M., and Barthwal, M. K. (2009) Models to study atherosclerosis. A mechanistic insight. Curr. Vasc. Pharmacol.7,75–109

39. Shrive, A. K., Cheetham, G. M. T., Holden, D., Myles, D. A. A., Turnell, W. G., Volanakis, J. E., Pepys, M. B., Bloomer, A. C., and Greenhough, T. J.(1996) Three-dimensional structure of human C-reactive protein. Nat. Struct. Biol.3,346–354

40. Srinivasan, N., White, H. E., Emsley, J., Wood, S. P., Pepys, M. B., and Blundell, T. L. (1994) Comparative analyses of pentraxins. Implications for protomer assembly and

ligand binding.Structure2,1017–1027

41. Thompson, D., Pepys, M. B., and Wood, S. P. (1999) The physiological structure of human C-reactive protein and its complex with phosphocholine. Structure Fold. Des.7,169–177
42. Agrawal, A., Xu, Y., Ansardi, D., Macon, K. J., and Volanakis, J. E. (1992) Probing the phosphocholine-binding site of human C-reactive protein by site-directed mutagenesis. J. Biol. Chem.267,25352–25358
43. Suresh, M. V., Singh, S. K., Ferguson, D. A., Jr., and Agrawal, A. (2006) Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. J. Immunol.176,4369–4374
44. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest.34,1345–1353
45. Brown, A. J., Leong, S. L., Dean, R. T., and Jessup, W. (1997) 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. J. Lipid Res.38,1730–1745
46. Pangburn, M. K., and Müller-Eberhard, H. J. (1983) Kinetic and thermodynamic analysis of the control of C3b by the complement regulatory proteins factors H and I. Biochemistry22,178–185
47. Guex, N., and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer. An environment for comparative protein modeling. Electrophoresis18,2714–2723
48. Kumar, S., and Nussinov, R. (2002) Relationship between ion pair geometries and electrostatic strengths in proteins. Biophys. J. 83,1595–1612

49. Costantini, S., Colonna, G., and Facchiano, A. M. (2008) ESBRI. A web server for evaluating salt bridges in proteins.Bioinformation3,137–138

50. Sheu, B. C., Lin, Y. H., Lin, C. C., Lee, A. S., Chang, W. C., Wu, J. H., Tsai, J. C., and Lin, S. (2010) Significance of the pH-induced conformational changes in the structure of C-reactive protein measured by dual polarization interferometry. Biosensors. Bioelectron.26,822–827

51. Miyazawa, K., Kiyono, S., and Inoue, K. (1988) Modulation of stimulus dependent human platelet activation by C-reactive protein modified with active oxygen species. J. Immunol.141,570–574

52. Wang, M. Y., Ji, S. R., Bai, C. J., El Kebir, D., Li, H. Y., Shi, J. M., Zhu, W., Costantino, S., Zhou, H. H., Potempa, L. A., Zhao, J., Filep, J. G., and Wu, Y. (2011) A redox switch in C-reactive protein modulates activation of endothelial cells. FASEB J.25,3186–3196

53. Schroeder, B. O., Wu, Z., Nuding, S., Groscurth, S., Marcinowski, M., Beisner, J., Buchner, J., Schaller, M., Stange, E. F., and Wehkamp, J. (2011) Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1. Nature469,419–423

54. Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008) Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. Science 321,952–956

55. Mold, C., Kingzette, M., and Gewurz, H. (1984) C-reactive protein inhibits pneumococcal activation of the alternative pathway by increasing the interaction between factor H and C3b. J. Immunol.133,882–885

56. Jarva, H., Jokiranta, T. S., Hellwage, J., Zipfel, P. F., and Meri, S. (1999) Regulation of complement activation by C-reactive protein. Targeting the complement inhibitory

activity of factor H by an interaction with short consensus repeat domains 7 and 8–11. J. Immunol.163,3957–3962

57. Sjöberg, A. P., Trouw, L. A., Clark, S. J., Sjölander, J., Heinegård, D., Sim, R. B., Day, A. J., and Blom, A. M. (2007) The factor H variant associated with age-related macular degeneration (His-384) and the non-disease associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. J. Biol. Chem.282,10894–10900
58. Skerka, C., Lauer, N., Weinberger, A. A. W. A., Keilhauer, C. N., Sühnel, J., Smith, R., Schlötzer-Schrehardt, U., Fritsche, L., Heinen, S., Hartmann, A., Weber, B. H. F., and Zipfel, P. F. (2007) Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol. Immunol.44,3398–3406
59. Suresh, M. V., Singh, S. K., Ferguson, D. A., Jr., and Agrawal, A. (2007) Human C-reactive protein protects mice from Streptococcus pneumonia infection without binding to pneumococcal C-polysaccharide. J. Immunol.178,1158–1163
60. Agrawal, A., Suresh, M. V., Singh, S. K., and Ferguson, D. A., Jr. (2008) The protective function of human C-reactive protein in mouse models of Streptococcus pneumoniae infection. Endocr. Metab. Immune Disord. Drug Targets8,231–237
61. Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. Nature 475,324–332
62. Rodriguez, W., Mold, C., Marnell, L. L., Hutt, J., Silverman, G. J., Tran, D., and Du Clos, T. W. (2006) Prevention and reversal of nephritis in MRL/lpr mice with a single injection of C-reactive protein. Arthritis Rheum.54,325–335
63. Carlucci, F., Cook, H. T., Garg, A., Pepys, M. B., and Botto, M. (2010) Lack of effect of a single injection of human C-reactive protein on murine lupus or nephrotoxic nephritis.

Arthritis Rheum.62,245–249

64. Szalai, A. J., Weaver, C. T., McCrory, M. A., van Ginkel, F. W., Reiman, R. M., Kearney, J. F., Marion, T. N., and Volanakis, J. E. (2003) Delayed lupus onset in (NZB x NZW)F1 mice expressing a human C-reactive protein transgene. Arthritis Rheum.48,1602–1611
65. Gaitonde, S., Samols, D., and Kushner, I. (2008) C-reactive protein and systemic lupus erythematosus. Arthritis Rheum.59,1814–1820

CHAPTER 4

Hydrogen peroxide modifies C-reactive protein structure and exposes a hidden functional site

Avinash Thirumalai and Alok Agrawal

Department of Biomedical Sciences, James H Quillen College of Medicine,
East Tennessee State University, Johnson City, Tennessee 37614, USA.

Key Words: C-reactive protein, inflammation, atherosclerosis, H_2O_2, low-density lipoprotein

Reference: This chapter consists of data from preliminary experiments which have not been published.

Abstract

C-reactive protein (CRP) is an evolutionarily conserved protein involved in the response to systemic inflammation. CRP is a plasma protein that is also deposited at sites of localized inflammation, such as atherosclerotic lesions. Atherosclerotic lesions are characterized by dysregulation of the redox environment due to the generation of reactive oxygen species (ROS). In this study, we used hydrogen peroxide (H_2O_2) as a prototypical ROS and investigated its influence on the binding specificities of CRP. In the presence of H_2O_2, CRP gained the ability to bind immobilized ox-LDL in a H_2O_2 dose-dependent manner. The ox-LDL binding ability was retained even when H_2O_2 was dialyzed out, indicating that H_2O_2-induced modification in CRP exposed a hidden ox-LDL binding site. H_2O_2-induced binding of CRP was not restricted to ox-LDL, as H_2O_2-CRP bound a variety of immobilized and structurally altered proteins. H_2O_2-treatment did not disrupt the phosphocholine-binding site in the CRP pentamer. HD2.4 reactivity, gel filtration profile and 1-anilinonaphthalene-8-sulfonic acid-binding fluorescence assays showed that H_2O_2-treatment did not alter the CRP pentamer, but only induced a local structural change. The exact nature and mechanism of H_2O_2-induced modification of CRP structure and function requires further study. However, our results suggest that the function of CRP is dependent on the environmental context, and that it might play an important role in inflammatory diseases that involve deposition and structural modification of proteins.

Introduction

Human C-reactive protein (CRP) is a member of the evolutionarily conserved pentraxin family of proteins that participates in the response to systemic inflammation (reviewed in (1, 2)). The CRP molecule is a pentamer made up of five identical, non-covalently associated subunits arranged symmetrically around a central pore. At physiological pH, CRP binds to molecules and cells that contain exposed phosphocholine (PCh) moieties in a Ca^{2+}-dependent manner. Under certain conditions CRP can adopt a different pentameric configuration, which exposes a hidden binding site and enables it to bind to immobilized, aggregated or conformationally altered proteins(3).

CRP is a plasma protein that is also found deposited at sites of localized inflammation, such as in atherosclerotic lesions(4-7). Atherosclerosis is a disease of chronic inflammation that is initiated by the deposition, and subsequent modification of low-density lipoproteins (LDL) in the artery wall. Excessive cholesterol and lipid uptake from modified LDLs by macrophages & monocytes converts them to pro-inflammatory foam cells – the key event in the pathogenesis of atherosclerosis (reviewed in (8, 9)). The functions of CRP in atherosclerosis are not very clear. The binding of CRP to the atherogenic forms of LDL, oxidized LDL (ox-LDL) and enzymatically modified LDL (E-LDL) has been studied previously(7, 10-15). CRP binds E-LDL in a Ca^{2+}-dependent and PCh-inhibitable manner at pH 7.0, and this binding is increased dramatically at acidic pH(16, 17). CRP does not bind ox-LDL at physiological pH. However, acidic pH induces a reversible conformational change exposing a hidden binding site and enables CRP to bind to immobilized ox-LDL(3). We have also shown that CRP-bound E-LDL does not induce foam-cell formation(17). However,

studies using animals models of atherosclerosis have shown that human CRP is neither atherogenic nor atheroprotective(18-25), the reasons for which are not clear.

Atherosclerotic lesions are characterized by acidosis(26-28) and increased O_2 and energy demand(29, 30), which result in production of reactive oxygen species (ROS), and subsequent deregulation of the extracellular redox environment(31-33). In addition to increasing inflammatory cell signaling(34), these changes enhance LDL deposition, modification and macrophage uptake, contributing to the progression of atherosclerosis(35-38). Such an inflammatory environment may not have developed in the animal models used to study the function of human CRP in atherosclerosis; CRP may not have undergone structural modification enabling it to efficiently bind atherogenic ox-LDL. To investigate the role of CRP in atherosclerosis using these animal models, we need a structurally altered CRP that can bind to atherogenic ox-LDL at physiological conditions. For this purpose we have previously used site-directed mutagenesis to mimic the acidic pH-induced changes in CRP, and generated a mutant CRP that could bind ox-LDL at physiological pH(39). In this study, we present the results of preliminary studies investigating the effects of redox free radicals on CRP – ox-LDL interactions.

Experimental Procedures

Purification of CRP

Native CRP was purified from pleural fluid in three steps: Ca^{2+}-dependent affinity chromatography on a PCh-conjugated-Sepharose column(Pierce), followed by anion-exchange chromatography on a MonoQ column(GE Healthcare), and gel filtration on a Superose 12 column(GE Healthcare), as described previously(16). On the day of the experiments, CRP was re-purified by gel filtration on a Superose 12 column(GE Healthcare) to remove any form of modified CRP that might have been generated due to storage of CRP. Re-purified CRP was stored in 10 mm Tris-HCl, pH 7.2, 150 mm NaCl(TBS) containing 2 mM $CaCl_2$ at 4°C and was used within 1 week.

Preparation and purification of ROS-modified CRP

Freshly purified CRP was treated with 1% H_2O_2 and the Fe^{2+}-ascorbate solution as described before(40). Briefly, CRP (350µg/ml), 30 µM $FeSO_4$ and 1 mM sodium ascorbate were dissolved in 10 mM Tris-Cl, 150 mM NaCl (pH 7.4). This mixture was incubated at 37ºC for 1 h with rapid shaking. The reaction was then stopped by adding 300 µM diethylenetriamine-pentaacetic acid. The solution was dialyzed O/N against 10 mm Tris-HCl, pH 7.2, 150 mm NaCl (TBS) containing 2 mM $CaCl_2$, and then used as FA-CRP. To prepare H_2O_2-CRP, 500 µg/ml of CRP was incubated with 1% H_2O_2 10 mM Tris-Cl, 150 mM NaCl (pH 7.2) solution containing 2 mM $CaCl_2$ for a minimum of 1 h at 37°C. It was then dialyzed O/N against 10 mm Tris-HCl, pH 7.2, 150 mm NaCl (TBS) containing 2 mM $CaCl_2$.

Preparation of Ox-LDL and Ac-LDL

Native LDL was isolated from human plasma by sequential ultracentrifugation (1.019<d>1.063 g/ml), as described previously(41). Ox-LDL was prepared by treating LDL with 20μM $CuCl_2$ in PBS for 12 h at 37 °C(42). The Cu^{2+}-mediated oxidation was terminated by adding EDTA to the reaction at a final concentration of 0.5 mM. Following dialysis against PBS, ox-LDL was passed through chelex-100 resin (Bio-Rad) to remove any traces of Cu^{2+}, filter sterilized, and stored in the dark at 4 °C. Acetylated LDL (Ac-LDL) was also prepared as described previously(43). 0.5 mL of 0.15 M NaCl solution containing 14 mg of freshly isolated LDL protein was added to 0.5 mL saturated sodium acetate. The reaction mixture was placed in an ice-water bath, and over a period of 1 h, aliquots (2 μL) of acetate anhydride were added with continuous stirring. After addition of acetic anhydrate equal to 1.5 times the mass of LDL used, mixture was stirred for a further 30 min. After dialysis for 24 h at 4°C against 2 L of 0.15 M NaCl, 0.3 EDTA and pH 7.4, Ac-LDL was filter sterilized and stored in the dark at 4°C. The degree of LDL modification was evaluated by agarose gel electrophoresis. The degree of oxidation was also determined by TBARS assay (Cayman Chemical Co.), a colorimetric assay using malondialdehyde as a standard. Ox-LDL used in this study resulted in 30.5 nmoles malondialdehyde/mg of protein. Protein concentrations of the ox-LDL and Ac-LDL preparations were measured using the microBCA protein assay kit (Pierce).

Ox-LDL-binding assay

Microtiter wells were coated with 10 μg/ml ox-LDL in TBS and incubated overnight at 4 °C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. Freshly purified CRP or modified CRP (depending on the assay) was diluted in TBS containing 0.1%

gelatin, 0.02% Tween 20, and 2 mM $CaCl_2$ (TBS-Ca), added in duplicate wells and incubated for 2 h at 37 °C. As mentioned in the figure legends, this step was performed in the presence of varying concentrations of H_2O_2. After the CRP incubation step, the wells were washed with TBS-Ca. Immunoaffinity purified polyclonal rabbit anti-CRP antibody (1 µg/ml), diluted in TBS-Ca, was used(1 h at 37°C) to detect bound CRP. Immunoaffinity purified polyclonal rabbit anti-CRP antibody was purified from the rabbit anti-human CRP antiserum (Sigma) by affinity chromatography on a CRP-conjugated agarose column prepared by using the AminoLink Immobilization kit(Pierce), as described previously(44). In some assays, monoclonal anti-CRP antibodies (mAb) were used to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG(GE Healthcare) and HRP-conjugated goat anti-mouse IgG(Thermo Scientific), diluted in TBS-Ca, were used (1 h at 37°C) as secondary antibodies. Color was developed and absorbance was read at 405 nm in a microtiter plate reader(Molecular Devices).

PnC and PCh-BSA binding assay

Binding activity of CRP for PCh was evaluated by using PnC(Statens Serum Institut) and PCh-BSA(Sigma) as ligands. Microtiter wells were coated (350 µl/well) with 10 µg/ml PnC or 10 µg/ml PCh-BSA in TBS O/N at 4°C. CRP diluted in TBS-Ca was added (100 µl/well) in duplicate wells. After incubating the plates for 2 h at 37°C, the wells were washed with appropriate TBS-Ca. The anti-CRP monoclonal antibody HD2.4 (0.5 µg/ml), diluted in TBS-Ca, was used (100 µl/well, 1 h at 37°C) to detect bound CRP. HRP-conjugated goat anti-mouse IgG, diluted in TBS-Ca, were used (100 µl/well, 1 h at 37°C) as the secondary antibody. Color was developed, and the absorbance was read at 405 nm in a microtiter plate reader.

Protein ligand-binding assay

This assay was used to determine the binding of CRP to proteins other than ox-LDL and was performed as described for the ox-LDL-binding assay, except that the microtiter wells were coated with 10 µg/ml complement factor H (purified from normal human plasma) (45), LDL, acetylated LDL, or ovalbumin diluted in TBS (350 µl/well) overnight at 4°C. To test binding to IgG immune complexes, microtiter wells were first coated with 10 µg/ml ovalbumin in TBS and incubated O/N at 4°C. 100 µl/well of 1:1000 dilution of polyclonal goat α-ovalbumin antibody (MP Biomedicals #0855303) was then added and incubated for 1 h at 37°C. Wells were then blocked with 0.5% gelatin, and CRP samples were added as before for binding.

Gel filtration

Gel filtration analysis of CRP at pH 7.0 was carried out on a Superose12 column. The column was equilibrated and eluted with TBS (pH 7.0) containing 2 mM$CaCl_2$ at a flow rate of 0.3 ml/min. Fractions(60 fractions, 0.25 ml each) were collected, and absorbance at 280 nm was measured to locate the elution volume of CRP from the column.

1-Anilinonaphthalene-8-sulfonic acid (ANS)-binding fluorescence assay

The hydrophobic fluorescent probe ANS (8-Anilininaphthalene-1-sulfonic acid) was purchased from AnaSpec, Inc. ANS-binding fluorescence assays were performed as described previously(3). CRP (50 µg/ml) in TBS containing 2 mM $CaCl_2$ was mixed with ANS at a final concentration of 100 µM. The fluorescence intensity of the binding of ANS to CRP was measured by using excitation and emission wavelengths of 390 nm and 460 nm respectively, in a spectrofluorometer (Fluostar Galaxy, BMG Lab Technologies).

Results

Hydrogen peroxide induces the binding of CRP to ox-LDL

To determine the effect of oxidizing agents on CRP – ox-LDL binding, we first performed ox-LDL binding assays using CRP modified by two oxidizing agents: 1% hydrogen peroxide (H_2O_2) and the Fe^{2+}-ascorbate system (40). As shown in Fig 4.1A, the binding of CRP to ox-LDL was not changed in the presence of the Fe^{2+}-ascorbate system at pH 7.0. However, 1% H_2O_2-modified CRP (H_2O_2-CRP) strongly bound immobilized ox-LDL at physiological pH. The binding of CRP to ox-LDL at pH 7.0 and pH 5.0 were used as negative and positive controls respectively. We then tested the binding of CRP to ox-LDL in different concentrations of H_2O_2. As shown in Fig 4.1B, the binding of CRP to immobilized ox-LDL increased in the presence of H_2O_2, and this increased in a H_2O_2 dose-dependent manner. H_2O_2-induced binding to ox-LDL was specific to CRP, as H_2O_2 had no effect on the binding of BSA to ox-LDL. Dialyzed and freshly re-purified H_2O_2-CRP retained its ox-LDL binding activity at pH 7.0

H_2O_2-CRP binds to a variety of proteins immobilized to microtiter plates

Similar to its interaction with immobilized ox-LDL, 1% H_2O_2 treatment of CRP caused it to bind to various immobilized proteins (Fig 4.2). H_2O_2-CRP bound to unmodified native LDL, and showed increased binding to Ac-LDL, another form of modified LDL.H_2O_2-CRP also bound to immobilized Factor H, ovalbumin and ovalbumin – goat α-ovalbumin complexes (IgG immune complex). This suggests that similar to acidic pH, H_2O_2 treatment exposed a hidden binding site in CRP that enabled it to bind deposited and conformationally changed proteins.

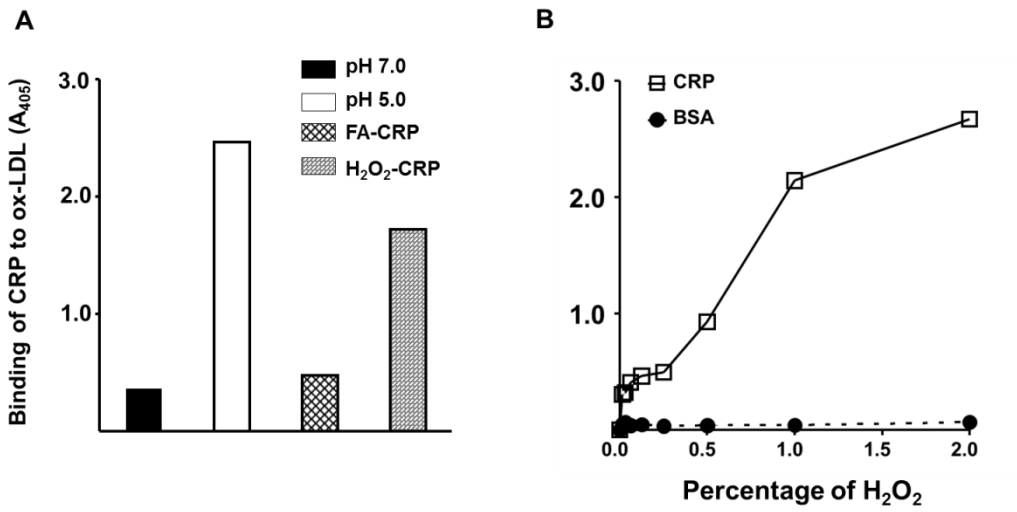

Figure 4.1. H_2O_2 induces binding of CRP to ox-LDL in a dose dependent manner. *A*, CRP (10 µg/ml) diluted in TBS-Ca (pH 7.0 and 5.0), FA-CRP, and H_2O_2-CRP in TBS-Ca pH 7.0 buffers were added to ox-LDL-coated wells. Bound CRP was detected using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm. *B*, CRP (10 µg/ml), diluted in TBS-Ca pH 7.0 buffer containing H_2O_2 dilutions in the range of (0.156 – 2.0%) was added to ox-LDL-coated wells. Bound CRP was detected as in A. Representative results from two experiments are shown.

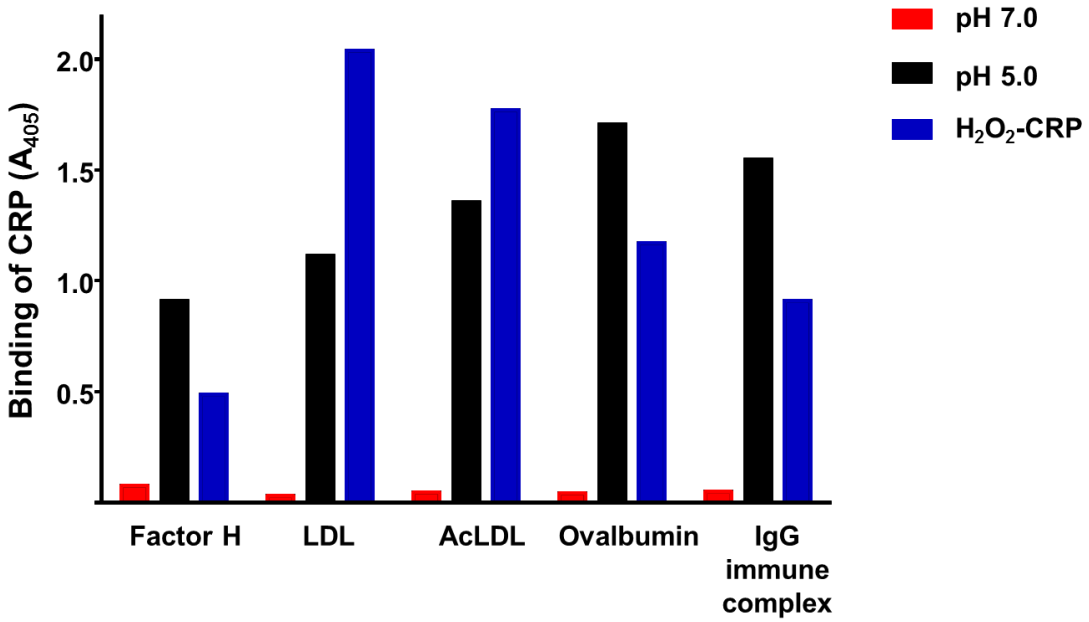

Figure 4.2. Binding of H_2O_2-CRP to immobilized protein ligands. 10 µg/ml CRP diluted in TBS-Ca (pH 7.0 and 5.0), and H_2O_2-CRP in TBS-Ca pH 7.0 buffers were added to wells coated with Factor H, LDL, AcLDL, and ovalbumin. To test binding to IgG-immune complexes, ovalbumin – α-ovalbumin were added to wells as described earlier. Bound CRP was detected using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm.

The phosphocholine binding site in CRP is not disrupted by H_2O_2 treatment

We next tested whether treatment with 1% H_2O_2 had an effect on the PCh-binding site of CRP. For this, we used the PCh-containing pneumococcal C-polysaccharide (PnC) and purified PCh-BSA molecules as ligands in the binding assay. Serial dilutions of native CRP and re-purified H_2O_2-CRP in TBS-Ca pH 7.0 buffer were made, and added to microtiter wells coated with 10 µg/ml of PnC or PCh-BSA. After incubation at 37ºC for 1 h, the bound CRP was measured using the monoclonal HD 2.4 as per standard procedure. As shown in Fig. 4.3, pre-treatment with 1% H_2O_2 had no effect on the binding of CRP to PCh-containing ligands. The HD2.4 monoclonal antibody binds only to pentameric CRP, and this assay also indicates that H_2O_2-CRP retains its pentameric structure.

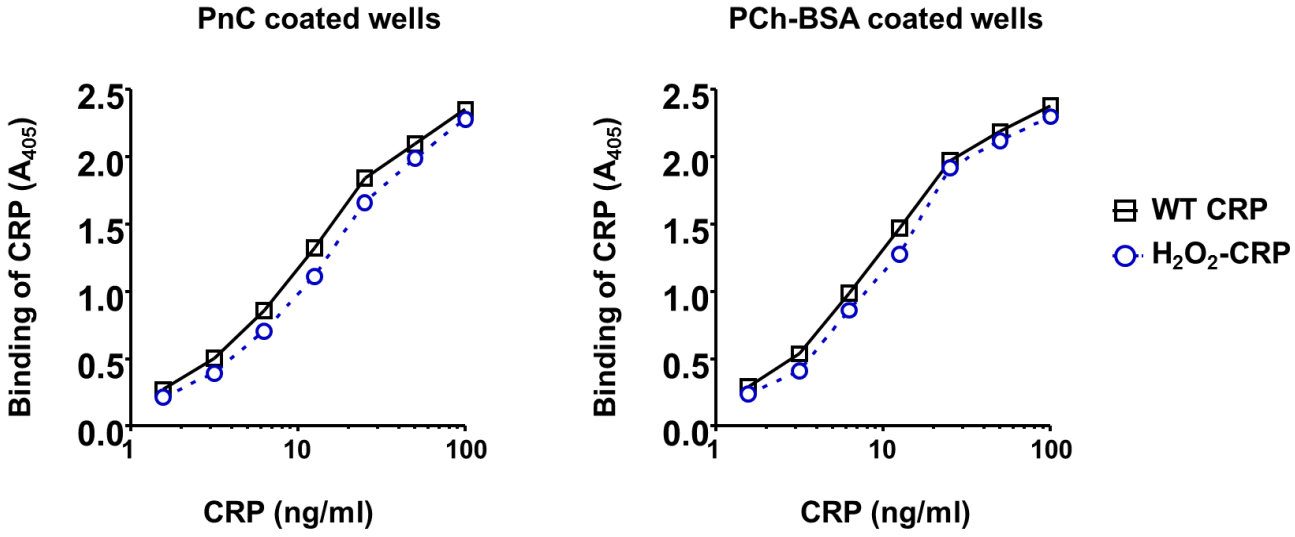

Figure 4.3. Binding of H_2O_2-CRP to PCh-containing ligands. Serial dilutions of native CRP and H_2O_2-CRP were made in TBS-Ca pH 7.0 buffers and added to wells coated with 10 µg/ml PnC or PCh-BSA. Bound CRP was detected using a monoclonal HD2.4 antibody and HRP-conjugated goat anti-mouse IgG. The absorbance of the color was read at 405 nm. The experiment was repeated twice, and a representative result is shown.

H_2O_2-treatment does not monomerize or aggregate CRP

Gel filtration and ANS-binding assays were used to determine whether H_2O_2-treatment caused monomerization or aggregation of CRP. Two aliquots of 1 mg/ml of freshly purified CRP were made. One aliquot was treated with 1% H_2O_2 at 37ºC for 2 h, while the other was just incubated at 37ºC for 2 h. After this time, the two aliquots were separately dialyzed against TBS-Ca pH 7.0 buffer at 4ºC overnight. Following dialysis, the CRP samples were concentrated using a centrifuge filter, and re-purified by gel filtration. As shown in Fig. 4.4, the elution profiles of native CRP and 1% H_2O_2-treated CRP are identical, suggesting that the pentameric structure of the CRP molecule remained intact.

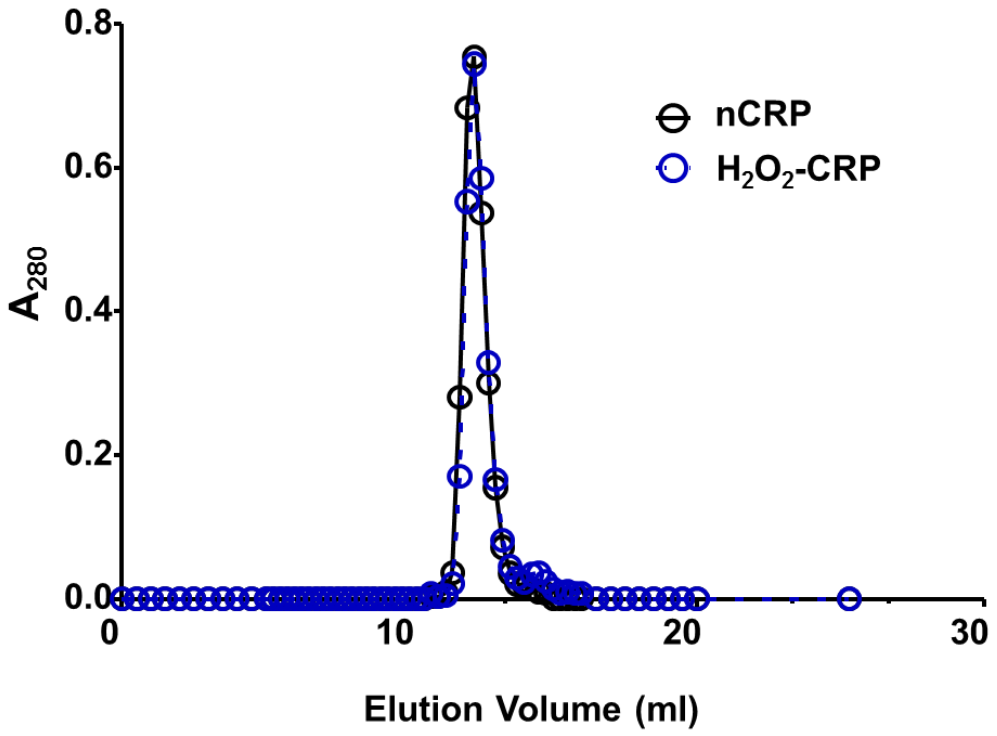

Figure 4.4. Gel filtration elution profile of CRP. After treatment, native CRP and H_2O_2-CRP were dialyzed separately and concentrated using a centrifuge filter. The concentrated CRP samples were re-purified by gel filtration. Absorbance of fractions at 280 nm was plotted to get the elution profile.

We have previously shown that acidic pH changed the overall hydrophobicity of the CRP pentamer, and induced only a local structural change. To determine whether 1% H_2O_2 treatment also induced a similar change in hydrophobicity, ANS-binding fluorescence assays were performed. As before, CRP at pH 7.0 showed minimal ANS binding at pH 7.0, but this was strongly increased on a shift to acidic pH. H_2O_2-CRP also showed an intense ANS fluorescence, but not as much as that of native CRP at pH 5.0 (Fig 4.5). If CRP is monomerized or aggregated, it cannot bind to ANS and show increased fluorescence(3). This indicates that H_2O_2 increases hydrophobicity and causes only a local structural change in the CRP pentamer.

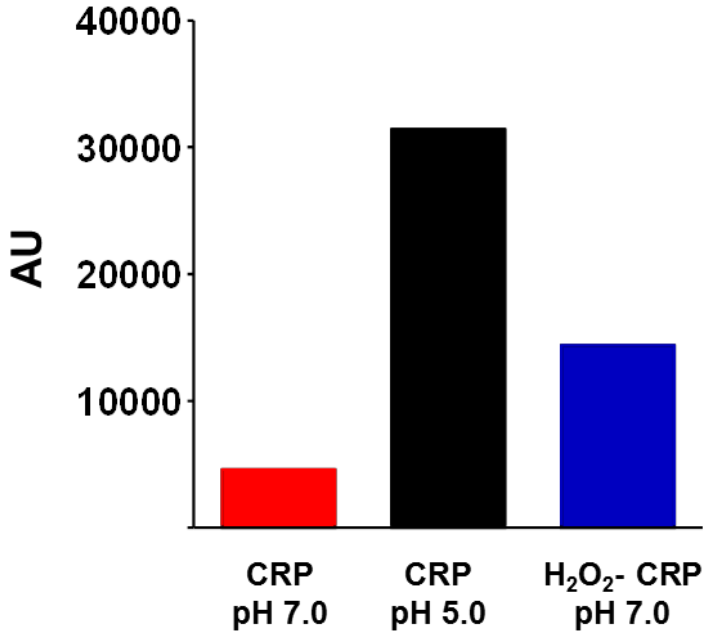

Fig. 4.5. ANS-binding fluorescence of CRP samples: CRP at pH 7.0 (red bar), pH 5.0 (black bar) and 1% H_2O_2-treated CRP. All samples were dialyzed and re-purified by gel filtration prior to use in the ANS-binding assay. A representative result from two experiments is shown.

Discussion

The aim of this study was to determine whether reactive oxygen species could modify CRP and thus generate a CRP molecule that binds to ox-LDL at physiological pH. The major results from our preliminary studies are:

1. CRP gains the ability to bind immobilized ox-LDL in the presence of H_2O_2. The ox-LDL binding ability is retained even after H_2O_2 has been dialyzed out, and the modified CRP has been re-purified.
2. H_2O_2-CRP (1% H_2O_2-treated CRP) binds to not only ox-LDL, but also to a variety of immobilized, aggregated or conformationally altered proteins.
3. The PCh-binding site in CRP is not disrupted by H_2O_2 treatment. H_2O_2-CRP retains its pentameric structure, but shows an increase in hydrophobicity indicating that H_2O_2 treatment induces a local structural change.

Pitfalls

Firstly, there are only preliminary results as the experiments have not been repeated a sufficient number of times. Unlike our studies with acidic pH-induced modification of CRP, or those with the E42Q CRP mutant, all required experiments to elucidate the nature of H_2O_2-mediated change in CRP structure have not been performed. It is not yet clear whether the H_2O_2-mediated change in CRP binding is valid, or is an experimental artifact due the presence of minute amounts of monomerized CRP (mCRP). Experiments on the reversibility of H_2O_2-mediated changes, or whether only a partial loosening of the CRP pentamer is sufficient for ox-LDL binding have yet to be performed. The nature of the structural changes induced by H_2O_2 are

also not completely clear. Furthermore, whether H_2O_2-induced binding of CRP to immobilized ox-LDL has any physiological relevance (i.e., on foam cell formation) is yet to be determined.

Although these are only preliminary results and experiments have not been repeated a sufficient number of times, they provide encouraging hints into the functions of CRP during atherosclerosis. Atherosclerotic lesions are characterized by not just acidic pH, but also a dysregulated redox environment, both of which are known to cause protein modifications, enhance protein-protein interactions and subsequent foam cell formation(reviewed in (26, 32, 33, 46). In a non-atherosclerosis context, changes in the redox environment have been shown previously to modify CRP function. ROS-mediated CRP modification regulates the stimulus-dependent activation of platelets(40), while the reduction of the single intra-subunit disulfide bond in CRP has been shown to induce its pro-inflammatory functions(47). Additionally, CRP is also not the only innate immune system protein regulated by changes in the redox environment (30, 48, 49).

In animal models of atherosclerosis, WT CRP is neither atheroprotective nor proatherosclerotic (reviewed in (46)). These animal models may not have a sufficiently inflammatory microenvironment (acidosis, ROS generation) at lesion sites like in humans. In such a scenario, administration of human CRP will be ineffective, as the CRP is not structurally modified, and cannot bind atherogenic LDLs to prevent foam cell formation. If reactive oxygen species can modify CRP to permanently expose the hidden ox-LDL binding site, they can be used as a tool to study the role of human CRP in animal models of atherosclerosis (similar to E42Q and mutant CRP created by site-directed mutagenesis). Our ultimate goal is to design small-molecule compounds that change the structure of endogenous native CRP to enable ox-

LDL binding, and prevent the progression of atherosclerosis.

Acknowledgements

This work was supported, in whole or in part, by National Institutes of Health Grants R01HL071233 (to A. A.) and ETSU Research Development Committee Major Grant (to A.A.).

References

1. Black, S., Kushner, I., and Samols, D. (2004) C-reactive Protein. *J. Biol. Chem*. 279, 48487–90.

2. Agrawal, A. (2005) CRP after 2004. *Mol. Immunol*. 42, 927–30.

3. Hammond, D. J., Singh, S. K., Thompson, J. a, Beeler, B. W., Rusiñol, A. E., Pangburn, M. K., Potempa, L. a, and Agrawal, A. (2010) Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J. Biol. Chem*. 285, 36235–44.

4. Hatanaka, K., Li, X. A., Masuda, K., Yutani, C., and Yamamoto, A. (1995) Immunohistochemical localization of C-reactive protein-binding sites in human atherosclerotic aortic lesions by a modified streptavidin-biotin-staining method. *Pathol. Int*. 45, 635–41.

5. Kushner, I., and Kaplan, M. H. (1961) Studies of acute phase protein. I. An immunohistochemical method for the localization of Cx-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. *J. Exp. Med*. 114, 961–974.

6. Bhakdi, S., Torzewski, M., Paprotka, K., Schmitt, S., Barsoom, H., Suriyaphol, P., Han, S.-R., Lackner, K. J., and Husmann, M. (2004) Possible protective role for C-reactive protein in atherogenesis: complement activation by modified lipoproteins halts before detrimental terminal sequence. *Circulation* 109, 1870–6.

7. Torzewski, M., Rist, C., Mortensen, R. F., Zwaka, T. P., Bienek, M., Waltenberger, J., Koenig, W., Schmitz, G., Hombach, V., and Torzewski, J. (2000) C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler. Thromb. Vasc. Biol*. 20, 2094–2099.

8. Ross, R. (1999) Atherosclerosis--an inflammatory disease. *N. Engl. J. Med*. 340, 115–26.

9. Moore, K. J., and Tabas, I. (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341–355.

10. Bhakdi, S., Torzewski, M., Klouche, M., and Hemmes, M. (1999) Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler. Thromb. Vasc. Biol*. 19, 2348–54.

11. Chang, M.-K., Binder, C. J., Torzewski, M., and Witztum, J. L. (2002) C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13043–8.

12. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci.* 76, 333–337.

13. Singh, U., Dasu, M. R., Yancey, P. G., Afify, A., Devaraj, S., and Jialal, I. (2008) Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats. *J. Lipid Res*. 49, 1015–23.

14. Taskinen, S., Kovanen, P. T., Jarva, H., Meri, S., and Pentikäinen, M. O. (2002) Binding of C-reactive protein to modified low-density-lipoprotein particles: identification of cholesterol as a novel ligand for C-reactive protein. *Biochem. J*. 367, 403–12.

15. Van Tits, L., de Graaf, J., Toenhake, H., van Heerde, W., and Stalenhoef, A. (2005) C-reactive protein and annexin A5 bind to distinct sites of negatively charged phospholipids present in oxidized low-density lipoprotein. *Arterioscler. Thromb. Vasc. Biol*. 25, 717–22.

16. Singh, S. K., Hammond, D. J., Beeler, B. W., and Agrawal, A. (2009) The binding of C-reactive protein, in the presence of phosphoethanolamine, to low-density lipoproteins is due to phosphoethanolamine-generated acidic pH. *Clin. Chim. Acta*. 409, 143–144.

17. Singh, S. K., Suresh, M. V, Prayther, D. C., Moorman, J. P., Rusiñol, A. E., and Agrawal, A. (2008) C-reactive protein-bound enzymatically modified low-density lipoprotein does not transform macrophages into foam cells. *J. Immunol*. 180, 4316–22.

18. Trion, A., De Maat, M. P. M., Jukema, J. W., Van Der Laarse, A., Maas, M. C., Offerman, E. H., Havekes, L. M., Szalai, A. J., Princen, H. M. G., and Emeis, J. J. (2005) No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-leiden/human C-reactive protein transgenic mice. *Arterioscler. Thromb. Vasc. Biol*. 25, 1635–1640.

19. Torzewski, M., Reifenberg, K., Cheng, F., Wiese, E., Küpper, I., Crain, J., Lackner, K. J., and Bhakdi, S. (2008) No effect of C-reactive protein on early atherosclerosis in LDLR-/- / human C-reactive protein transgenic mice. *Thromb. Haemost*. 99, 196–201.

20. Tennent, G. A., Hutchinson, W. L., Kahan, M. C., Hirschfield, G. M., Gallimore, J. R., Lewin, J., Sabin, C. A., Dhillon, A. P., and Pepys, M. B. (2008) Transgenic human CRP is not pro-atherogenic, pro-atherothrombotic or pro-inflammatory in apoE-/- mice. *Atherosclerosis* 196, 248–55.

21. Paul, A., Ko, K. W. S., Li, L., Yechoor, V., McCrory, M. A., Szalai, A. J., and Chan, L. (2004) C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 109, 647–55.

22. Ortiz, M. A., Campana, G. L., Woods, J. R., Boguslawski, G., Sosa, M. J., Walker, C. L., and Labarrere, C. A. (2009) Continuously-infused human C-reactive protein is neither proatherosclerotic nor proinflammatory in apolipoprotein E-deficient mice. *Exp. Biol. Med. Maywood NJ* 234, 624–631.

23. Kovacs, A., Tornvall, P., Nilsson, R., Tegnér, J., Hamsten, A., and Björkegren, J. (2007) Human C-reactive protein slows atherosclerosis development in a mouse model with human-like hypercholesterolemia. *Proc. Natl. Acad. Sci. U. S. A.* 104, 13768–73.

24. Koike, T., Kitajima, S., Yu, Y., Nishijima, K., Zhang, J., Ozaki, Y., Morimoto, M., Watanabe, T., Bhakdi, S., Asada, Y., Chen, Y. E., and Fan, J. (2009) Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation* 120, 2088–94.

25. Hirschfield, G. M., Gallimore, J. R., Kahan, M. C., Hutchinson, W. L., Sabin, C. A., Benson, G. M., Dhillon, A. P., Tennent, G. A., and Pepys, M. B. (2005) Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8309–8314.

26. Leake, D. S. (1997) Does an acidic pH explain why low density lipoprotein is oxidised in atherosclerotic lesions? *Atherosclerosis* 129, 149–157.

27. Naghavi, M., John, R., Naguib, S., Siadaty, M. S., Grasu, R., Kurian, K. C., van Winkle, W. B., Soller, B., Litovsky, S., Madjid, M., Willerson, J. T., and Casscells, W. (2002) pH Heterogeneity of human and rabbit atherosclerotic plaques; a new insight into detection of vulnerable plaque. *Atherosclerosis* 164, 27–35.

28. Silver, I. A., Murrills, R. J., and Etherington, D. J. (1988) Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp. Cell Res*. 175, 266–276.

29. Björnheden, T., Levin, M., Evaldsson, M., and Wiklund, O. (1999) Evidence of hypoxic areas within the arterial wall in vivo. *Arterioscler. Thromb. Vasc. Biol*. 19, 870–6.

30. Rajamäki, K., Nordström, T., Nurmi, K., Åkerman, K. E. O., Kovanen, P. T., Öörni, K., and Eklund, K. K. (2013) Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. *J. Biol. Chem.* 288, 13410–9.

31. Hulsmans, M., and Holvoet, P. (2010) The vicious circle between oxidative stress and inflammation in atherosclerosis. *J. Cell. Mol. Med*. 14, 70–8.

32. Kondo, T., Hirose, M., and Kageyama, K. (2009) Roles of oxidative stress and redox regulation in atherosclerosis. *J. Atheroscler. Thromb*. 16, 532–8.

33. Ottaviano, F. G., Handy, D. E., and Loscalzo, J. (2008) Redox regulation in the extracellular environment. *Circ. J*. 72, 1–16.

34. Patel, R. P., Moellering, D., Murphy-Ullrich, J., Jo, H., Beckman, J. S., and Darley-Usmar, V. M. (2000) Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. *Free Radic. Biol. Med*. 28, 1780–94.

35. Lähdesmäki, K., Öörni, K., Alanne-Kinnunen, M., Jauhiainen, M., Hurt-Camejo, E., and Kovanen, P. T. (2012) Acidity and lipolysis by group V secreted phospholipase A(2) strongly increase the binding of apoB-100-containing lipoproteins to human aortic proteoglycans. *Biochim. Biophys. Acta* 1821, 257–67.

36. Oörni, K., and Kovanen, P. T. (2006) Enhanced extracellular lipid accumulation in acidic environments. *Curr. Opin. Lipidol*. 17, 534–540.

37. Plihtari, R., Kovanen, P. T., and Öörni, K. (2011) Acidity increases the uptake of native LDL by human monocyte-derived macrophages. *Atherosclerosis* 217, 401–6.

38. Sneck, M., Kovanen, P. T., and Oörni, K. (2005) Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans. *J. Biol. Chem.* 280, 37449–54.

39. Singh, S. K., Thirumalai, A., Hammond, D. J., Pangburn, M. K., Mishra, V. K., Johnson, D. A., Rusiñol, A. E., and Agrawal, A. (2012) Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis. *J. Biol. Chem.* 287, 3550–8.

40. Miyazawa, K., Kiyono, S., and Inoue, K. (1988) Modulation of stimulus-dependent human platelet activation by C-reactive protein modified with active oxygen species. *J. Immunol*. 141, 570–4.

41. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34, 1345–53.

42. Brown, A. J., Leong, S. L., Dean, R. T., and Jessup, W. (1997) 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *J. Lipid Res*. 38, 1730–45.

43. Zha, X., Tabas, I., Leopold, P. L., Jones, N. L., and Maxfield, F. R. (1997) Evidence for Prolonged Cell-Surface Contact of Acetyl-LDL Before Entry Into Macrophages. *Arterioscler. Thromb. Vasc. Biol*. 17, 1421–1431.

44. Agrawal, A., Simpson, M. J., Black, S., Carey, M. P., and Samols, D. (2002) A C-reactive protein mutant that does not bind to phosphocholine and pneumococcal C-polysaccharide. *J. Immunol*. 169, 3217–22.

45. Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Lehtinen, M. J., Lindeman, N., Anderson, D. H., Johnson, P. T., Järvelä, I., Jokiranta, T. S., Hageman, G. S., Immonen, I., and Meri, S. (2007) Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *J. Immunol*. 178, 3831–6.

46. Agrawal, A., Gang, T. B., and Rusiñol, A. E. (2014) Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflamm*. 2014, 319215.

47. Wang, M.-Y., Ji, S.-R., Bai, C.-J., El Kebir, D., Li, H.-Y., Shi, J.-M., Zhu, W., Costantino, S., Zhou, H.-H., Potempa, L. A., Zhao, J., Filep, J. G., and Wu, Y. (2011) A redox switch in C-reactive protein modulates activation of endothelial cells. *FASEB J.* 25, 3186–96.

48. Dimitrov, J. D., Kaveri, S. V, and Lacroix-Desmazes, S. (2013) Gain of function of immunoglobulins after partial unfolding or cofactor binding. *Mol. Immunol*. 55, 195–6.

49. Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008) Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–6.

CHAPTER 5

SUMMARY

The major findings of our studies on determining the role of Oct-1 in regulating CRP gene expression were:

1. Overexpression of Oct-1 inhibited (IL-6+IL-1β)-induced CRP promoter driven luciferase activity. This inhibition occurred irrespective of the length of the promoter used, suggesting that Oct-1 acts as a transcriptional repressor of CRP expression. The 157bp proximal promoter is sufficient for the Oct-1 mediated repressive action.
2. The -59 to -66 region is crucial for full (IL-6+IL-1β)-induced CRP expression, as deletion of Oct-1 site within this region drastically reduced (IL-6+IL-1β)-induced CRP promoter driven luciferase activity. This was an expected result as the Oct-1 site overlaps the binding site for the IL-1β induced transcription factor NF-κB.
3. Overexpression of Oct-1 inhibited (IL-6+IL-1β)-induced CRP promoter driven luciferase activity even in the absence of the Oct-1 binding site, indicating that Oct-1 mediated inhibition of CRP expression was not mediated solely by the binding of Oct-1 to its cognate site.
4. C/EBPβ-induced CRP promoter driven luciferase activity was reduced when the Oct-1 site was mutated despite the C/EBPβ binding site not overlapping with the Oct-1 binding site. This suggests that for full C/EBPβ-induced CRP expression C/EBPβ has to interact with another transcription factor bound to the -59 to -66 region.
5. Oct-1 overexpression inhibited the residual C/EBPβ-induced CRP promoter driven luciferase activity even in the absence of the Oct-1 binding site, further indicating that

the repressive effects of Oct-1 on CRP gene expression were not mediated solely by Oct-1 binding to its cognate site on the promoter.

The main drawback of our experimental design was in our use of the mutated Oct-1 promoter constructs, which were made by complete deletion of the -59 to -66 region on the CRP proximal promoter. This abolished the binding of Oct-1 to the CRP promoter, but also the binding of the transcription factors HNF-1, HNF-3 and the NF-κB p50-p65 heterodimer, whose binding sites overlap with the Oct-1 binding site. Our efforts to specifically abolish the binding of Oct-1 to the CRP promoter but retain the HNF-1, HNF-3 and NF-κB binding activities have not been successful so far. Secondly, the binding of transcription factors to the CRP promoter was studied using a short 45 bp fragment of the CRP proximal promoter. This does not give us a complete picture of transcription factor – CRP promoter interactions *in vivo*. It is necessary to use techniques like chromatin immunoprecipitation (ChIP) for a more accurate understanding of transcription factor – promoter interactions.

Our studies also showed that Oct-1 mediated inhibition, and C/EBPβ-induced CRP gene expression occurred even without direct binding of these proteins to their cognate sites on the promoter. This suggests that both Oct-1 and C/EBPβ partly mediate their effects indirectly by interacting with other transcription factors bound to the promoter. Which transcription factors Oct-1 and C/EBPβ interact with, and what changes occur to these complexes on cytokine induction remain to be determined.

The major results from our studies on using site-directed mutagenesis to make a mutant CRP that binds ox-LDL under physiological conditions can be summarized as follows:

1. Mutation of Glu^{42} → Gln resulted in a CRP mutant that showed significant binding to

ox-LDL at physiological pH. Furthermore, for maximal binding the E42Q mutant required a pH less acidic than that required by WT CRP. The E42Q mutant was also more efficient at binding immobilized ox-LDL at any given pH.

2. Acidic pH does not monomerize or aggregate WT and mutant CRP. It also does not disrupt the global protein structure, but only induces a local structural change in hydrophobicity, thus reducing CRP's affinity to Ca^{2+}. These pH-dependent changes are reversible and suggest that the CRP pentamer undergoes a transition between 2 conformational forms at sites of localized inflammation.
3. E42Q bound to not just immobilized ox-LDL with higher avidity than WT CRP but also to other immobilized, conformationally altered or aggregated proteins when both were exposed to acidic pH.

The results of our studies using H_2O_2 as a modifier of CRP structure and function can be summarized as follows:

1. CRP gains the ability to bind immobilized ox-LDL in the presence of H_2O_2. The ox-LDL binding ability is retained even after H2O2 has been dialyzed out, and the modified CRP has been repurified.
2. 1% H_2O_2-treated CRP (H_2O_2-CRP) binds to not only ox-LDL but also a variety of immobilized, aggregated, or conformationally altered proteins.
3. The PCh-binding site in CRP is not disrupted by H_2O_2 treatment. H_2O_2-CRP retains its pentameric structure, but shows an increase in hydrophobicity indicating that H_2O_2 treatment induces a local structural change.

Our results validate the approach of using site-directed mutagenesis to study *in vitro* the interactions of CRP – modified LDL as they might occur at inflammatory sites. Only one amino acid substitution was sufficient to significantly increase CRP – ox-LDL binding, raising the possibility that more such substitutions will give us a CRP pentamer permanently in an 'active' confirmation. Efforts on constructing more such mutant CRPs and on making a mutant CRP that is more effective than E42Q in binding to ox-LDL are currently underway in our lab.

In addition to an acidic pH, atherosclerotic lesion sites are also characterized by free radical induced changes in the redox environment, a known modifier of protein structure and function (Patel *et al.* 2000; Ottaviano *et al.* 2008; Kondo *et al.* 2009; Hulsmans and Holvoet 2010; Papaharalambus and Griendling 2010). Such an environment may induce structural and conformational changes in CRP, enabling it to bind to deposited ox-LDL. Previous studies have shown that CRP can be modified by redox changes and that these changes significantly affect its function (Miyazawa *et al.* 1988; Wang *et al.* 2011). Such conformational changes are necessary not just for the function of CRP but also for the functions of other molecules involved in host defense (Tada *et al.* 2008; Schroeder *et al.* 2011; Dimitrov *et al.* 2013). Our preliminary studies using H_2O_2 treatment of CRP indicate that ROS may induce structural alterations in CRP at atherosclerotic lesions. These changes appear to be similar to that induced by acidic pH, i.e., the CRP pentamer remains intact, but a local structural change exposes the hidden ox-LDL binding site. It remains to be seen if these ROS-induced changes are reversible or if they involve a permanent structural alteration. ROS-induced CRP binding to ox-LDL involves a loosening of the CRP pentamer, but whether our results are accurate, or if they are an experimental artifact due to the presence of trace amounts of mCRP remains to be seen. We are currently continuing these studies in the lab.

Our lab has previously shown that native CRP binds to E-LDL in a Ca^{2+}-dependent manner at physiological pH, and that such CRP-bound E-LDL did not transform macrophages into foam cells (Singh *et al.* 2008). We could not previously extend this study to examine the effect of CRP on ox-LDL induced foam cell formation as CRP does not bind ox-LDL at physiological pH. Using modified CRPs - mutant CRP like E42Q and CRP modified by inflammatory mediators - we are also now extending our previous studies to determine if CRP can prevent ox-LDL induced foam cell formation.

Previous studies have shown that WT CRP had no significant affect in animal models of atherosclerosis (reviewed in Agrawal *et al.* 2010; Agrawal *et al.* 2014). The development of atherosclerosis in these animal models differs in many respects from that seen in humans (Daugherty 2002; Libby *et al.* 2011). It is possible that a sufficiently inflammatory environment did not develop at atherosclerotic lesion sites in these animals – CRP did not undergo any structural change and so could not bind ox-LDL. The E42Q CRP binds ox-LDL at a less acidic pH than WT CRP and even when $[Ca^{2+}]$ was 20-fold lower than normal – it should therefore be able to bind ox-LDL *in vivo* in these animal models even if they lack an inflammatory environment at the atherosclerotic lesion site. We propose to use mutant CRP like E42Q CRP as a tool to test our hypothesis that CRP has to undergo a structural modification to bind to ox-LDL, prevent ox-LDL induced foam cell formation, and inhibit the progression of atherosclerosis.

To summarize, our studies indicate that CRP has a dual function depending on the environmental context. In circulation CRP remains as an unchanged native pentamer and binds to PCh-containing molecules in a Ca^{2+}-dependent manner. Ligand bound CRP activates the classical complement pathway and mediates clearance of pathogens and other cellular debris

from circulation. At sites of localized inflammation CRP undergoes a reversible structural modification that exposes a hidden binding site and can now bind to deposited, aggregated, and amyloidogenic proteins. In the case of ox-LDL the binding of such modified CRP to ox-LDL may prevent its uptake by macrophages and subsequent foam cell formation, thereby slowing or inhibiting atherogenesis. Such modified CRP may function not just during atherosclerosis but also in other inflammatory diseases involving deposition of proteins at sites of localized inflammation and in which CRP has been implicated (Szalai *et al.* 2003; Carlucci *et al.* 2010).

REFERENCES

Abernethy, T. J., and Avery, O. T. (1941). The occurrence during acute infections of a protein not normally present in the blood: I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of Pneumococcus. *J. Exp. Med.* 73, 173–82.

Agrawal, A., and Volanakis, J. E. (1994) Probing the C1q-binding site on human C-reactive protein by site-directed mutagenesis. *J. Immunol*. 152, 5404–10.

Agrawal, A., Lee, S., Carson, M., Narayana, S. V, Greenhough, T. J., and Volanakis, J. E. (1997) Site-directed mutagenesis of the phosphocholine-binding site of human C-reactive protein: role of Thr76 and Trp67. *J. Immunol*. 158, 345–50.

Agrawal, A., Cha-Molstad, H., Samols, D., and Kushner, I. (2001) Transactivation of C-reactive protein by IL-6 requires synergistic interaction of CCAAT/enhancer binding protein beta (C/EBP beta) and Rel p50. *J. Immunol*. 166, 2378–2384.

Agrawal, A., Shrive, A. K., Greenhough, T. J., and Volanakis, J. E. (2001) Topology and structure of the C1q-binding site on C-reactive protein. *J. Immunol*. 166, 3998–4004.

Agrawal, A., Cha-Molstad, H., Samols, D., and Kushner, I. (2003) Overexpressed nuclear factor-kappaB can participate in endogenous C-reactive protein induction, and enhances the effects of C/EBPbeta and signal transducer and activator of transcription-3. *Immunology* 108, 539–47.

Agrawal, A., Suresh, M. V, Singh, S. K., and Ferguson, D. A. (2008) The protective function of human C-reactive protein in mouse models of Streptococcus pneumoniae infection. *Endocr Metab Immune Disord Drug Targets* 8, 231–237.

Agrawal, A., Singh, P. P., Bottazzi, B., Garlanda, C., and Mantovani, A. (2009) Pattern recognition by pentraxins. *Adv Exp Med Biol* 653, 98–116.

Agrawal, A., Hammond, D. J., and Singh, S. K. (2010) Atherosclerosis-related functions of C-reactive protein. *Cardiovasc. Hematol. Disord. drug targets.* 10, 235–240.

Agrawal, A., Gang, T. B., and Rusiñol, A. E. (2014) Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflamm*. 2014, 319215.

Bang, R., Marnell, L., Mold, C., Stein, M.-P., Clos, K. T. Du, Chivington-Buck, C., and Clos, T. W. Du (2005) Analysis of binding sites in human C-reactive protein for Fc{gamma}RI, Fc{gamma}RIIA, and C1q by site-directed mutagenesis. *J. Biol. Chem*. 280, 25095–102.

Bhakdi, S., Torzewski, M., Klouche, M., and Hemmes, M. (1999) Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler. Thromb. Vasc. Biol*. 19, 2348–54.

Bhat, R., Weaver, J. A., Sterling, K. M., and Bresnick, E. (1996) Nuclear transcription factor Oct-1 binds to the 5'-upstream region of CYP1A1 and negatively regulates its expression. *Int. J. Biochem. Cell Biol*. 28, 217–27.

Black, S., Agrawal, A., and Samols, D. (2003) The phosphocholine and the polycation-binding sites on rabbit C-reactive protein are structurally and functionally distinct. *Mol. Immunol*. 39, 1045–54.

Blaschke, F., Takata, Y., Caglayan, E., Collins, A., Tontonoz, P., Hsueh, W. A., and Tangirala, R. K. (2006) A nuclear receptor corepressor-dependent pathway mediates suppression of cytokine-induced C-reactive protein gene expression by liver X receptor. *Circ. Res*. 99(12), e88–99.

Carlucci, F., Cook, H. T., Garg, A., Pepys, M. B., and Botto, M. (2010) Lack of effect of a single injection of human C-reactive protein on murine lupus or nephrotoxic nephritis. *Arthritis Rheum*. 62, 245–9.

Castell, J. V, Gómez-Lechón, M. J., David, M., Fabra, R., Trullenque, R., and Heinrich, P. C. (1990) Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology* 12, 1179–86.

Cha-Molstad, H., Young, D. P., Kushner, I., and Samols, D. (2007) The interaction of C-Rel with C/EBPbeta enhances C/EBPbeta binding to the C-reactive protein gene promoter. *Mol. Immunol*. 44, 2933–2942.

Chang, M.-K., Binder, C. J., Torzewski, M., and Witztum, J. L. (2002) C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13043-13048.

Darlington, G. J., Wilson, D. R., and Lachman, L. B. (1986) Monocyte-conditioned medium, interleukin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. *J. Cell Biol*. 103, 787–793.

Daugherty, A. (2002) Mouse models of atherosclerosis. *Am. J. Med. Sci*. 323, 3–10.

Dong, B., Huang, C., Li, D., and Zhao, F.-Q. (2009) Oct-1 functions as a transactivator in the hormonal induction of beta-casein gene expression. *Mol. Cell. Biochem*. 328, 93–9.

Dimitrov, J. D., Kaveri, S. V, and Lacroix-Desmazes, S. (2013) Gain of function of immunoglobulins after partial unfolding or cofactor binding. *Mol. Immunol*. 55, 195–6.

Gabay, C., and Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med*. 340, 448–54.

Gaboriaud, C., Juanhuix, J., Gruez, A., Lacroix, M., Darnault, C., Pignol, D., Verger, D., Fontecilla-Camps, J. C., and Arlaud, G. J. (2003) The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. *J. Biol. Chem*. 278, 46974–82.

Ganapathi, M. K., Schultz, D., Mackiewicz, A., Samols, D., Hu, S. I., Brabenec, A., Macintyre, S. S., and Kushner, I. (1988) Heterogeneous nature of the acute phase response. Differential regulation of human serum amyloid A, C-reactive protein, and other acute phase proteins by cytokines in Hep 3B cells. *J. Immunol.* 141, 564–569.

Gang, T. B., Hammond, D. J., Singh, S. K., Ferguson, D. A., Mishra, V. K., and Agrawal, A. (2012) The Phosphocholine-binding pocket on C-reactive protein is necessary for initial protection of mice against pneumococcal infection. *J. Biol. Chem.* 287, 43116–25.

Ganter, U., Arcone, R., Toniatti, C., Morrone, G., and Ciliberto, G. (1989) Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. *EMBO J.* 8, 3773–9.

Grimm, A. A., Brace, C. S., Wang, T., Stormo, G. D., and Imai, S. (2011) A nutrient-sensitive interaction between Sirt1 and HNF-1α regulates Crp expression. *Aging Cell* 10, 305–17.

Haka, A. S., Grosheva, I., Chiang, E., Buxbaum, A. R., Baird, B. A., Pierini, L. M., and Maxfield, F. R. (2009) Macrophages create an acidic extracellular hydrolytic compartment to digest aggregated lipoproteins. *Mol. Biol. Cell* 20, 4932–4940.

Hammond, D. J., Singh, S. K., Thompson, J. a, Beeler, B. W., Rusiñol, A. E., Pangburn, M. K., Potempa, L. and Agrawal, A. (2010) Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J. Biol. Chem.* 285, 36235–44.

Hatanaka, K., Li, X. A., Masuda, K., Yutani, C., and Yamamoto, A. (1995) Immuno-histochemical localization of C-reactive protein-binding sites in human atherosclerotic aortic lesions by a modified streptavidin-biotin-staining method. *Pathol. Int.* 45, 635–41.

Hirschfield, G. M., Gallimore, J. R., Kahan, M. C., Hutchinson, W. L., Sabin, C. A., Benson, G. M., Dhillon, A. P., Tennent, G. A., and Pepys, M. B. (2005) Transgenic human C-reactive protein is not pro-atherogenic in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8309–8314.

Hulsmans, M., and Holvoet, P. (2010) The vicious circle between oxidative stress and inflammation in atherosclerosis. *J. Cell. Mol. Med*. 14, 70–8.

Hurlimann, J., Thorbecke, G. J., and Hochwald, G. M. (1966) The liver as the site of C-reactive protein formation. *J. Exp. Med*. 123, 365–78.

Inamoto, S., Segil, N., Pan, Z. Q., Kimura, M., and Roeder, R. G. (1997) The cyclin-dependent kinase-activating kinase (CAK) assembly factor, MAT1, targets and enhances CAK activity on the POU domains of octamer transcription factors. *J. Biol. Chem*. 272, 29852–8.

Kleemann, R., Gervois, P. P., Verschuren, L., Staels, B., Princen, H. M. G., and Kooistra, T. (2003) Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. *Blood* 101, 545–551.

Koike, T., Kitajima, S., Yu, Y., Nishijima, K., Zhang, J., Ozaki, Y., Morimoto, M., Watanabe, T., Bhakdi, S., Asada, Y., Chen, Y. E., and Fan, J. (2009) Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation* 120, 2088–94.

Kondo, T., Hirose, M., and Kageyama, K. (2009) Roles of oxidative stress and redox regulation in atherosclerosis. *J. Atheroscler. Thromb*. 16, 532–8.

Kovacs, A., Tornvall, P., Nilsson, R., Tegnér, J., Hamsten, A., and Björkegren, J. (2007) Human C-reactive protein slows atherosclerosis development in a mouse model with human-like hypercholesterolemia. *Proc. Natl. Acad. Sci. U. S. A.* 104, 13768–73.

Kramer, F., Torzewski, J., Kamenz, J., Veit, K., Hombach, V., Dedio, J., and Ivashchenko, Y. (2008) Interleukin-1beta stimulates acute phase response and C-reactive protein synthesis by inducing an NFkappaB- and C/EBPbeta-dependent autocrine interleukin-6 loop. *Mol. Immunol*. 45, 2678–89.

Kushner, I., and Kaplan, M. H. (1961) Studies of acute phase protein. I. An immunohistochemical method for the localization of Cx-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. *J. Exp. Med*. 114, 961–974.

Kushner, I., and Feldmann, G. (1978) Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. *J. Exp. Med*. 148, 466–77.

Kushner, I. (1982). The phenomenon of the acute phase response. *Ann. N. Y. Acad. Sci.* 389, 39–48.

Kushner, I., and Elyan, M. (2008) Why does C-reactive protein predict coronary events? *Am. J. Med*. 121(7), e11.

Lähdesmäki, K., Öörni, K., Alanne-Kinnunen, M., Jauhiainen, M., Hurt-Camejo, E., and Kovanen, P. T. (2012) Acidity and lipolysis by group V secreted phospholipase A(2) strongly increase the binding of apoB-100-containing lipoproteins to human aortic proteoglycans. *Biochim. Biophys. Acta* 1821, 257–67.

Leake, D. S. (1997) Does an acidic pH explain why low density lipoprotein is oxidised in atherosclerotic lesions? *Atherosclerosis* 129, 149–157.

Li, S. P., and Goldman, N. D. (1996) Regulation of human C-reactive protein gene expression by two synergistic IL-6 responsive elements. *Biochemistry* 35, 9060–8.

Libby, P., and Ridker, P. M. (2004) Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. *Am. J. Med*. 116 Suppl , 9S–16S.

Libby, P., Ridker, P. M., and Hansson, G. K. (2011) Progress and challenges in translating the biology of atherosclerosis. *Nature* 473, 317–25.

Macleod, C. M., and Avery, O. T. (1941a).The occurrence during acute infections of a protein not normally present in the blood: II. Isolation and properties of the reactive protein. *J. Exp. Med*. 73, 183–90.

Macleod, C. M., and Avery, O. T. (1941b).The occurrence during acute infections of a protein not normally present in the blood: III. Immunological properties of the C-reactive Protein and its differentiation from normal blood proteins. *J. Exp. Med*. 73, 183–90.

Mold, C., Nakayama, S., Holzer, T. J., Gewurz, H., and Du Clos, T. W. (1981) C-reactive protein is protective against Streptococcus pneumoniae infection in mice. *J. Exp. Med.* 154, 1703–8.

Moore, K. J., and Tabas, I. (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341–355.

Miyazawa, K., Kiyono, S., and Inoue, K. (1988) Modulation of stimulus-dependent human platelet activation by C-reactive protein modified with active oxygen species. *J. Immunol*. 141, 570–4.

Naghavi, M., John, R., Naguib, S., Siadaty, M. S., Grasu, R., Kurian, K. C., van Winkle, W. B., Soller, B., Litovsky, S., Madjid, M., Willerson, J. T., and Casscells, W. (2002) pH Heterogeneity of human and rabbit atherosclerotic plaques; a new insight into detection of vulnerable plaque. *Atherosclerosis* 164, 27–35.

Nakshatri, H., Nakshatri, P., and Currie, R. A. (1995) Interaction of Oct-1 with TFIIB. Implications for a novel response elicited through the proximal octamer site of the lipoprotein lipase promoter. *J. Biol. Chem*. 270, 19613–23.

Nishikawa, T., Hagihara, K., Serada, S., Isobe, T., Matsumura, A., Song, J., Tanaka, T., Kawase, I., Naka, T., and Yoshizaki, K. (2008) Transcriptional complex formation of c-Fos, STAT3, and hepatocyte NF-1 alpha is essential for cytokine-driven C-reactive protein gene expression. *J. Immunol.* 180, 3492–501.

Ochrietor, J. D., Harrison, K. A., Zahedi, K., and Mortensen, R. F. (2000) Role of STAT3 and C/EBP in cytokine-dependent expression of the mouse serum amyloid P-component (SAP) and C-reactive protein (CRP) genes. *Cytokine* 12, 888–899.

Oörni, K., and Kovanen, P. T. (2006) Enhanced extracellular lipid accumulation in acidic environments. *Curr. Opin. Lipidol*. 17, 534–540.

Ortiz, M. A., Campana, G. L., Woods, J. R., Boguslawski, G., Sosa, M. J., Walker, C. L., and Labarrere, C. A. (2009) Continuously-infused human C-reactive protein is neither proatherosclerotic nor proinflammatory in apolipoprotein E-deficient mice. *Exp. Biol. Med*. 234, 624–631.

Ottaviano, F. G., Handy, D. E., and Loscalzo, J. (2008) Redox regulation in the extracellular environment. *Circ. J*. 72, 1–16.

Papaharalambus, C. A., and Griendling, K. K. (2007) Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc. Med*. 17, 48–54.

Patel, D. N., King, C. A., Bailey, S. R., Holt, J. W., Venkatachalam, K., Agrawal, A., Valente, A. J., and Chandrasekar, B. (2007) Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J. Biol. Chem*. 282, 27229–38.

Patel, R. P., Moellering, D., Murphy-Ullrich, J., Jo, H., Beckman, J. S., and Darley-Usmar, V. M. (2000) Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. *Free Radic. Biol. Med*. 28, 1780–94.

Pepys, M. B., and Hirschfield, G. M. (2003) C-reactive protein: a critical update. *J. Clin. Invest*. 111, 1805–12.

Poli, V., and Cortese, R. (1989) Interleukin 6 induces a liver-specific nuclear protein that binds to the promoter of acute-phase genes. *Proc. Natl. Acad. Sci. U. S. A*. 86, 8202–8206.

Plihtari, R., Kovanen, P. T., and Öörni, K. (2011) Acidity increases the uptake of native LDL by human monocyte-derived macrophages. *Atherosclerosis* 217, 401–6.

Préfontaine, G. G., Walther, R., Giffin, W., Lemieux, M. E., Pope, L., and Haché, R. J. (1999) Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. *J. Biol. Chem*. 274, 26713–9.

Ramji, D. P., Vitelli, A., Tronche, F., Cortese, R., and Ciliberto, G. (1993) The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBP delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. *Nucleic Acids Res*. 21, 289–294.

Reiner, A. P., Barber, M. J., Guan, Y., Ridker, P. M., Lange, L. A., Chasman, D. I., Walston, J. D., Cooper, G. M., Jenny, N. S., Rieder, M. J., Durda, J. P., Smith, J. D., Novembre, J., Tracy, R. P., Rotter, J. I., Stephens, M., Nickerson, D. A., and Krauss, R. M. (2008)

Polymorphisms of the HNF1A gene encoding hepatocyte nuclear factor-1 alpha are associated with C-reactive protein. *Am. J. Hum. Genet*. 82, 1193–201.

Reynolds, G. D., and Vance, R. P. (1987) C-reactive protein immune-histochemical localization in normal and atherosclerotic human aortas. *Arch. Pathol. Lab. Med*. 111, 265–269.

Rodriguez, W., Mold, C., Marnell, L. L., Hutt, J., Silverman, G. J., Tran, D., and Du Clos, T. W. (2006) Prevention and reversal of nephritis in MRL/lpr mice with a single injection of C-reactive protein. *Arthritis Rheum*. 54, 325–35.

Ross, R. (1999) Atherosclerosis--an inflammatory disease. *N. Engl. J. Med*. 340, 115–26.

Roux, K. H., Kilpatrick, J. M., Volanakis, J. E., and Kearney, J. F. (1983) Localization of the phosphocholine-binding sites on C-reactive protein by immunoelectron microscopy. *J. Immunol.* 131, 2411–5.

Schroeder, B. O., Wu, Z., Nuding, S., Groscurth, S., Marcinowski, M., Beisner, J., Buchner, J., Schaller, M., Stange, E. F., and Wehkamp, J. (2011) Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1. *Nature* 469, 419–23.

Shakya, A., Kang, J., Chumley, J., Williams, M. A., and Tantin, D. (2011) Oct1 is a switchable, bipotential stabilizer of repressed and inducible transcriptional states. *J. Biol. Chem*. 286, 450–9.

Shrive, A. K., Cheetham, G. M., Holden, D., Myles, D. A., Turnell, W. G., Volanakis, J. E., Pepys, M. B., Bloomer, A. C., and Greenhough, T. J. (1996) Three dimensional structure of human C-reactive protein. *Nat. Struct. Biol*. 3, 346–354.

Silver, I. A., Murrills, R. J., and Etherington, D. J. (1988) Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp. Cell Res*. 175, 266–276.

Simons, J. P., Loeffler, J. M., Al-Shawi, R., Ellmerich, S., Hutchinson, W. L., Tennent, G. A., Petrie, A., Raynes, J. G., de Souza, J. B., Lawrence, R. A., Read, K. D., and Pepys, M. B. (2014) C-reactive protein is essential for innate resistance to pneumococcal infection. *Immunology* 142, 414–20.

Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986) A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* 319, 154–8.

Singh, P. P., Voleti, B., and Agrawal, A. (2007) A novel RBP-J kappa-dependent switch from C/EBP beta to C/EBP zeta at the C/EBP binding site on the C-reactive protein promoter. *J. Immunol*. 178, 7302–9.

Singh, S. K., Suresh, M. V, Praythe r, D. C., Moorman, J. P., Rusiñol, A. E., and Agrawal, A. (2008) Phosphoethanolamine-complexed C-reactive protein: a pharmacological-like macromolecule that binds to native low-density lipoprotein in human serum. *Clin. Chim. Acta*. 394, 94–8.

Singh, S. K., Suresh, M. V, Praythe r, D. C., Moorman, J. P., Rusiñol, A. E., and Agrawal, A. (2008) C-reactive protein-bound enzymatically modified low-density lipoprotein does not transform macrophages into foam cells. *J. Immunol*. 180, 4316–22.

Singh, S. K., Suresh, M. V, Hammond, D. J., Rusiñol, A. E., Potempa, L. a, and Agrawal, A. (2009) Binding of the monomeric form of C-reactive protein to enzymatically-modified low-density lipoprotein: effects of phosphoethanolamine. *Clin. Chim. Acta*. 406, 151–5.

Sneck, M., Kovanen, P. T., and Oörni, K. (2005) Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans. *J. Biol. Chem*. 280, 37449–54.

Ström, A. C., Forsberg, M., Lillhager, P., and Westin, G. (1996) The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. *Nucleic Acids Res*. 24, 1981–6.

Sun, H., Koike, T., Ichikawa, T., Hatakeyama, K., Shiomi, M., Zhang, B., Kitajima, S., Morimoto, M., Watanabe, T., Asada, Y., Chen, Y. E., and Fan, J. (2005) C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. *Am. J. Pathol*. 167, 1139–48.

Suresh, M. V, Singh, S. K., Ferguson, D. A., and Agrawal, A. (2006) Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J. Immunol*. 176, 4369–74.

Suresh, M. V, Singh, S. K., Ferguson, D. A., and Agrawal, A. (2007) Human C-reactive protein protects mice from Streptococcus pneumoniae infection without binding to pneumococcal C-polysaccharide. *J. Immunol*. 178, 1158–63.

Szalai, A. J., Briles, D. E., and Volanakis, J. E. (1995) Human C-reactive protein is protective against fatal Streptococcus pneumoniae infection in transgenic mice. *J. Immunol*. 155, 2557–63.

Szalai, A. J., Agrawal, A., Greenhough, T. J., and Volanakis, J. E. (1999) C-reactive protein: structural biology and host defense function. *Clin. Chem. Lab. Med*. 37, 265–70.

Szalai, A. J., Weaver, C. T., McCrory, M. A., van Ginkel, F. W., Reiman, R. M., Kearney, J. F., Marion, T. N., and Volanakis, J. E. (2003) Delayed lupus onset in (NZB x NZW)F1 mice expressing a human C-reactive protein transgene. *Arthritis Rheum*. 48, 1602–11.

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008) Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–6.

Tennent, G. A., Hutchinson, W. L., Kahan, M. C., Hirschfield, G. M., Gallimore, J. R., Lewin, J., Sabin, C. A., Dhillon, A. P., and Pepys, M. B. (2008) Transgenic human CRP is not pro-atherogenic, pro-atherothrombotic or pro-inflammatory in apoE-/- mice. *Atherosclerosis* 196, 248–55.

Teupser, D., Weber, O., Rao, T. N., Sass, K., Thiery, J., and Fehling, H. J. (2011) No reduction of atherosclerosis in C-reactive protein (CRP)-deficient mice. *J. Biol. Chem*. 286, 6272–6279.

Thompson, D., Pepys, M. B., and Wood, S. P. (1999) The phsiological structure of human C-reactive protein and its complex with phosphocholine. *Struct. London Engl*. 1993 7, 169–177.

Toniatti, C., Demartis, A., Monaci, P., Nicosia, A., and Ciliberto, G. (1990) Synergistic trans-activation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites. *EMBO J*. 9, 4467–75.

Torzewski, M., Reifenberg, K., Cheng, F., Wiese, E., Küpper, I., Crain, J., Lackner, K. J., and Bhakdi, S. (2008) No effect of C-reactive protein on early atherosclerosis in LDLR-/- / human C-reactive protein transgenic mice. *Thromb. Haemost*. 99, 196–201.

Volanakis, J. E. (2001) Human C-reactive protein: expression, structure, and function. *Mol. Immunol*. 38, 189–97.

Voleti, B., and Agrawal, A. (2005) Regulation of basal and induced expression of C-reactive protein through an overlapping element for OCT-1 and NF-kappaB on the proximal promoter. *J. Immunol*. 175, 3386–90.

Voss, J. W., Wilson, L., and Rosenfeld, M. G. (1991) POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. *Genes Dev*. 5, 1309–20.

Wang, P., and Jin, T. (2010) Oct-1 functions as a sensor for metabolic and stress signals. *Islets* 2, 46–8.

Wang, M.-Y., Ji, S.-R., Bai, C.-J., El Kebir, D., Li, H.-Y., Shi, J.-M., Zhu, W., Costantino, S., Zhou, H.-H., Potempa, L. A., Zhao, J., Filep, J. G., and Wu, Y. (2011) A redox switch in C-reactive protein modulates activation of endothelial cells. *FASEB J*. 25, 3186–96.

Wang, Y., Ripperger, J., Fey, G. H., Samols, D., Kordula, T., Wetzler, M., Van Etten, R. A., and Baumann, H. (1999) Modulation of hepatic acute phase gene expression by epidermal

growth factor and Src protein tyrosine kinases in murine and human hepatic cells. *Hepatology* 30, 682–697.

Yap, S. H., Moshage, H. J., Hazenberg, B. P., Roelofs, H. M., Bijzet, J., Limburg, P. C., Aarden, L. A., and van Rijswijk, M. H. (1991) Tumor necrosis factor (TNF) inhibits interleukin (IL)-1 and/or IL-6 stimulated synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. *Biochim. Biophys. Acta* 1091, 405–408.

Yother, J., Volanakis, J. E., and Briles, D. E. (1982) Human C-reactive protein is protective against fatal Streptococcus pneumoniae infection in mice. *J. Immunol*. 128, 2374–6.

Zhang, D., Jiang, S. L., Rzewnicki, D., Samols, D., and Kushner, I. (1995) The effect of interleukin-1 on C-reactive protein expression in Hep3B cells is exerted at the transcriptional level. *Biochem. J.* 310 (Pt 1), 143–8.

Zhang, D., Sun, M., Samols, D., and Kushner, I. (1996) STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J. Biol. Chem*. 271, 9503–9.

Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H., and Kaufman, R. J. (2006) Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. *Cell* 124, 587–599.

Zhou, D. X., and Yen, T. S. (1991) The ubiquitous transcription factor Oct-1 and the liver-specific factor HNF-1 are both required to activate transcription of a hepatitis B virus promoter. *Mol. Cell. Biol*. 11, 1353–9.

Zwilling, S., Annweiler, A., and Wirth, T. (1994) The POU domains of the Oct1 and Oct2 transcription factors mediate specific interaction with TBP. *Nucleic Acids Res*. 22, 1655–62.

VITA

AVINASH THIRUMALAI

Education:

East Tennessee State University, Johnson City, Tennessee.
PhD – Biomedical Sciences, 2014

Madurai Kamaraj University, Madurai, India.
M.Sc. – Biotechnology, 2002

St. Joseph's College, Bangalore University, Bangalore, India.
B.Sc. – Microbiology, Chemistry, Zoology, 2000

Professional Experience:

Graduate Research Assistant, James H Quillen College of Medicine, East Tennessee State University, Department of Biomedical Sciences, 2009 -2014

Senior Curator, Molecular Connections Pvt. Ltd., Bangalore, India, 2006 – 2009

Research Student, Centre for Molecular Neurobiology, Hamburg, Germany, 2003-2004

Research Student, European Neuroscience Institute, Göttingen, Germany, 2002-2003

Publications:

Thirumalai A, Voleti B, Hammond DJ Jr, and Agrawal A. (2012). Oct-1 acts as a transcriptional repressor on the C-reactive protein promoter. *Molecular Immunology*; 52(3-4):242-8.

Thirumalai A, Singh SK, Hammond DJ Jr, Pangburn MK, Mishra VK, Johnson DA, Rusiñol AE, and Agrawal A. (2012). Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis. *Journal of Biological Chemistry*; 287(5):3550-8.