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Regulation of Nrf2 Transactivation Domain Activity by p160 RAC3/SRC3 and Other Nuclear Co-Regulators

Wen Lin[1], Guoxiang Shen[1], Xiaoling Yuan[1], Mohit R. Jain[1], Siwang Yu[1], Aihua Zhang[2], J. Don Chen[2] and Ah-Ng Tony Kong[1,*]

[1]Department of Pharmaceutics, Ernest-Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA

[2]Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-5635, USA

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Transcription factor NF-E2-related factor 2 (Nrf2) regulates the induction of Phase II detoxifying enzymes and anti-oxidant enzymes in response to many cancer chemopreventive compounds. In this study, we investigated the role of receptor associated coactivator (RAC3) or steroid receptor coactivator-3 (SRC3) and other nuclear co-regulators including CBP/p300 (CREB-binding protein), CARM1 (Coactivator-associated arginine methyltransferase), PRMT1 (Protein arginine methyl-transferase 1), and p/CAF (p300/CBP-associated factor) in the transcriptional activation of a chimeric Gal4-Nrf2-Luciferase system containing the transactivation domain (TAD) of Nrf2 in HepG2 cells. The results indicated that RAC3 up-regulated the transactivation activity of Gal4-Nrf2-(1-370) in a dose-dependent manner. The enhancement of transactivation domain activity of Gal4-Nrf2-(1-370) by RAC3 was dampened in the presence of dominant negative mutants of RAC3. Next we studied the effects of other nuclear co-regulators including CBP/p300, CARM1, PRMT1 and p/CAF, and the results showed that they had different level of positive effects on this transactivation domain activity of Gal4-Nrf2-(1-370). But importantly, synergistic effects of these co-regulators in the presence of RAC3/SRC3 on the transactivation activity of Gal4-Nrf2-(1-370) were observed. In summary, our present study showed for the first time that the 160 RAC3/SRC3 is involved in the functional transactivation of TAD of Nrf2 and that the other nuclear co-regulators such as CBP/p300, CARM1, PRMT1 and p/CAF can also transcriptionally activate this TAD of Nrf2 and that they could further enhance the transactivation activity mediated by RAC3/SRC3.

Keywords: CARM1, CBP/p300, Nrf2, p/CAF, PRMT1, RAC3/SRC3

Introduction

Transcription factor NF-E2-related factor 2 (Nrf2) was demonstrated to regulate the expression of genes encoding antioxidant and phase II detoxifying enzymes, including glutathione S-transferase GST), NADP(H): quinine oxidoreductase (NQO), UDP-glucuronosyltransferase (UGT), heme oxygenase-1 (HO-1) and γ-glutamylcysteine synthetase (γGCS) (Zhang *et al*., 1992; Morse and Stoner, 1993; Venugopal and Jaiswal, 1996; Itoh *et al*., 1997; Chan *et al*., 2001). As a member of the CNC family of bZIP protein, Nrf2 is mainly sequestered in the cytoplasm by tethering to a cytoskeleton-binding protein (Moi *et al.,* 1994) called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1). Upon challenges by oxidative and/or chemical stress induced by reactive oxygen species (ROS), electrophiles as well as many cancer chemopreventive agents, Nrf2 appears to be released from Keap1 binding and enters the nucleus (Itoh *et al.,* 1999; Chan *et al.,* 2001; Nakaso *et al.,* 2003). Upon entering the nucleus, Nrf2 will heterodimerize with small Maf-F/G/K protein, through the leucine zipper dimerization domain and bound to the ARE (antioxidant-response-element) promoter via its DNA binding domain. The ARE has been found to be located in the 5'-flanking region of more than 100 genes than 100

Abbreviations: Nrf2 (NF-E2-related factor 2), RAC3 (Receptor associated coactivator 3), steroid receptor coactivator-3 (SRC3), CBP/p300 (CREB-binding protein), CARM1 (Coactivator-associated arginine methyltransferase), PRMT1 (Protein arginine methyl-transferase 1), p/CAF (p300/CBP-associated factor)

*To whom correspondence should be addressed.
Tel: 1-732-445-3831 ext. 228; Fax: 1-732-445-3134
E-mail: kongt@rci.rutgers.edu

genes including many antioxidant/phase II enzyme genes (Itoh *et al*., 1997; Nguyen *et al.,* 2000; Jain *et al*., 2005). In addition, after Nrf2/Maf bind to the ARE sequence on target genes, they will also recruit and partner with other nuclear regulatory factors that can modulate (increase or decrease) gene transcription. Recent study indicated that CBP (CREB-binding protein) can bind directly to the Nrf2 transactivation domains Neh4 and Neh5 (Katoh *et al.,* 2001), and that CBP showed synergistic stimulation with Raf on the transactivation activity of Gal4-Nrf 2 (1-370) and full length Nrf2 (Shen *et al*., 2004).

Gene transcription activation by transcription factors is accomplished through the recruitment of a series of coactivators (Spiegelman and Heinrich, 2004). Coactivator proteins fulfill their functions through protein-protein interactions that bridge transcription factors and basal transcription machinery and through their chromatin remodeling activities that facilitate the assembly of a transcription initiation complex (McManus and Hendzel, 2001; Xu and O'Malley, 2002). The nuclear receptor-associated coactivator-3 (RAC3) or steroid receptor coactivator-3 (SRC3) is a transcriptional coactivator for nuclear receptors and other transcription factors (Li *et al.,* 1997; Belandia and Parker, 2000; Chen *et al.,* 2000; Leo *et al.,* 2000; Soutoglou *et al.,* 2000; Werbajh *et al.,* 2000). RAC3/SRC3 (also known as ACTR,P/CIP and TRAM-1) functions mainly through interaction with transcription factors and recruitment of histone acetylases (HATs) (such as CBP/p300, and p/CAF) and histone methyltransferase (such as CARM-1, PRMT-1) to the promoter for chromatin remodeling and DNA transcription (Chen *et al.,* 1997; Chen *et al.,* 1999; Chen *et al.,* 2000; Stallcup *et al.,* 2000; Demarest *et al.,* 2002; Stallcup *et al.,* 2003; Yadav *et al.,* 2003). Members of SRC family have been shown to enhance gene transactivation by interacting with a wide variety of transcription factors such as serum response factor (Kim *et al.,* 1998), AP-1 (Kim *et al.,* 1997), STAT (Korzus *et al.,* 1997) and NF-κB (Werbajh *et al.,* 2000). We hypothesized that similar to these transcription factors, SRC family protein may also be involved in Nrf-2-mediated transactivation.

In this study, we tested whether RAC3/SRC3 could participate in the stimulation of transactivation activity of Gal4-Nrf 2 (1-370) with other coactivators. We found that RAC3/SRC3 alone could enhance the transactivation activity of Gal4-Nrf2-(1-370) in a dose-dependant manner. Site-directed mutagenesis on the LXXLL nuclear receptor recognition motif of RAC3/SRC3 decreased the transactivation activity of Gal4-Nrf2-(1-370). Furthermore, we found that CBP, p/CAF, CARM-1 and PRMT1 could further enhance the RAC3/SRC3-mediated transactivation activity on Gal4-Nrf2. Taken together, we showed for the first time that the p160 coactivator RAC3/SRC3 could stimulate the transactivation activity of Gal4-Nrf 2 (1-370) and this could be further enhanced by the coregulators CBP, p/CAF, CARM-1, and PRMT1.

Material and Methods

Cell culture. Human hepatoma HepG2 cells were obtained from ATCC (Manassas, VA) and maintained in F-12 medium supplemented with 10% fetal bovine serum, 1.17 g/liter of sodium bicarbonate, 0.1 units/ml insulin, 0.5 × minimal essential medium amino acid, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were split every 3 days.

Generation of Gal4-Nrf2 chimeras and expression construct. The Gal4-Nrf2 chimera (1-370) was amplified from the Nrf2 template (589 amino acids) by polymerase chain reaction using the following primers: 5'-GGTACCTGGATTTGATTGACATACTT-3' (sense) and 5'-TCTAGATTCCAGGGGCACTATCTAGCTCTTC-3' (antisense); as described by us previously (Shen *et al.,* 2004). CBP was kindly provided by Robert. H. Goodman (Vollum Institute). CARM1 cDNA was kindly provided by Dr. Michael Stallup, University of Southern California. p/CAF, RAC3/SRC3 and its mutants (the two consecutive leucines within each of the LXXLL motifs in the RAC3/SRC3-ID) was described previously (Li *et al.,* 1997).

Transient transfection and reporter gene activity assays. HepG2 cells were plated in 6-well plates at a density of 4.0×10^5 cells/well. Twenty-four hours after plating, cells were transfected with expression plasmids using LipofectAMINE 2000 (Invitrogen) according to the instruction provided by the manufacturer. In brief, cell culture medium was changed to OPTI-MEM medium before each transfection. Total amount of DNA transfected in each well was adjusted to 4 μg by using empty vector pcDNA 3.1. Cells were incubated with transfection mixtures for 5 h and then cultured in fresh F-12 medium for an additional 36 h before harvesting. Luciferase activity was determined according to the manufacturer's protocol (Promega). Briefly, cells were washed once with ice-cold phosphate-buffered saline (pH 7.4) and harvested in 1 × Reporter lysis buffer (Promega). After centrifugation, a 20 μl supernatant was assayed for luciferase activity with a Sirius luminometer (Berthold Detection Systems). The luciferase activity was normalized by protein concentration, determined by BCA protein assay (Pierce). Data were statistically analyzed by ANOVA, followed by unpaired Student's *t*-test.

Results

Up-regulation of Gal4-Nrf2(1-370) chimera transactivation activity by RAC3/SRC3. To assess the functional significance of the role of RAC3/SRC3 on Gal4-Nrf2 transcriptional activity, the Gal4-Nrf2 was coexpressed with different doses of RAC3/SRC3 in HepG2 cells. Co-transfection of RAC3/SRC3 resulted in a 1.3-fold, 2.2-fold, 4.1-fold, and 11.8-fold activation of Gal4-Nrf2-(1-370), at doses of 1.0 μg, 2.0 μg, 3.0 μg and 4.0 μg, respectively (Fig. 1A), suggesting that the Gal4-Nrf2-(1-370) transactivation activity increased in a dose-dependent manner by RAC3/SRC3. In order to verify whether RAC3/SRC3 alone increases Gal4-luciferase

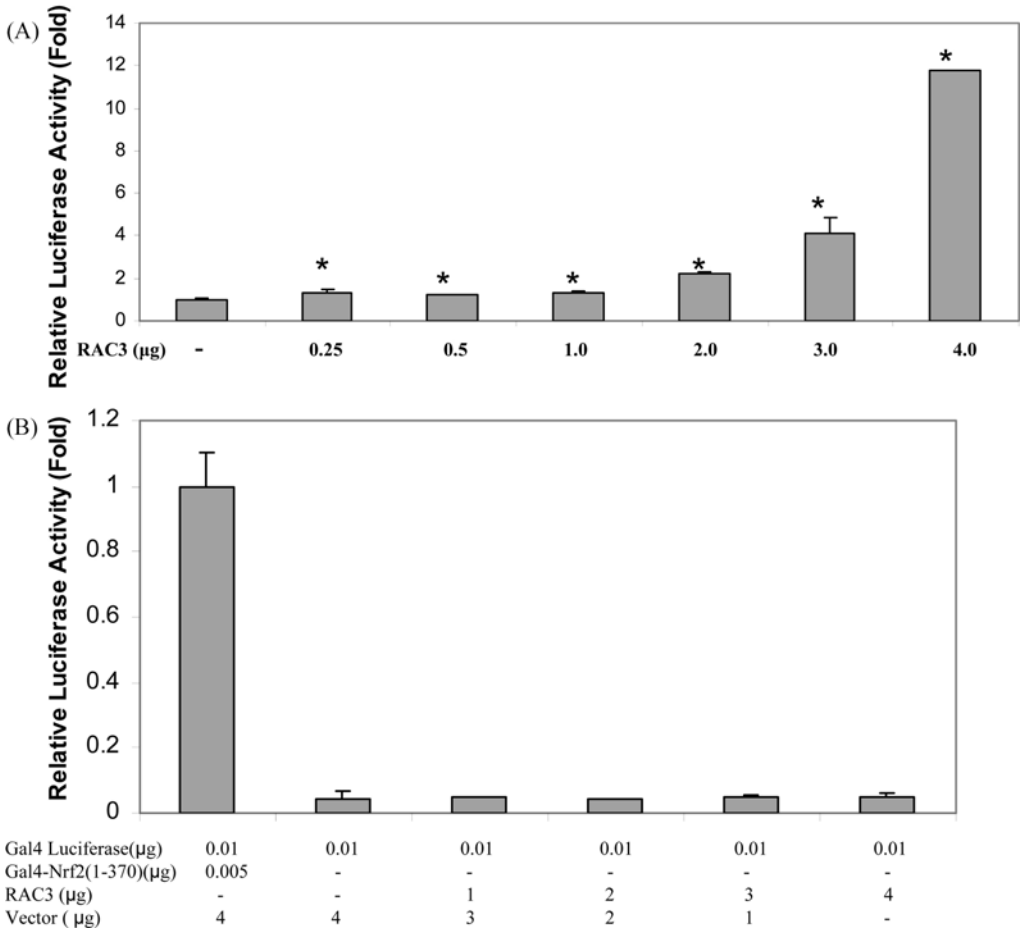

Fig. 1. Regulation of Gal4-Nrf2 chimera transactivation activity by RAC3. HepG2 cells were transiently transferred with 10 ng of Gal4-Luc reporter and 5 ng of Gal4-Nrf2- (1-370), together with indicated amounts (μg) of RAC3. Empty vector (pcDNA3.1) was used to make up the total DNA to a 4 μg/well. Luciferase activity was normalized by protein concentration, determined by BCA protein assay. The luciferase activity of cells transfected with emptor vector was used as control, and its luciferase activity was arbitrarily set as 1. The data shown are means of three independent experiments performed in duplicate ± S.D.*, $p < 0.05$ *versus* control.

reporter activity non-specifically, the effect of RAC3/SRC3 on the Gal4-luciferase reporter activity was tested, and the results show that there was no significant changes in the Gal4-luciferase activity, as compared with the control group (Fig. 1B).

Effect of RAC3/SRC3 mutants on the regulation of Gal4-Nrf2 chimera transactivation activity. Within the RAC3/SRC3 coding region, there are six specific motifs sharing a consensus sequence of LXXLL or LLXXL (Li *et al.,* 1997). These motifs are required for coactivation function (Leo *et al.,* 2000). RAC3/SRC3 mutant M2 (LL688/9AA) and M3 (LL741/2AA) are located in the receptor-interacting domain (RID). RAC3/SRC3 mutant M4 (DLV1036/7/8EAA) has mutation in C-terminal transcriptional activation domain (AD). We compared the effect of wild type RAC3/SRC3 construct with their mutant forms on the induction of Gal4-Nrf2-(1-370) transactivation activity. As shown in Fig. 2, at doses of 1.0 μg and 2.0 μg, there were no significant differences between their effects on the Gal4-Nrf2-(1-370) transactivation activity. When RAC3/SRC3 and its mutants were overexpressed at 4.0 μg, all the RAC3/SRC3 mutants significantly attenuated Gal4-Nrf2-(1-370) transactivation activity (Fig. 2), suggesting the specificity of this activation. This specificity was further confirmed by our co-immunoprecipitation experiments showing that the full lengths of Nrf2 and RAC3/SRC3 could be co-immunoprecipitated (unpublished observations).

Enhancement of RAC3/SRC3-mediated regulation of Gal4-Nrf2 chimera transactivation activity by CBP and p/CAF. Next we tested whether the histone acetylation proteins, CBP or p/CAF had any activity on Gal4-Nrf2-(1-370), and found that a 2.0- and a 2.4-fold increase of RAC3/SRC3-mediated transactivation, respectively in HepG2 cells (Fig. 3). In addition, we investigated whether CBP and p/CAF could induce RAC3/SRC3 mediated Gal4-Nrf2 activation in these cells. Cotransfection of both CBP and RAC 3 induced Gal4-Nrf2-(1-370) transactivation activity about 5.7-fold. Cotransfection of both p/CAF and RAC3/SRC3 enhanced Gal4-Nrf2-(1-370) transactivation activity to about 7.8-fold (Fig. 3). These results suggested that CBP and p/CAF could enhance with RAC 3 to induce Gal4-Nrf2-(1-370) transactivation activity in HepG2 cells.

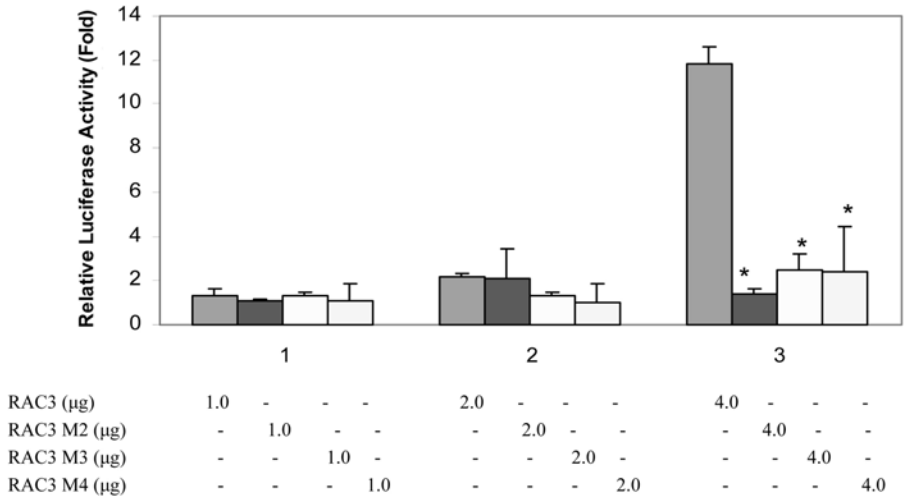

Fig. 2. Regulation of Gal4-Nrf2 chimera transactivation activity by RAC3 and its mutants. HepG2 cells were transiently transfected with 10 ng of Gal4-Luc reporter and 5 ng of Gal4-Nrf2- (1-370), together with indicated amounts (μg) of SRC-3. Empty vector (pcDNA3.1) was used to make up the total DNA to a 4 μg/well. Luciferase activity was normalized by protein concentration, determined by BCA protein assay. The luciferase activity of cells transfected with RAC3 vector was used as control. The data shown are means of three independent experiments performed in duplicate ± S.D.*, $p < 0.05$ *versus* control.

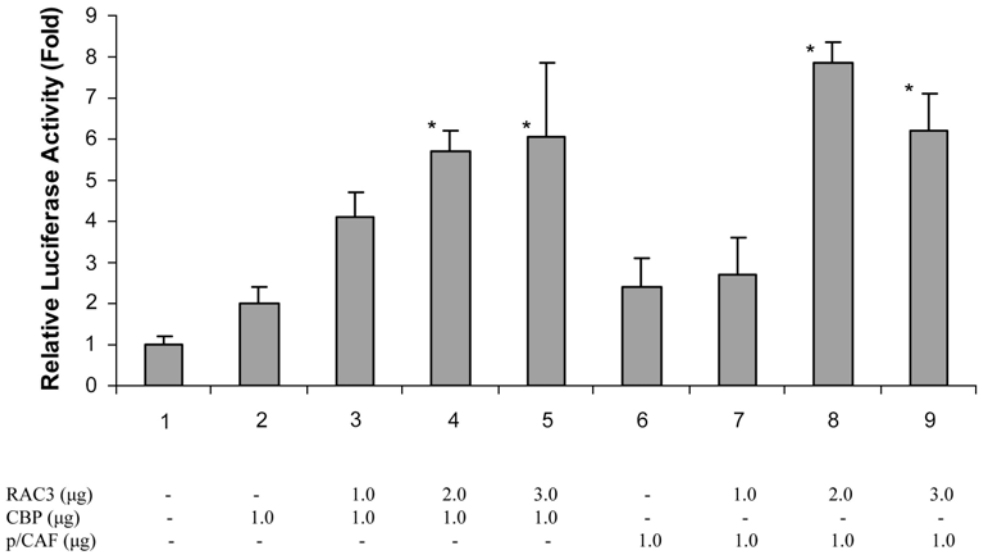

Fig. 3. Regulation of Gal4-Nrf2 chimera transactivation activity by RAC3, CBP and p/CAF. HepG2 cells were transiently transferred with 10 ng of Gal4-Luc reporter and 5 ng of Gal4-Nrf2- (1-370), together with indicated amounts (μg) of SRC-3, CBP and p/CAF. Empty vector (pcDNA3.1) was used to make up the total DNA to a 4 μg/well. Luciferase activity was normalized by protein concentration, determined by BCA protein assay. The luciferase activity of cells transfected with emptor vector was used as control, and its luciferase activity was arbitrarily set as 1. The data shown are means of three independent experiments performed in duplicate ± S.D.*, $p < 0.05$ *versus* CBP 1.0 μg or p/CAF 1.0 μg.

Enhancement of RAC3/SRC3-mediated regulation of Gal4-Nrf2 chimera transactivation activity by CARM-1, PRMT1. CARM1 belongs to the PRMT family of arginine-specific protein methyltransferases, which share a 330 amino acid-region that contains the methyltransferase activity (Lin *et al.,* 1996; Chen *et al.,* 1999). Once bound to the promoter, CARM1 exerts its effect through methylation of histones and enhances protein-protein interactions (Teyssier *et al.,* 2002). PRMT1 is the predominant member of family of protein arginine methyltransferases (PRMT). We found that both CARM1 and PRMT1 induced the transactivation activity of Gal4-Nrf2-(1-370) (Fig. 4). Cotransfection of CARM1 and RAC3/SRC3 further enhanced the Gal4-Nrf2-(1-370) transactivation activity significantly. Similar effect was also observed with the coexpression of PRMT1 and RAC3/SRC3 on Gal4-Nrf2 (1-370) transactivation activity in HepG2 cells (Fig. 4), which suggested that a potential synergistic stimulation of CARM1 and PRMT1 on RAC3/SRC3-mediated Gal4-Nrf2-(1-370) transactivation activity.

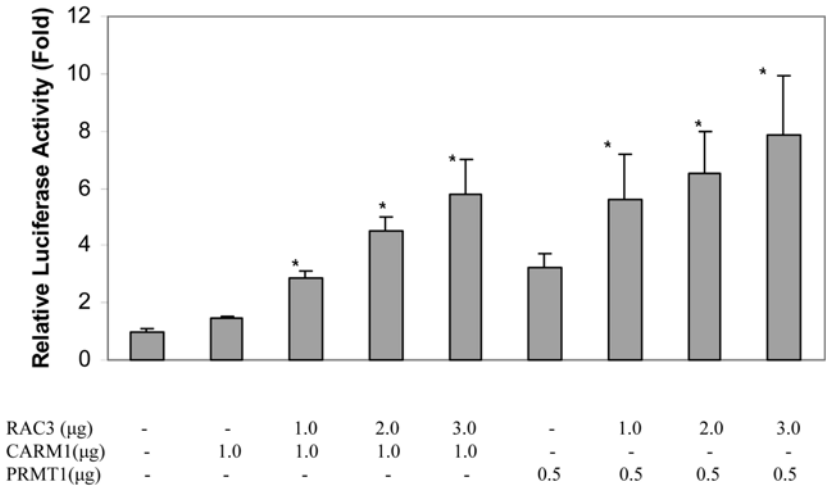

Fig. 4. Regulation of Gal4-Nrf2 chimera transactivation activity by RAC3, CARM1 and PRMT1. HepG2 cells were transiently transfected with 10 ng of Gal4-Luc reporter and 5 ng of Gal4-Nrf2- (1-370), together with indicated amounts (μg) of RAC3, CARM1 and PRMT1. Empty vector (pcDNA3.1) was used to make up the total DNA to a 4 μg/well. Luciferase activity was normalized by protein concentration, determined by BCA protein assay. The luciferase activity of cells transfected with empty vector was used as control, and its luciferase activity was arbitrarily set as 1. The data shown are means of three independent experiments performed in duplicate ± S.D.*, $p < 0.05$ *versus* control.

Discussion

Nrf2 is an important transcription regulator of ARE-mediated gene expression of antioxidant and phase II detoxifying enzymes in response to a variety of stimuli including ROS, oxidants, electrophiles, xenobiotics and many dietary cancer chemopreventive compounds. Once Nrf2 enters the nucleus, the mechanism by which Nrf2 recruits the transcriptional machinery remains unknown. Our present study shows that the nuclear receptor coactivator RAC3/SRC3 enhances the transactivation activity of the chimeric Gal4-Nrf2-(1-370). The Gal4-Nrf2-(1–370) construct, which contains the Nrf2 transactivation domain (TAD), has very high basal transactivation activity, similar to to the full-length Nrf2's transcriptional activity on the ARE (Shen, Hebbar *et al.,* 2004). We also show that the histone acetylation related co-activators CBP and p/CAF, and histone methylation related co-activators CARM-1 and PRMT-1 could further enhance the transactivation activity of Gal4-Nrf2 (1-370) mediated by RAC3/SRC3, suggesting their positive roles in this system.

The p160 coactivators contribute to transcriptional activation by bringing other associated coregulatory proteins to the promoter. The p160 coactivator complex includes either of the two related proteins p300 or CBP, which bind to the activation domain-1 (AD-1) of p160 coactivators and function as coactivators for many DNA-binding transcriptional activators (Li *et al.,* 1997). CBP and p300 contribute to chromatin remodeling by acetylating histones, and also acetylate other components of the transcription initiation complex (Chen *et al.,* 1999). CBP and p300 can also bind directly to the basal transcription factors and may thereby facilitate the assembly of the transcription initiation complex (Chen *et al.*, 1997). Along with acetylation of histones by CBP/p300, methylation of histone H3 and possibly other proteins in the transcription initiation complex by CARM1 could also be important to help remodel the chromatin structure and recruit RNA polymerase II. CARM1 methylates histone H3 and PRMT1 methylates histone H4 (Stallcup *et al.,* 2000; Ma *et al.,* 2001; An *et al.,* 2004). We found that CARM1 or PRMT1 cotransfected with Gal4-Nrf2 slightly increased Gal4-Nrf2 (1-370) transactivation activity, while coexpression of RAC3/SRC-3 and CARM1 significantly stimulated Gal4-Nrf2 (1-370) transactivation activity.

From the above results, it could be postulated that Nrf2 would be able to recruit the basic transcriptional control machinery to efficiently transcribe the target genes, and these include the various co-activators such as the p300 family [CBP/p300/pCAF], the p160 family of co-activators [SRC1/TIF2/RAC3], as well as the CARM1/PRMT1 proteins, as described previously by Rosenfeld and Glass (Rosenfeld and Glass, 2001). Future studies focusing on the biochemical, molecular and functional interactions between these proteins complexes will be needed to arrive at a more physiologically relevant model of Nrf2-mediated transcription of critical genes including cancer chemopreventive genes as well as genes involved in inflammation, cardiovascular diseases and central nervous system. In summary, our current study shows for the first time that the p160 coactivator RAC3/SRC3 could stimulate the transactivation activity of Gal4-Nrf 2 (1-370) and that this transactivation activity could be further enhanced by the coregulators such as CBP, p/CAF, CARM-1 and PRMT1.

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