DNA sequence determinants of nuclear protein binding to the *c-Ha-ras* antioxidant/electrophile response element in vascular smooth muscle cells: identification of Nrf2 and heat shock protein 90 β as heterocomplex components

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Abstract The antioxidant/electrophile response element (ARE/EpRE) is a *cis*-acting element involved in redox regulation of *c-Ha-ras* gene. Protein binding to the ARE/EpRE may be credited to deoxyribonucleic acid sequence; therefore, studies were conducted to evaluate the influence of internal and flanking regions to the 10-bp human *c-Ha-ras* ARE/EpRE core (hHaras10) on nuclear protein binding in oxidant-treated vascular smooth muscle cells. A protein doublet bound to an extended oligonucleotide comprising the ARE/EpRE core in genomic context (hHaras27), whereas a single complex bound to hHaras10. Protein binding involved specific interactions of 25- and 23-kDa proteins with hHaras10, and binding of 80-, 65-, and 55-kDa proteins to hHaras27. Competition assays with *hNQO*₁ and *rGSTA2* confirmed the specificity of deoxyribonucleic acid–protein interactions and indicated preferred binding of p25 and p23 to the *c-Ha-ras* ARE/EpRE. “NNN” sequences within the core afforded unique protein-binding profiles to the *c-Ha-ras* ARE/EpRE. In addition, Nrf2 and heat shock protein 90 β (p80) were identified as components of the *c-Ha-ras* ARE/EpRE heterocomplex. We conclude that both internal bases and flanking sequences regulate nuclear protein recruitment and complex assembly on the *c-Ha-ras* ARE/EpRE.

INTRODUCTION

Antioxidant/electrophile response elements (ARE/EpRE) mediate the basal and inducible expression of *c-Ha-ras* protooncogene, as well as the Phase-II detoxifying enzymes glutathione *S*-transferase A1, A2, and A3 (GSTA1, GSTA2, GSTA3), NADPH:quinone oxidoreductase (NQO₁), and γ -glutamylcysteine synthetase (γ GCS) (Rushmore et

al 1991; Favreau and Pickett 1995; Venugopal and Jaiswal 1996; Bral and Ramos 1997; Mulcahy et al 1997; Kang et al 2000; Jowsey et al 2003). The ARE/EpRE core sequence was initially defined as 5'-TGACNNNGC-3', where N represents any nucleotide (Rushmore et al 1991). It was later modified and extended to 5'-TMANNRT GAYNNGCRWWW-3' (Wasserman and Fahl 1997), and further defined in the NQO₁ promoter as 5'-GAGTCA CAGTGAGTCGGCAAAATT-3' (Nioi et al 2003).

Transcriptional activity mediated by the ARE/EpRE is highly gene- and cell-specific, and influenced by a variety of chemical treatments. Consistent with this degree of biological complexity, the genomic contexts of the ARE/EpRE in different genes are also markedly different. Consensus ARE/EpRE core sequences are often present in

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conjunction with 12-O-tetradecanoylphorbol-13-acetate response elements (TRE) distal to, or within, the ARE/EpRE core that act coordinately to induce basal gene expression. Several proteins have been identified to interact with ARE/EpREs (Li and Jaiswal 1994; Vasiliou et al 1995; Venugopal and Jaiswal 1996; Itoh et al 1997; Montano et al 1998, 2000; Moinova and Mulcahy 1999; Chen and Ramos 2000; Nguyen et al 2000; Zipper and Mulcahy 2000; Holderman et al 2002; Sankaranarayanan and Jaiswal 2004). Among these, the transcription factor Nrf2 has received much attention as a critical mediator of the cellular response to stress in multiple cells types, including HepG2 and neuronal cell lines. Of note is the recent finding that Keap1 functions as a redox sensing protein that suppresses Nrf2 translocation until this interaction is disrupted by oxidative stress (Dinkova-Kostova et al 2002; Itoh et al 2003; McMahon et al 2003). The mechanisms of activation of ARE/EpRE protein binding have been widely studied and include stress-regulated shuttling mechanisms (Itoh et al 1999; Dinkova-Kostova et al 2002; Itoh et al 2003; McMahon et al 2003), as well as regulation by ERK, p38, phosphatidylinositol 3-kinase, and protein kinase C (Huang et al 2000; Kang et al 2000; Yu et al 2000a, 2000b; Zipper and Mulcahy 2000; Nguyen et al 2003). These regulatory pathways are highly dependent on chemical treatments because ARE/EpREs are responsive to planar aromatic compounds, β -naphthoflavone, phenolic antioxidants, thiol-containing compounds, and hydrogen peroxide (Miller et al 2000; Nguyen et al 2003).

In this study, aortic (vascular) smooth muscle cells (vSMCs) were used as a model to investigate the molecular interaction between redox signaling and atherogenesis, and specifically to define deoxyribonucleic acid (DNA) sequence determinants of nuclear protein binding to the *c-Ha-ras* ARE/EpRE. *c-Ha-ras* is activated in vSMCs via a redox-sensitive transcriptional mechanism that is partly controlled by ARE/EpRE sequences in the upstream regulatory region of the gene. Ras activation by chemical mimics of oxidative stress, such as benzo[a]pyrene (BaP), is associated with uncontrolled proliferation and dedifferentiation of vSMCs leading to formation of vascular lesions of atherosclerotic morphology (Ramos 1999; Kerzee and Ramos 2000). BaP is metabolized within the intracellular compartment to oxidative intermediates that compromise redox homeostasis and induce oxidative stress (Miller and Ramos 2001). Multiple protein complexes interact with the human (h) *c-Ha-ras* (hHaras) ARE/EpRE in vSMCs, as indicated by formation of a doublet upon BaP and oxidative metabolite challenge (Miller et al 2000). Several proteins directly interact with the *c-Ha-ras* ARE/EpRE and exhibit cross-linking profiles comparable with the ARE/EpRE in *GSTA2* and *NQO1*. However, some of these proteins are unique to *c-Ha-ras*. Flanking sequences distal to the core are necessary for

both protein binding and transcriptional activation via the ARE/EpRE of the *NQO1* and *GSTA2* genes (Nguyen et al 1994; Favreau and Pickett 1995; Xie et al 1995), and thus a similar paradigm may play a role in recruitment of specific *c-Ha-ras* ARE/EpRE protein complexes. Although the *c-Ha-ras* ARE/EpRE region consists only of a minimal core sequence that regulates protein complex assembly and transcriptional activity (Miller et al 2000), regions surrounding the core may influence protein recruitment and stabilization of binding. In addition, whereas the identity of the "NNN" region of the ARE/EpRE core is not considered critical for transactivation, these base pairs may influence protein recruitment and binding. Studies were therefore conducted to test the hypothesis that DNA sequences within, proximal to, and distal to the *c-Ha-ras* ARE/EpRE core influence protein complex assembly. Studies were also conducted to identify proteins that participate in redox-regulated formation of *c-Ha-ras* ARE/EpRE protein complexes. Our results indicate that flanking regions influence protein recruitment to the core sequence, whereas alterations in base pair identity within the NNN group of nucleotides of the *c-Ha-ras* ARE/EpRE core help to define protein binding characteristics to this element. Through immunodepletion and ultraviolet (UV) cross-linking experiments, we also found that Nrf2 interacts with the ARE/EpRE in *c-Ha-ras* and that Hsp90 β is part of the heterocomplex interacting with the ARE/EpRE.

MATERIALS AND METHODS

Reagents

BaP (98% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), and prepared in dimethyl sulfoxide (DMSO). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise noted.

Oligonucleotides

Oligonucleotide sequences used in this study are shown within each figure. hHaras, hNQO₁, and rGSTA2 oligonucleotides were used to assess protein binding to ARE/EpRE motifs located in each promoter. Poly dIdC was also used to assess protein-binding specificity. The double-stranded oligonucleotide containing the ARE/EpRE motif of the hHaras promoter (hHaras27) was prepared as described previously (Bral and Ramos 1997; Miller et al 2000), filled in using the Klenow fragment of DNA polymerase I, and end-labeled using [γ -³²P]ATP (New England Nuclear Research Products, Boston, MA, USA) and T4 polynucleotide kinase (Promega, Madison, WI, USA). This resulted in a 27-bp oligonucleotide. The oligonucle-

otide sequence hNQO₁(33) was synthesized (Genosys, The Woodlands, TX, USA) and prepared as described previously (Miller et al 2000). The double-stranded oligonucleotides hHaras10, hNQO₁(10), hNQO₁(19), rGST(PROX), rGST(DIST), rGST(25), and rGST(41) were synthesized to contain blunted ends upon annealing (Gene Technologies Laboratory, Texas A&M University, College Station, TX, USA, and Genosys). Cross-linking probes were synthesized by annealing the top strand of each oligonucleotide with their respective primers and synthesizing the opposite strand in a Klenow reaction with 0.1 mM deoxyadenosine triphosphate, 0.1 mM deoxyguanosine triphosphate, 0.1 mM bromodeoxyuridine triphosphate (BrdUTP), 1 mM dithiothreitol (DTT), and 100 µCi [α -³²P]deoxycytidine triphosphate for 1 hour at 37°C. Primer sequences included: 5'-TCTCGCTC-3' (hHaras27); 5'-TCTCGC-3' (hHaras27); and 5'-GCTCTG-3' (hHaras10). Because the top and bottom strands of hHaras27 were not originally synthesized to contain blunted ends, the hHaras cross-linking probe is 4 bp shorter on the 3' end. Labeled oligonucleotides were purified using Sephadex G-25 spin columns (Roche, Indianapolis, IN, USA).

Cell culture

Primary cultures of vSMCs were isolated from female C57BL/6 mouse aorta and maintained under standard conditions as described (Ramos and Cox 1993). Cells were grown in Medium 199 (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 2 mM glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B (GIBCO). Subcultures were prepared by trypsinization (GIBCO) of subconfluent primary cultures. Cells were seeded at 75–85 cells/mm² onto 100-mm plates, allowed to acclimate 48 hours, and challenged with DMSO (control) or BaP (3 µM) for 3 hours. A non-treated control was used in some experiments to control for nonspecific changes in redox balance. DMSO concentrations in all experiments were kept below 0.1%, levels that solubilized BaP in aqueous media without overt changes in ARE protein binding to ARE/EpRE (Miller et al 2000).

Nuclear cell extract preparation

Nuclear extracts were prepared as described previously (Miller et al 2000). In brief, cells were washed twice with ice-cold HEGD (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid-Cl, pH 7.6, 1 mM DTT, 1.5 mM ethylenediamine-tetraacetic acid [EDTA], 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and scraped from plates using 1 mL HEGD. Cells were then transferred to

a Dounce homogenizer and lysed with 20 strokes. Nuclei were pelleted at 14 000 rpm, 2 minutes, 4°C, in a variable speed microfuge, and the supernatant was discarded. Nuclei were resuspended in 50 µL HEGDK (HEGD plus 0.5 M KCl) and incubated on ice for 2–3 hours to extract nuclear proteins. Nuclear ghosts were removed by centrifugation at 14 000 rpm, 10 minutes, 4°C, in microfuge, and supernatant was quick frozen in liquid nitrogen and stored at –80°C. Protein concentration was determined by the method of Bradford (Bradford 1976).

Electrophoretic mobility shift assays

For electrophoretic mobility shift assay (EMSA) reactions, 5 µg of nuclear protein was incubated with 10-fmol double-stranded [γ -³²P]-labeled oligonucleotide at room temperature for 20 minutes. For competition experiments, nuclear extracts were incubated with cold oligonucleotide for 30 minutes, followed by 20 minutes incubation with labeled oligonucleotide. Binding reactions were performed in 0.25× HEGDK supplemented with 1 mM DTT, 20 µg bovine serum albumin, and 50 ng poly dIdC in a total volume of 20 µL. On the basis of concentration finding and optimization experiments, 50 ng poly dIdC was used to minimize nonspecific DNA-protein interactions. Loading dyes were added (15% Ficoll [Type 400; Amersham Pharmacia Biotech, Piscataway, NJ, USA], 0.25% bromophenol blue, 0.25% xylene cyanol FF) and reactions were immediately loaded on a 7% nondenaturing polyacrylamide gel (except where noted) with 0.5× TBE buffer (50 mM Tris, 45 mM boric acid, 1 mM Na₂EDTA, pH 8.3) and electrophoresed at 25 mA. Gels were dried, exposed to a phosphor screen, and analyzed using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

UV cross-linking

Equilibrium binding reactions were prepared as described above using 5 µg nuclear protein and 10⁵ counts per minute (CPM) cross-linking probe. The reaction was allowed to attain equilibrium for 20 minutes at room temperature in noncompetition experiments, followed by UV irradiation at 254 nm for 30 minutes on ice. For competition experiments, nuclear extracts were incubated with cold oligonucleotide for 30 minutes, followed by 20 minutes incubation with labeled oligonucleotide, and UV irradiation at 254 nm for 30 minutes on ice. After loading dyes were added, samples were boiled 5 minutes and subsequently run on denaturing sodium dodecyl sulfate (SDS) gels (10% resolving) along with rainbow molecular weight markers (Amersham Pharmacia). Gels were dried and markers manually labeled with [γ -³²P]ATP, followed

by exposure to a phosphor screen and analysis using a STORM phosphorimager (Molecular Dynamics).

In-gel digestion-extraction

Proteins obtained by polyethyleneimine (PEI) purification to identify ARE/EpRE binding proteins were resolved on a 10% SDS-polyacrylamide gel electrophoresis gel, excised, and subjected to reduction/alkylation digestion and extraction. The PEI purification used has been described previously (Holderman et al 2002). Slices were washed for 30 minutes in 1 mL of 30% methanol and then washed for 60 minutes in 500 μ L of 100 mM ammonium bicarbonate. Protein was reduced by the addition of 160 μ L of 100 mM ammonium bicarbonate containing 2.5 mM DTT and incubated for 30 minutes at 60°C. After incubation, 10 μ L of a 100 mM solution of iodoacetamide was added and incubated for an additional 30 minutes at room temperature in the dark. The gel slice was washed in 500 μ L of wash buffer (50% acetonitrile and 50 mM Tris, pH 9.2) for 60 minutes at room temperature, minced, and subsequently washed with 50 μ L of acetonitrile, dried in a SpeedVac (Thermo Savant, Holbrook, NY, USA) without heat, rehydrated in 10 μ L of 100 mM ammonium bicarbonate containing 0.25 μ g of either modified trypsin (Promega, Madison, WI, USA) or EndoLysC (Waco Biochemicals, Waco, TX, USA), and incubated for 12 hours at 30°C. After digestion, peptides were extracted with 150 μ L of 0.1% trifluoroacetic acid (TFA), 60% acetonitrile for 60 minutes at room temperature. The slices were reextracted with an additional 150 μ L of extraction solution, and the supernatants combined before concentration by SpeedVac and submission for microsequence analysis.

Peptide purification

Peptides generated by in-gel digestion were purified using a Hewlett Packard 1100 high-performance liquid chromatography system (HPLC) (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and separated on a 218TP5215 (2.1 $\times$ 150 mm) C18 reversed phase column (Vydak, Hesperia, CA, USA) at a flow rate of 0.2 mL/min. Eluent A was 0.05% aqueous TFA and eluent B was 0.04% TFA in acetonitrile. The chromatography was carried out with 95% A for 5 minutes, followed by a linear gradient from 5% to 50% B over 90 minutes, and peptides collected manually.

Protein sequencing

Automated Edman chemistry was performed on a Hewlett Packard G1000A automated protein sequencer. Samples were bound to a C18 resin. Liquid samples were loaded in 2% TFA and the column subsequently washed

to remove solvents and salts before mounting into the Hewlett Packard G1000A. All samples were preceded by phenylthiohydantoin amino acid standards for calibration purposes.

Immunodepletion-immunoprecipitation

Nuclear extracts from vSMCs were prepared as described above and protein concentrations measured by the method of Bradford (Bradford 1976). For immunodepletion, 200 μ g of nuclear protein was combined with 1 μ g antibody or immunoglobulin-G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1 $\times$ Tris-EDTA buffer to a volume of 50 μ L, and incubated with mixing overnight at 4°C. A 50% slurry of Protein A sepharose beads to a volume of 50 μ L, and incubated with mixing overnight at 4°C. A 50% slurry of Protein A sepharose beads was added to the protein-antibody mixture and incubated at room temperature 4 hours with mixing. Samples were then centrifuged at 14 000 rpm for 5 minutes at 4°C. Supernatant was collected as immunodepleted sample. Protein A sepharose beads were washed 3 times with TBS (0.1 M Tris, pH 7.5, 0.15 M NaCl), and resuspended in 40 μ L HEGDK as immunoprecipitated samples.

Western blotting

Immunodepleted and immunoprecipitated protein samples were analyzed by Western blotting to determine effective depletion. For electrophoresis, loading dye was added to approximately 40 μ L sample, which was then boiled 5 minutes, loaded onto a 12% denaturing Tris-Cl gel (Bio-Rad, Hercules, CA, USA) and electrophoresed at 20 mA in 1 $\times$ SDS running buffer with appropriate molecular weight markers (Amersham-Pharmacia). Gels were then electroblotted onto nitrocellulose membrane (Bio-Rad) at 10 V, overnight at 4°C. Membranes were removed from the transfer apparatus and equilibrated in 5% Blotto solution (5% nonfat dry milk in TTBS [TBS with 0.1% Tween-20]) overnight at 4°C or at room temperature for 2 hours. Membranes were then incubated at room temperature for 1 hour in 1:200 rabbit polyclonal Nrf2-5% Blotto for primary antibody incubation, followed by washing 6 times, 5 minutes each, with 1% Blotto. For secondary antibody incubations, membranes were incubated at room temperature, 1 hour, in 1:2000 goat anti-rabbit IgG-HRP:5% Blotto. Membranes were then washed again 6 times, 5 minutes each, with 1% Blotto, and rinsed 3 times with TBS, 5 minutes each. For chemiluminescence detection, membranes were incubated for 5 minutes with a 1:4 dilution (in TBS) of Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA), sealed under plastic, and exposed to Biomax film (Kodak, Rochester, NY, USA).

RESULTS

Flanking regions to the *c-Ha-ras* ARE/EpRE core influence complex assembly

Multiple protein complexes of varying inducibility interact with the hHaras ARE/EpRE in vSMCs upon BaP and oxidative metabolite challenge (Miller et al 2000). The formation of different complexes may involve recruitment of different proteins to DNA as a function of internal or flanking DNA sequences to the ARE/EpRE core. To test this hypothesis, experiments were first designed to compare protein-binding profiles of the *c-Ha-ras* ARE/EpRE core sequence (hHaras10) relative to an extended oligonucleotide sequence in genomic context (hHaras27). A doublet (C1 and C2) was resolved when hHaras27 was incubated with nuclear extracts of vSMCs (Fig 1A). Only a singlet that comigrated with C1 was resolved when extracts were incubated with hHaras10. Oligonucleotide competition assays showed that protein binding to hHaras10 was ablated by excess cold hHaras27 and hHaras10 oligonucleotides, consistent with sequence similarities in the core region (Fig 1A). However, only C1 of hHaras27 was effectively competed by cold hHaras10 oligonucleotide, suggesting that comigrating C1 complexes interact solely with the core ARE/EpRE. Thus, protein binding to the ARE/EpRE is influenced by core and flanking sequences.

Absence of the ARE/EpRE core eliminates protein binding

To examine protein complex formation in the absence of a core ARE/EpRE, a mutant hHaras27 oligonucleotide was synthesized (hHaras27WOUC). This oligonucleotide consisted of the core flanking regions of hHaras27, with a fully mutated ARE/EpRE core region inserted in normal context. The acronym WOUC stands for "without BrdU in the core." When a cross-linking probe is prepared using the top strand of hHaras27WOUC along with its respective primer, no BrdU cross-linking site within the mutant core region is present, such that nonspecific protein interactions are minimized during cross-linking. Gel shift analysis to compare vSMC nuclear protein binding to hHaras27 and hHaras27WOUC showed that the mutant oligonucleotide could not compete protein binding to hHaras27 (Fig 1B). Protein binding to a [³²P]-labeled hHaras27WOUC probe was nonspecific showing that mutation of the 10-bp *c-Ha-ras* ARE/EpRE core sequence eliminates specific protein interactions at this site.

Protein binding to hHaras27 exhibits core region and flanking region specificity

UV cross-linking analysis revealed that proteins of 80, 65, 40, 25, and 23 kDa bound to hHaras27, 2 of which (p80

Fig 1. Comparison and specificity of nuclear protein binding to hHaras27, hHaras10, and hHaras27WOUC. Electrophoretic mobility shift assay (EMSA) was performed as described in Materials and Methods. FP indicates reaction run without extract. D indicates extracts from dimethyl sulfoxide-treated cells. B indicates extracts from cells treated with benzo[a]pyrene (BaP) (3 μ M). All competition reactions were run with nuclear extracts from cells treated with BaP (3 μ M). (A) Competition EMSA to examine specificity of protein complex formation to [³²P]-labeled hHaras27 and [³²P]-labeled hHaras10. Specific complexes were identified by competition with unlabeled oligonucleotides (1–3). (B) Protein binding and competitive EMSA were performed against a [³²P]-labeled hHaras27 probe and a [³²P]-labeled hHaras27WOUC probe as indicated. Specific complexes were identified by competition with unlabeled oligonucleotides (1–3). Arrows indicate specific complexes identified. Results are representative of 3 separate experiments.

and p65) interacted specifically with the sequence (Fig 2). p25 and p23 protein binding did not exhibit specificity for hHaras27 but was effectively competed by hHaras10. When hHaras10 was used as the cross-linking probe, only p25 and p23 bound specifically, suggesting that p80 and p65 favor flanking sequences, whereas p25 and p23 preferentially interact with the ARE/EpRE core sequence. Competition assays were performed to determine whether deletion sequences from human (h) NQO₁ and rat (r) GSTA2 competed covalent protein binding to hHaras27 and hHaras10. When nuclear extracts from BaP-treated vSMCs were incubated with [³²P]-labeled hHaras27 and competed with a molar excess of unlabeled sequences, larger proteins (p80, p65, p55) were competed by extended sequences (hHaras27, hNQO₁[19], hNQO₁[33],

rGST[25], and rGST[41]) (Fig 3A). Smaller proteins (p25 and p23) were only competed specifically by hHaras10. In a similar experiment using hHaras10 as the [32 P]-labeled cross-linking probe, p25 and p23 were the only proteins that bound with specificity to hHaras10 (Fig 3B). Binding was not competed by any other sequences, indicating that the larger proteins favor the extended sequences. The lack of competition of protein binding to hHaras10 by any other core or extended oligonucleotide sequences indicates that p25 and p23 preferentially interact with the *c-Ha-ras* core sequence. The core sequences of hHaras10, hNQO₁(10), and rGST(PROX) represent the minimal core ARE/EpRE sequence as originally defined (Rushmore et al 1991). The only difference between these sequences is the identity of the 3 "N" base pairs within the core. Whereas point mutations of these base pairs in the rat and mouse GSTA1 ARE/EpRE sequences have no effect on either basal or inducible transcriptional activity (Rushmore et al 1991; Wasserman and Fahl 1997), the NNN sequence may influence protein binding to the *c-Ha-ras* ARE/EpRE.

Mutations in NNN affect protein complex formation

To evaluate the role of NNN base pairs on the entire 27-bp *c-Ha-ras* ARE/EpRE sequence, oligonucleotides were synthesized containing the intact flanking regions of *c-Ha-ras* ARE/EpRE, along with the core sequence of the hNQO₁, rNQO₁, and rGSTA2 (27NQOh, 27NQOr, 27GSTr). In addition, an hHaras27 ARE/EpRE with GGG substituted for NNN (hHaras27NMUT) was synthesized. Gel shift analysis showed that BaP-induced protein binding of C1 was common to all oligonucleotides, except hHaras27WOUC (Fig 4A). C2 binding was detected in all cases except 27NQOr and 27GSTr, where a faster migrating complex was identified (C3). The specificity of vSMC nuclear protein complex formation to each of the 27-bp NNN mutant oligonucleotides was examined using competitive gel shift analysis (Fig 4B). Protein binding to [32 P]-labeled hHaras27 was ablated by hHaras27, hHaras27NMUT, 27NQOh, 27NQOr, and 27GSTr, whereas hHaras27WOUC was ineffective. Although a doublet was not resolved in this gel, a highly inducible complex was evident. Thus, protein complex formation on *c-Ha-ras* ARE/EpRE is influenced by the identity of NNN base pairs, although these bases do not confer absolute specificity.

Nrf2 is present in the *c-Ha-ras* ARE/EpRE protein-binding complex

Nrf2 is a prominent ARE/EpRE binding protein (Venugopal and Jaiswal 1996, 1998; Itoh et al 1997; Moinova and Mulcahy 1999; Wild et al 1999; Nguyen et al 2000;

Fig 2. Ultraviolet (UV) cross-linking profile and specificity of nuclear protein binding to hHaras27 and hHaras10. UV cross-linking was performed as described in Materials and Methods. P indicates reaction run without extract. Nuclear extracts from dimethyl sulfoxide (D) and benzo[a]pyrene (BaP) (B) (3 μ M) treated cells were incubated with [32 P]-labeled hHaras27 or [32 P]-labeled hHaras10 cross-linking probes as indicated. Specific complexes of BaP nuclear extracts were identified by competition with the indicated unlabeled oligonucleotides (1–3). MW indicates molecular weight markers. Arrows indicate specific proteins identified. Results are representative of 3 separate experiments.

Zhu and Fahl 2001; Bloom et al 2002; Holderman et al 2002; Jowsey et al 2003). Therefore, immunodepletion experiments were performed to determine whether Nrf2 is a component of the *c-Ha-ras* ARE/EpRE binding complex. Incubation of BaP-treated vSMC nuclear extracts with polyclonal Nrf2 antibody and protein A sepharose beads immunodepleted Nrf2 protein from nuclear extracts (Fig 5A). Nrf2 protein was identified in the crude extract, in extract incubated with nonspecific IgG, and in the Nrf2-immunoprecipitated sample. No Nrf2 protein was detected by Western blot in immunodepleted nuclear extracts. In the absence of Nrf2 protein, protein complexes were unable to bind to hHaras27 (Fig 5B). Nrf2 depletion results in the loss of p65 and p55, proteins present in non-depleted samples, and in extract incubated with nonspecific IgG (Fig 5C). Immunoprecipitated Nrf2 alone did not form a complex with hHaras27 (Fig 5 B,C), showing that Nrf2 alone cannot bind to the hHaras ARE/EpRE.

Sequence analysis of PEI-purified proposed ARE/EpRE binding proteins

To identify *c-Ha-ras* ARE/EpRE binding proteins, a predominant 80-kDa band was resolved from nuclear ex-

Fig 3. Comparison and specificity of nuclear protein binding to hHaras27 and hHaras10. Ultraviolet cross-linking was performed as described in Materials and Methods. Competitive cross-linking was performed against a [32 P]-labeled hHaras27 probe (A) and [32 P]-labeled hHaras10 probe (B). Specific complexes were identified by competition with unlabeled oligonucleotides (1–11). FP indicates reaction run without extract. D indicates extracts from dimethyl sulfoxide-treated cells. B indicates extracts from cells treated with benzo[a]pyrene (BaP) (3 μ M). All competition reactions were run with nuclear extracts from cells treated with BaP (3 μ M). Arrows indicate specific proteins. Results are representative of 3 separate experiments.

tracts of vSMCs after PEI purification (Holderman et al 2002). The size of this protein is consistent with preparative EMSA and UV cross-linking studies showing the presence of a predominant 80-kDa protein in ARE/EpRE protein complexes (Miller et al 2000; Holderman et al 2002). The PEI-purified protein fraction containing the 80-kDa protein was also found to contain proteins that bound specifically to the *c-Ha-ras* ARE/EpRE (Holderman et al 2002). Therefore, this 80-kDa protein was in-gel digested and extracted and sequenced by Edman chemistry. BLAST analysis of the Pro-Glu-Glu-Val-His-His-Gly-Glu sequence yielded a 100% match to heat shock protein 84 (Hsp84) and Hsp 90 β (Hsp90 β), as well as other matches (Table 1). Sequence matches to the Hsps were found at the N-termini of these proteins, and therefore correlate well with the N-terminal protein sequence obtained by Edman chemistry. The protein amount sequenced was over 5 pmol and was therefore considered a major target sequence as opposed to a contaminating sequence. A second sample of PEI-purified 80-kDa protein was digested with EndoLysC proteinase, separated by HPLC, and sequenced to obtain internal sequence. The

18-amino acid sequence obtained was Ile-Asp-Ile-Ile-Pro-Asn-Pro-Gln-Glu-Arg-Thr-Leu-Thr-Leu-Val-Asp-Thr-Gly. A SWISSPROT database search revealed a high degree of homology with Hsp90 β (Table 2). Thus, the 80-kDa protein isolated by PEI purification was identified as a Hsp. To follow-up on this finding, recombinant Hsp90 β protein was evaluated for its ability to interact directly or indirectly with the *c-Ha-ras* ARE/EpRE sequence. Binding of Hsp90 β alone to hHaras27 ARE/EpRE was not evident (data not shown), but combination of 50 ng Hsp90 β with crude nuclear extracts (10 or 7 μ g) and [32 P]-labeled hHaras27 decreased protein binding (Fig 6).

DISCUSSION

The present studies were conducted to test the hypothesis that internal and flanking DNA sequences to the *c-Ha-ras* ARE/EpRE core influence protein complex assembly. These experiments built on previous work showing differences in vSMC protein recruitment to ARE/EpREs of different promoters. Evidence was obtained indicating that flanking sequences to the core ARE/EpRE influence

Fig 4. "NNN" mutation effects on protein complex formation and specificity. Electrophoretic mobility shift assay (EMSA) was performed as described in Materials and Methods. (A) Protein complex formation to [³²P]-labeled 27-bp oligonucleotides with various NNN mutations (1–7). (B) Competitive EMSA against [³²P]-labeled hHaras27. Specific complexes were identified by competition with unlabeled oligonucleotides (1–7). FP indicates reaction run without extract. D indicates extracts from dimethyl sulfoxide-treated cells. B indicates extracts from cells treated with benzo[a]pyrene (3 μ M). Arrows indicate specific proteins for each oligonucleotide studied. Results are representative of 3 separate experiments.

vSMC nuclear protein binding. Differences in protein binding to the core relative to flanking regions were observed, with larger proteins showing preference for extended sequences, and smaller proteins interacting with the ARE/EpRE core. Only 1 protein complex was found to bind specifically to the core hHaras10 sequence. Extension of this sequence to 27 bp of proximal and distal flanking sequence allowed formation of 2 or more complexes. The assembly of these complexes does not likely

Fig 5. Presence of Nrf2 in the vascular smooth muscle cells (vSMC) *c-Ha-ras* antioxidant/electrophile response elements protein complex. Western blotting, immunodepletion (ID), immunoprecipitation (IP), electrophoretic mobility shift assay (EMSA), and ultraviolet (UV) cross-linking were all performed as described in Materials and Methods. (A) Western blot of Nrf2-immunodepleted vSMC nuclear extract. Crude = 40 μ g crude nuclear extract. ID immunoglobulin-G (IgG) = nuclear extract immunodepleted with nonspecific IgG. ID Nrf2 = nuclear extract immunodepleted with Nrf2 antibody. IP Nrf2 = immunoprecipitated Nrf2 from nuclear extract. MW = molecular weight. Arrow indicates Nrf2 protein. (B) EMSA of crude nuclear extract, ID Nrf2 and IP Nrf2. Numbers 1, 2, and 3 represent different sample preparations, all immunodepleted of Nrf2. FP = reaction run without extract. Arrows indicate specific complexes. (C) UV cross-linking analysis of crude, ID Nrf2, IP Nrf2, and ID IgG. Numbers 1 and 2 indicate different sample preps; sample 1 = experiment performed where Nrf2 was successfully immunodepleted; sample 2 = experiment performed where Nrf2 was not immunodepleted. Bracket indicates region of proteins eliminated by Nrf2 immunodepletion. Results are representative of 3 separate experiments.

involve differential phosphorylation because incubation with calf intestine alkaline phosphatase has no influence on protein binding (K. P. Miller and K. S. Ramos, unpublished observations).

A number of reports have shown that bZIP transcription factors such as Nrf2 dimerize with other proteins to form heterodimers of varying DNA binding affinity. For example, the TRE (TGACTCA) and cyclic adenosine 5' monophosphate (AMP) response element (CRE) (TGACGTCA) share similar sequences, but the Jun-Fos heterodimer exhibits higher affinity for TRE than CRE, whereas Jun-ATF preferentially binds to CRE (Ryseck and Bravo 1991). Such differences may explain the resolution of a protein-binding doublet that recognizes the *c-Ha-ras* ARE/EpRE. Interestingly, p25 and p23 proteins generally detected by UV cross-linking were not found in band excision studies (data not shown).

Evidence was also obtained that not all ARE/EpRE se-

Table 1 Best sequence matches obtained for an 80-kDa PEI-isolated protein. Sequence matches of known proteins to the sequence obtained for the 80-kDa PEI-isolated protein by Edman chemistry were identified using a BLAST database search. Amino acid identity ratio indicates the number of amino acids in a known protein sequence that match the proposed sequence^a

80-kDa PEI-isolated protein Seq: Pro-Glu-Glu-Val-His-His-Gly-Glu		
Sequence homology to:	Amino acid identities	% Homology
gi 194027: heat-shock protein hsp84 (mouse)	8/8	100
sp P34058 HS9B RAT: heat-shock protein Hsp 90β	8/9	89
gn1 PID e341171: Fixl protein	7/10	70
pir A46177: helix-loop-helix protein mC–fruit fly	6/10	60

^a PEI, polyethyleneimine.

Table 2 Best sequence matches obtained for an 80-kDa enzymatically digested PEI-isolated protein. Sequence matches of known proteins to the sequence obtained for the enzymatically digested 80-kDa PEI-isolated protein by Edman chemistry were identified by a BLAST database search. Amino acid identity ratio indicates the number of amino acids in a known protein sequence that match the proposed sequence^a

80-kDa enzymatically digested PEI-isolated protein Seq: Ile-Asp-Ile-Ile-Pro-Asn-Pro-Gln-Glu-Arg-Thr-Leu-Thr-Leu-Val-Asp-Thr-Gly		
Sequence homology to:	Amino acid identities	% Homology
sp P08238 HS9B HUMAN: heat shock protein Hsp 90β	20/20	100
gi 194027: heat-shock protein hsp84 (mouse)	20/20	100

^a PEI, polyethyleneimine.

Fig 6. Influence of heat shock protein 90β (Hsp90β) in protein complex formation to *c-Ha-ras* antioxidant/electrophile response elements. Electrophoretic mobility shift assay was performed as described in Materials and Methods. Hsp90β protein (50 ng) was coin-cubated with 20–3 μg of nuclear extract obtained from benzo[a]pyrene-treated (3 μM) vascular smooth muscle cells (+), and protein complex formation to [³²P]-labeled hHaras27 was assessed. Arrow indicates specific complex formed. Results are representative of 3 separate experiments.

quences behave in the same manner. Despite a conserved ARE/EpRE core sequence in the *c-Ha-ras* promoter, regions flanking the core strongly influenced the specificity of protein interactions. Differences in protein recruitment and binding specificity may be attributed to the presence, or lack thereof in the case of hHaras27, of other transcription factor binding sites proximal or distal to the ARE/EpRE core. *rGSTA2* contains a distal ARE-like site and *hNQO₁* contains a distal TRE-like site (Nguyen et al 1994). In fact, a palindromic sequence within the *hNQO₁* ARE/EpRE recognition motif is necessary for binding of Nrf2/MafK heterodimer and cannot be competed by the ARE/EpRE sequence of *rGSTA2*, which does not contain the palindromic sequence (Nguyen et al 2000). Differences within the ARE/EpRE core region may also be relevant. The core sequence was originally defined as 5'-

GTGACNNNGC-3', where N represented any nucleotide and did not influence transcriptional activity (Rushmore et al 1991; Wasserman and Fahl 1997). However, N nucleotides may influence protein recruitment to, and competition for, the ARE/EpRE core in a promoter-specific manner. Neither *hNQO₁*(10) or *rGSTPROX* could compete cross-linked protein binding to hHaras27 or hHaras10, although the ARE/EpRE core sequence elements were identical save for the NNN sequence.

To evaluate the characteristics of protein binding to the *c-Ha-ras* ARE/EpRE core, various oligonucleotide mutants were synthesized, representing the hHaras27 sequence, with changes in NNN bases made to match those of the *rNQO₁*, *hNQO₁*, and *rGSTA2* ARE/EpRE cores. This strategy served to determine whether NNN base pairs influence binding to the *c-Ha-ras* ARE/EpRE. In addition, full core sequence mutations within the hHaras27 oligonucleotide were performed to examine protein binding to the core sequence relative to flanking regions. Gel shift analyses using mutant oligonucleotides showed that mutation of the entire 10-bp core sequence eliminated site-specific protein interaction at this site. Mutations in NNN affected complex formation, but not binding specificity. Although the identity of the NNN nucleotides is not thought to influence transcriptional activity of ARE/EpRE regulated genes, our results show that these nucleotides may influence protein binding. Recent work by Nioi et al (2003) indicated that the second N position of the NNN in the mouse *NQO₁* ARE/EpRE significantly contributes to the function of the ARE/EpRE, induction by sulphoraphane, and full response of the ARE/EpRE to Nrf2. In addition, a point mutation of the third N po-

sition of the NNN within the core ARE/EpRE retains comparable function with the wild-type sequence but competes protein binding to the wild-type ARE/EpRE (Nioi et al 2003). These observations are consistent with our findings and support the hypothesis that sequence differences within the ARE/EpRE core of *c-Ha-ras* confer specificity of protein complex formation.

Not surprisingly, Nrf2 was identified as a member of the *c-Ha-ras* ARE/EpRE complex in vSMCs. Nrf2 is a bZIP transcription factor that heterodimerizes with small Maf proteins to bind ARE/EpRE or Maf-recognition element (MARE) sequences in all cells examined to date. Nrf2 is a cytosolic protein sequestered in the cytosol by the actin-associated protein Keap1 (or the similar protein INrf2) and requires translocation to the nucleus where it binds regulatory sequences (Itoh et al 1999; Dhakshinamoorthy and Jaiswal 2001; Nguyen et al 2004). Keap1 serves as a redox sensor that suppresses Nrf2 translocation until this interaction is inhibited by oxidative stress. A role for Nrf2 in the regulation of *c-Ha-ras* in vSMCs by oxidative stress is consistent with recent studies by Liu et al. (2004) showing Nrf2 binding to the ARE/EpRE of the heme oxygenase-1 (HO-1) gene in vSMCs after endoplasmic reticulum stress. Furthermore, Li et al (2004) have shown that diesel exhaust particle fractions enriched in polycyclic aromatic hydrocarbon (PAHs) induce expression of HO-1 through Nrf2 activation of ARE/EpRE.

Small Maf proteins form heterodimers with Nrf2 that bind ARE/EpRE and MARE. The size of these proteins (18–20 kDa) is similar to the 23- and 25-kDa proteins identified in UV cross-linking experiments, suggesting that these low-molecular weight proteins may in fact be Maf proteins. Interestingly, a homologue of MafG has been identified in hamster fibroblasts treated with H₂O₂. This homologue, adapt66, is redox sensitive and may implicate Maf or Maf-like proteins as stress-regulated proteins that bind ARE/EpREs (Crawford et al 1996).

Using a PEI-purified fraction containing proteins that specifically bind to hHaras27, Hsp90 β was identified as an ARE/EpRE interacting protein. Hsps function as molecular chaperones for proper protein folding and participate in the repair or degradation of denatured proteins after heat shock (Benjamin and McMillan 1998). Hsps also provide a mechanism for holding receptor proteins in ligand competent states and chaperoning these proteins to their sites of action. Hsp90 β is a constitutively expressed Hsp, but a number of stresses, including oxidative stress, induce its expression (Jornot et al 1991). Interestingly, oxidative stress-induced Hsp90 α and cyclophilin may play a role in promoting vSMC growth (Liao et al 2000). In addition, the chaperone functions of Hsp90 β are highly influenced by signal transduction cascades such as the serine/threonine and tyrosine kinase cascades that have been shown to mediate ARE/EpRE

binding protein activation. It is likely that detection of Hsp90 β by the PEI isolation method reflects its function as a chaperone with ARE/EpRE binding proteins pulled down with the PEI resin. As such, we previously have isolated and sequenced the zinc finger transcription factor Phi AP3 as a putative ARE/EpRE-binding protein (Holderman et al 2002). Because zinc finger proteins are known to interact with Hsp chaperones, this offers another possibility of the role of Hsp90 β in our system. It remains to be determined whether Hsp90 β interacts with Nrf2 in complex formation and interaction with ARE/EpREs. This seems a reasonable possibility given that both estrogen receptor and aryl hydrocarbon receptor, 2 proteins that complex with Hsp90, are present in ARE/EpRE protein complexes (Montano et al 1998; Chen and Ramos 2000).

In view of the putative role of Hsp90 β in the formation of ARE/EpRE complexes in vSMCs, proteins that normally interact with Hsp90 β were researched to determine whether their DNA-binding sequences are present in hHaras27. The glucocorticoid receptor (GR) is 1 such protein that complexes to Hsp90 β in the cytoplasm. Hsp90 binds to unliganded GR to open the steroid hormone-binding pocket of the receptor (Young et al 2001). Indeed, a GR-like response element (GRE), defined as TGTCT (where Y = C or T) and often found as an imperfect palindrome separated by 3 bp (Falkner et al 2001) was identified in hHaras27, with the half sites underlined and ARE/EpRE core sequence in bold: (5'-AGCTCCTGGGT **GACAGAGCGAGA**AAGCT-3'). Thus, Hsp90 β may be present in the PEI-isolated fraction as a member of a steroid receptor heterocomplex. Interestingly, a recent study showed that Hsp90 β binds to the GRE as a member of the GR heterocomplex and promotes disassembly of the transcriptional regulatory complex (Freeman and Yamamoto 2002). These findings are consistent with our own findings showing that Hsp90 β inhibits hHaras27 ARE/EpRE protein binding. Studies have also shown protein-protein interactions between GR and c-jun and c-fos, AP-1 proteins that bind ARE/EpRE sequences (Pfahl 1993; Cato and Wade 1996). GR and AP-1 proteins interact on the proliferin gene, where the GR target sequence is an overlapping GRE and TRE. A similar overlap is also found between a GRE half site and a TRE-like element within the *c-Ha-ras* ARE/EpRE core. Falkner et al (2001) have also described the regulation of rGSTA2 via sequences within the ARE/EpRE by both the GR and pregnane X receptors. In addition, ER α and ER β were found to mediate hNQO₁ gene expression through binding to ARE/EpRE and protein-protein interaction with hPMC2 (Montano et al 1998, 2000; Bianco et al 2004). Two overlapping estrogen response element (ERE)-like half sites are found in hHaras27 (5'-AGCTCCTGGGT**GACAGAGCGAGA**AAGCT-3'), with the ERE defined as 2 half sites

separated by a string of N base pairs (5'-GGTCAnnn TGACC-3') (Klinge 2001). Overall, these possibilities offer interesting mechanisms of steroid receptor binding and activation through ARE/EpRE sites.

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