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Identification of Albumin Precursor Protein, Phi AP3, and α-Smooth Muscle Actin as Novel Components of Redox Sensing Machinery in Vascular Smooth Muscle Cells

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ABSTRACT

Aerobic organisms are continually subjected to environmental stressors that compromise redox homeostasis and induce cellular injury. In vascular smooth muscle cells (vSMCs), the activation/repression of redox-regulated genes after environmental stress often involves protein binding to *cis*-acting antioxidant response elements (AREs). The present study was conducted to identify proteins that participate in redox-regulated protein binding to human c-Ha-ras and mouse glutathione *S*-transferase A1 AREs in vSMCs after oxidant injury. Challenge of vSMCs with 0.3 or 3 μM hydrogen peroxide, 3-methylcholanthrene, benzo[*a*]pyrene-7,8-diol, 3-hydroxy benzo[*a*]pyrene, and benzo[*a*]pyrene-3,6-quinone induced concentration-related increases in ARE protein binding. The profiles of ARE complex assembly were comparable, but exhibited chemical specificity. Pretreatment with 0.5 mM *N*-acetylcysteine inhibited activation of ARE protein binding in hydrogen peroxide-treated cells. Preparative electrophoretic mobility shift assays coupled to Western analysis identified NF-E2-related proteins 1 and 2 and JunD in complexes assembled on AREs. Polyethylenimine affinity and sequence-specific serial immobilized DNA affinity chromatography followed by N-terminal sequencing identified albumin precursor protein, phi AP3, and α-smooth muscle actin as members of the ARE signaling pathway. Sequence analysis of albumin protein revealed homology to the redox-regulated transcription factors Bach1 and 2, as well as cytoskeletal and molecular motor proteins. These results implicate albumin precursor protein, phi AP3, and α-smooth muscle actin as participants in redox sensing in vSMCs, and suggest that protein complex assembly involves interactions between leucine zipper and zinc finger transcription factors with cytoskeletal proteins.

Aerobic organisms are continually subjected to environmental stressors that compromise redox homeostasis and induce cellular injury. As part of the adaptive response to environmental stress, aerobes have evolved a variety of proteins that sense changes in redox status and convey signals to the nucleus that regulate gene expression. For example, bacteria express OxyR, a transcriptional regulator activated through disulfide bond formation and deactivated by glutaredoxin-1 (Zheng et al., 1998). In addition, several oxidative stress-activated transcription factors have been identified in *Saccharomyces cerevisiae*, including Yap1, Yap2, and Gcn4, all members of the basic leucine zipper family of proteins. These yeast factors are homologous to mammalian c-Jun, and act through DNA sequences termed stress responsive elements (Marchler et al., 1993). Higher eukaryotes have a similar defense strategy, whereby Rel, AP-1, and NF-E2-related factors (Nrfs) are recruited to the nucleus to interact with various *cis*-acting DNA elements and modulate transcription in response to redox stress.

One prominent redox-sensing mechanism in mammalian cells involves activation of protein binding to a *cis*-acting element sequence known as the antioxidant response element (ARE). ARE sequences are present in the 5′-untranslated region of genes involved in redox homeostasis (Li and Jaiswal, 1992), growth regulation (Bral and Ramos, 1997), and drug metabolism (Favreau and Pickett, 1991; Liu and Pickett, 1996). Extensive characterization of ARE sequences identified 5′-GTGACNNNGC-3′ as the minimal ARE core

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ABBREVIATIONS: Nrf, NF-E2-related factor; ARE, antioxidant response element; BaP, benzo[*a*]pyrene; CNC, Cap'n'Collar; Keap-1, Kelch-associated protein-1; vSMC, vascular smooth muscle cell; BaPQ, BaP 3,6-quinone; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; GSTA1, glutathione *S*-transferase A1; PEI, polyethylenimine; PAGE, polyacrylamide gel electrophoresis; SIDAC, serial immobilized DNA affinity chromatography; PVDF, polyvinylidene difluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; BLAST, basic local alignment search tool; DBP, vitamin D-binding protein; hHa-ras, human Ha-ras.

sequence required for inducible expression (Rushmore et al., 1991; Wasserman and Fahl, 1997). Subsequent studies have shown that flanking sequences exert a significant effect on basal and inducible gene expression, and the consensus core sequence was redefined as 5′-TMANNRTGAYNNNGCR-3′ (Wasserman and Fahl, 1997; Holderman et al., 2000). The transcriptional response mediated through ARE sequences is complex, because induction profiles vary according to cell type, gene context, and chemical treatment. Sequence homologies between AREs, 12-*O*-tetradecanoylphorbol-13-acetate-response elements, and Maf recognition elements add complexity to the integration of ARE-regulated responses. Although variations in ARE responsiveness have been documented, regulation through this element is often exerted by polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene (BaP) and its reactive metabolites, or phenolic antioxidants such as *tert*-butyl hydroquinone, hydrogen peroxide, and antiestrogens (Bral and Ramos, 1997; Montano et al., 1998; Moehlenkamp and Johnson, 1999; Miller et al., 2000). Current efforts are directed toward identification and characterization of the multiprotein ARE complex assembly in different gene contexts, cell types, and chemical treatments.

Several ARE-interacting proteins have been identified, including the Cap'n'Collar (CNC) proteins Nrf1 and Nrf2 (Ishii et al., 2000; Nguyen et al., 2000); small Maf proteins; JunB, C, and D (Itoh et al., 1999); Fos-related factors Fra-1 and Fra-2 (Jeyapaul and Jaiswal, 2000); the structurally related proteins Kelch-associated protein-1 (Keap-1) and Bach2 (Oyake et al., 1996; Jeyapaul and Jaiswal, 2000); as well as estrogen receptors α and β (Montano et al., 1998). Many ARE binding proteins belong to the leucine zipper family of transcription factors and dimerize with similar or related proteins for gene activation/repression. For example, protein complex assembly on the ARE of the NQO_1 gene in HepG2 cells includes Nrf2 and c-Jun and/or JunD (Nguyen et al., 2000). Itoh et al. (1999) have described a repression mechanism in response to electrophilic stress involving Nrf2 and a novel cytoplasmic factor termed Keap-1. Using the yeast two-hybrid assay, Keap-1 was identified as an Nrf2 binding protein that precludes translocation of Nrf2 to the nucleus. Addition of pro-oxidants can overcome this inhibition and allow activation of ARE-regulated transcription. Keap-1 is a homolog of the *Drosophila melanogaster* actin-binding protein Kelch, suggesting that Nrf2 and Keap-1 coordinate with the actin framework in response to redox stress.

Cell- and promoter-specific patterns of ARE protein binding indicate that multiple proteins interact with this DNA sequence, and that complex assembly involves recruitment of proteins for interaction with the basal transcriptional machinery. Although final outcomes of ARE protein binding and gene regulation may be comparable, different proteins may be involved in the biological response. Therefore, the present studies were conducted to identify proteins that participate in redox-regulated protein binding in vascular smooth muscle cells (vSMCs) treated with BaP, a pro-oxidant that generates reactive oxygen species after cellular metabolism, or its quinone metabolite BaP 3,6-quinone (BaPQ) (Miller et al., 2000). Immunochemical methods and N-terminal sequencing identified Nrf1 and Nrf2, JunD, albumin precursor protein, phi AP3, and α-smooth muscle actin as participants in redox signaling in vSMCs.

Materials and Methods

Cell Culture. Cultures of C57/BL6 mouse aortic vSMCs were grown in Media 199 (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen). For chemical treatments, vSMCs were challenged with either 3 μM BaP or BaPQ at 37°C and 5% CO_2 for 3 h. BaPQ is a primary oxidative metabolite of BaP that directly activates ARE signaling in vSMCs (Miller et al., 2000).

Electrophoretic Mobility Shift Assay (EMSA). Cultured cells were washed twice in cold buffer B (25 mM HEPES, 1 mM DTT, 1.5 mM EDTA, 10% glycerol, and 0.5 mM PMSF, pH 7.6) and harvested. Cells were Dounce homogenized with 30 strokes on ice and nuclei pelleted at 5000*g* at 4°C. The purity of nuclear preparation was evaluated by phase contrast microscopy. Nuclei were resuspended in buffer B plus 0.5 M KCl, and protein extracted on ice for 1 h. Nuclear ghosts were removed by centrifugation at 12,000*g* for 10 min and the supernatant removed and stored at −80°C. EMSA incubations were performed using 5 μg of vSMC nuclear extract in buffer B + KCl (25 mM HEPES pH 7.6, 1.5 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 M KCl) with 2 mM DTT, 40 ng of poly(dIdC), and 20 μg of BSA incubated with 10 fmol of [^{32}P]ATP-labeled probe at room temperature for 20 min. Oligonucleotides used in EMSA reactions included human Ha-ras (hHa-ras) ARE: 5′-AGCTCCTGGGTGACAGAGCGAGAAGCT-3′ and mouse GSTA1 ARE: 5′-GATCTAATGGTGACAAAGCAACTT-3′ (ARE cores underlined). Human Ha-ras and mouse GSTA1 ARE sequences were chosen because they are representative of the redox-regulated transcriptional response in vSMCs and exhibit similar nuclear protein binding profiles (Holderman et al., 2000; Miller et al., 2000). In some experiments, an hHa-ras mutant oligonucleotide, 5′-CCTGGGGAGAAGAAAGAGAGGTAC-3′, was used as competitor. Samples were loaded onto a 7% acrylamide gel and run in 0.5× Tris borate-EDTA buffer at 25 mA for 30 min. Gels were dried and analyzed via PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA/Amersham Biosciences AB, Uppsala, Sweden).

Polyethyleneimine (PEI) Purification. PEI purification was carried out according to Worland and Wang (1989), with modifications. Briefly, vSMCs were seeded in 15-cm culture dishes at 75 cells/mm^2, treated for 3 h with 3 μM BaP (Sigma-Aldrich, St. Louis, MO), and nuclear protein harvested in buffer I (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM PMSF, and 1 mM β-mercaptoethanol) as a wash buffer, and buffer I containing 25 mM KCl as an extraction and storage buffer. Protein concentration was determined by the method of Bradford using BSA as a standard. PEI (10% v/v, pH 7.9; Sigma-Aldrich) was added to the nuclear protein extract to a final concentration of 0.1%, along with 1 mM PMSF, and incubated on ice 3 min. Prebaked Celite (2 h, 120°C and cooled) (10 g/100 ml) (Sigma-Aldrich) was added before incubation on ice for 5 min and centrifugation at 14,000 rpm for 10 min at 4°C. A 200-μl aliquot of buffer I containing 25 mM KCl was added to the pellet, which was then incubated on ice for 5 min, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and stored until use. This procedure was repeated with three sequential washes with buffer I containing 500 mM KCl, three washes with buffer I containing 1 M KCl, and two washes with buffer I containing 2 M KCl. All fractions were stored at −80°C for further analysis. Protein content was determined using a discontinuous SDS-PAGE 4% stacking and 8 or 10% resolving gel in a 1× SDS running buffer. Rainbow Molecular Weight Markers (Amersham Biosciences, Piscataway, NJ) were run to estimate molecular masses. All samples were boiled for 5 min, loaded onto the gel, and run at 120 V for stacking and 225 V for resolving gels. Coomassie blue or silver staining was used to identify proteins in each fraction.

Preparative EMSA, Protein Elution, and Characterization. vSMCs were seeded in 15-cm culture dishes at 125 cells/mm^2, challenged with 3 μM BaP for 3 h, and nuclear protein harvested as

described previously (Bral and Ramos, 1997). For preparative EMSA, 112.5 μg of nuclear protein was incubated with 30 fmol (3.75:1 ratio) of double-stranded γ-^{32}P-labeled hHa-ras ARE oligonucleotide at room temperature for 20 min. The binding reaction was supplemented with 1 mM DTT and 50 ng of poly(dIdC) in a total volume of 20 μl. For the nonradioactive preparative lane, 1 mg of nuclear protein was incubated with 270 fmol of blunted, double-stranded oligonucleotide (3.75:1), equivalent to nine individual reactions, and supplemented as described above. BSA, often used in EMSA reactions to stabilize protein/DNA interactions, was eliminated to avoid contaminant protein bands. No difference in band shift or intensity was noted in trial experiments when BSA was eliminated. Loading dyes were added [15% Ficoll type 400 (Amersham Biosciences), 0.25% bromphenol blue, 0.25% xylene cyanol FF], and reactions immediately loaded onto a 7% nondenaturing polyacrylamide gel using 0.5× Tris borate-EDTA running buffer (50 mM Tris, 45 mM boric acid, and 1 mM Na_2EDTA, pH 8.3) and electrophoresed at 25 mA. After electrophoresis, the radioactive lane was cut from the nonradioactive preparative lane, the two pieces lined up for reference and sealed in cellophane. The gel was analyzed via PhosphorImager (Storm; Molecular Dynamics) and the image printed to paper. The gel was lined up directly above the printed image, and the nonradioactive band excised corresponding to the migration of the visible radioactive band. The upper and lower areas of the gel bordering the nonradioactive band of interest were excised as controls, along with the radioactive band. The excised gel slices were cut into smaller pieces and incubated in 100 μl of 12× loading buffer (0.75 M Tris, 0.42 M SDS, 12% basal medium Eagle, 20% glycerol, and 0.2% bromphenol blue) for 15 min. The loading buffer was removed and boiled for 3 min. Gel slices were loaded into a preparative lane of a 4% stacking/8 or 10% resolving SDS-PAGE gel along with the loading buffer. Rainbow Molecular Weight Markers were run alongside protein samples for size estimates, and BSA standards were included for approximation of protein amounts.

Serial Immobilized DNA Affinity Chromatography (SIDAC). Aliquots of 0.50 mg of GSTA1 ARE half-site (5′-GATCTAATGGTGACAAAGCAACTT-3′) and complementary sequence were annealed, phosphorylated with polynucleotide kinase, and filled-in with Klenow to provide a site-specific sequence suitable for affinity chromatography. DNA was purified and ligated as described by Kerrigan and Kadonaga (1993). Extracted DNA was coupled to a 1-ml *N*-hydroxysuccinamide-activated agarose Hi-trap column (Amersham Biosciences) for 30 min at 4°C in coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3). Ethanolamine (0.5 M) was used to block any remaining *N*-hydroxysuccinamide groups, and the column was equilibrated with buffer Z (25 mM HEPES, 1 mM DTT, 20% glycerol, and 0.1% Nonidet P-40, pH 7.6). Nuclear extract from 3 μM BaP-treated vSMCs was brought to total volume with buffer Z and incubated with 2 ng/μl poly(dI/dC). Samples were loaded onto the column and incubated on ice for 30 min. Protein was eluted with a salt gradient (0–2.0 M KCl) and analyzed by 8% SDS-PAGE using silver stain.

Preparative EMSA-Western. EMSA conditions were performed as described above. Large-scale incubations were electrophoresed at 40 mA. The gel was removed from the plates, sealed in cellophane, and visualized by PhosphorImaging analysis (Storm; Molecular Dynamics). The EMSA gel was overlaid onto a PhosphorImager print and shifted complexes excised. Gel slices were minced in microcentrifuge tubes and suspended in sample buffer at a 1:3 ratio. Samples were boiled for 2 to 3 min, loaded onto SDS-PAGE, and electrophoresed as described above. Protein was transferred onto PVDF (Bio-Rad, Hercules, CA) membrane overnight at 4°C and processed for Western analysis according to manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, primary antibody was added at 1:250 and secondary at 1:1000 with two washes of Tris-buffered saline + 0.1% Tween between additions. Immunodetection was afforded by alkaline phosphatase-conjugated secondary antibodies and enzyme substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Two-Dimensional Electrophoresis. Crude or SIDAC nuclear protein samples were incubated in 2× volume of cold acetone on ice for 30 min. Precipitated protein was collected by centrifugation (15,000*g*) for 5 min at 4°C. Samples were dissolved in solubilization buffer [9.5 M urea, 4% CHAPS, 2.5 mM DTT, and 2% IPG buffer (3–10 NL or 4–7; Amersham Biosciences), and 0.01% bromphenol blue] and equilibrated for 30 min at room temperature before loading onto IPG Drystrips (Amersham Biosciences). Samples were focused for 100,000 vhr at 20°C on an IPGPhor (Amersham Biosciences). After focusing, IPG Drystrips were incubated in SDS-PAGE sample buffer containing 10 mg/ml DTT followed by incubation in sample buffer containing 25 mg/ml iodoacetamide. Proteins were separated on a 10% SDS slab gel and visualized by silver stain.

In-Gel Digestion/Extraction. Proteins were excised from 10% SDS-PAGE and subjected to reduction/alkylation digestion and extraction. Slices were washed for 30 min in 1 ml of 30% MeOH and then washed for 60 min in 500 μl of 100 mM ammonium bicarbonate. Protein was reduced by the addition of 160 μl of 100 mM ammonium bicarbonate containing 2.5 mM DTT and incubated for 30 min at 60°C. After incubation, 10 μl of a 100 mM solution of iodoacetamide was added and incubated for an additional 30 min at room temperature in the dark. The gel slice was washed in 500 μl of wash buffer (50% MeCN and 50 mM Tris, pH 9.2) for 60 min at room temperature, minced, and subsequently washed with 50 μl of MeCN, dried in a SpeedVac (Thermo Savant, Holbrook, NY) without heat, rehydrated in 10 μl of 100 mM ammonium bicarbonate containing 0.25 μg of either modified trypsin (Promega, Madison, WI) or EndoLys C (Waco Biochemicals, Waco, TX), and incubated for 12 h at 30°C. After digestion, peptides were extracted with 150 μl of 0.1% trifluoroacetic acid (TFA), 60% acetonitrile for 60 min at room temperature. The slices were re-extracted with an additional 150 μl 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 equipped with a diode array detector and separated on a 218TP5215 (2.1 × 150 mm) C18 reversed phase column (Vydak, Hesperia, CA) 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 min, followed by a linear gradient from 5 to 50% B over 90 min and peptides collected manually.

Protein Sequencing. Automated Edman chemistry was performed on a Hewlett Packard G1000A automated protein sequencer. Samples were either bound to a C18 resin (liquid samples) or inserted into an empty column space (PVDF electroblotted samples). 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.

Results

ARE Complex Assembly. Profiles of ARE complex assembly in response to 0.3 or 3 μM hydrogen peroxide, 3-methylcholanthrene, benzo[*a*]pyrene-7,8-diol, 3-hydroxy benzo[*a*]pyrene, and benzo[*a*]pyrene 3,6-quinone were evaluated by EMSA. These agents are known to activate ARE-regulated redox signaling in vSMCs (Miller et al., 2000). All chemical agents induced concentration-related increases in ARE protein binding, but profiles of protein complex assembly were specific for each chemical. In most instances, protein binding resolved as a doublet (denoted C1 and C2), except in vSMCs treated with 3-methylcholanthrene or benzo[*a*]pyrene-7,8-diol where three distinct complexes were re-

solved (Fig. 1A). C2 was the predominant complex in vSMCs treated with benzo[*a*]pyrene 3,6-quinone, indicating that different chemical oxidants induce comparable profiles of ARE complex assembly in vSMCs, but that complex assembly is oxidant-specific. The redox sensitivity of ARE complex assembly was confirmed in experiments showing that 0.5 mM *N*-acetylcysteine, a soluble antioxidant and inducer of glutathione in vSMCs (Kerzee and Ramos, 2000), inhibits hydrogen peroxide-induced protein binding to the ARE oligonucleotide (Fig. 1B).

PEI EMSA. PEI affinity purification has been used to successfully purify the chromatin-binding protein topoisomerase II (Worland and Wang, 1989). Crude vSMC nuclear protein extracts were eluted with increasing concentrations of salt, with most DNA-binding proteins eluted in 1 M KCl. After sequential protein elutions with 25 mM, 500 mM, 1 M, and 2 M KCl, vSMC protein fractions were electrophoresed on a discontinuous 10% SDS-PAGE gel. Fractions 6 to 8, which represent sequential 1 M KCl elutions, showed clean resolution of proteins by SDS-PAGE (Fig. 2A). Two prominent bands of 80 and 100 kDa were present in fraction 6, a finding consistent with previous UV cross-linking studies identifying an 80-kDa protein as an ARE-interacting protein (Bral and Ramos, 1997). A 42-kDa protein was also predominant in this fraction. Protein fraction 6 contained the highest amount of protein from the 1 M KCl elution and was

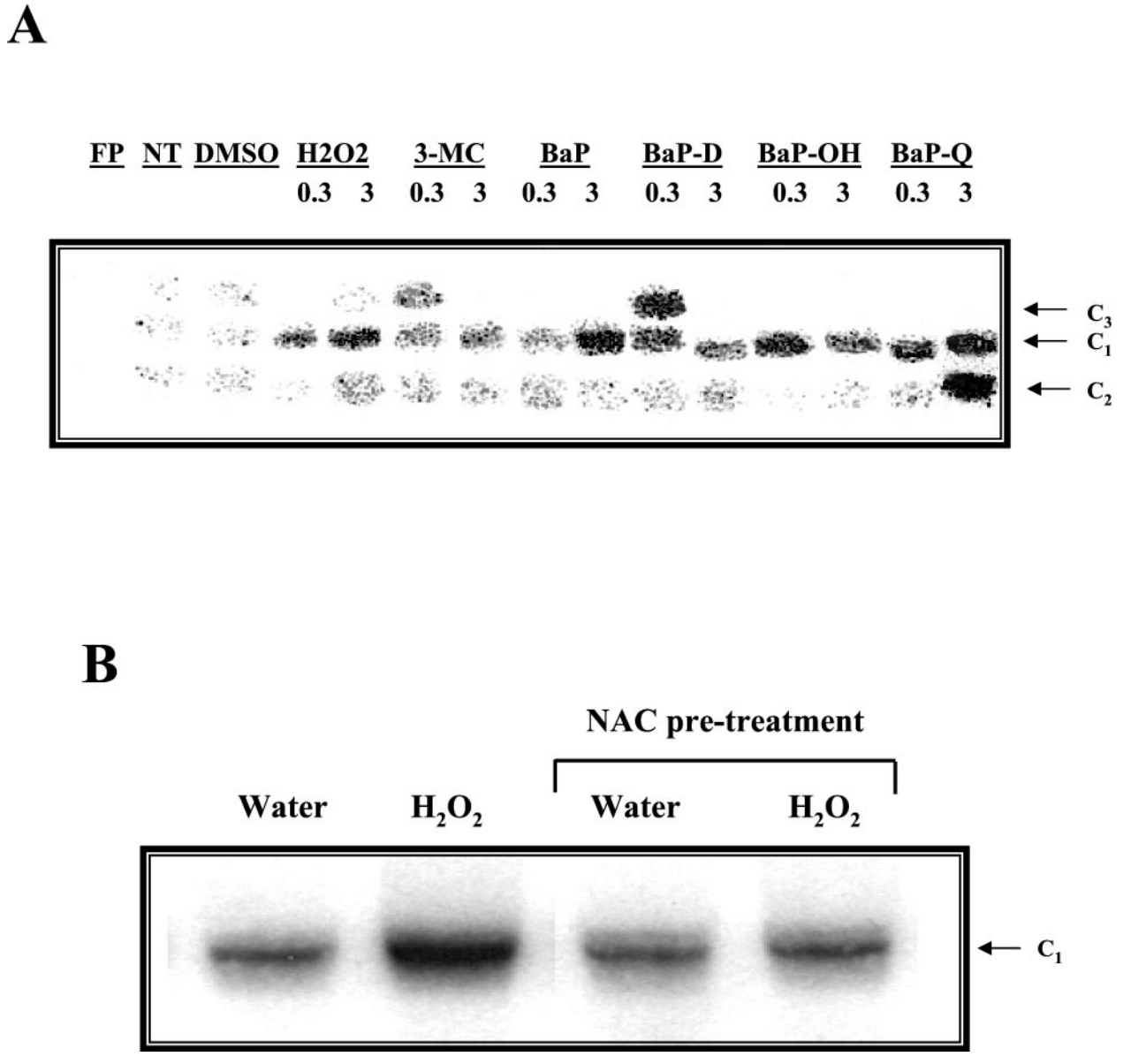

Fig. 1. Protein binding to hHa-ras ARE induced by various chemical oxidants. A, nuclear extracts of vSMCs (5 μg) treated with 0.3 and 3 μM hydrogen peroxide, 3-methylcholanthrene, BaP, BaP diol, 3-hydroxy BaP, or BaPQ or an equivalent volume of DMSO (control) were incubated at room temperature with 10 fmol of end-labeled ARE for 20 min. Protein/DNA complexes were resolved on a 7% nondenaturing polyacrylamide gel. FP, free probe; NT, no treatment. Arrows indicate specific complexes (C1, C2, and C3). Specific complexes were identified by competition with unlabeled specific (hHa-ras) oligonucleotide (data not shown). Results are representative of two separate experiments. B, nuclear extracts of vSMCs (5 μg) treated with water or 25 μM H_2O_2 alone or in the presence of *N*-acetylcysteine (NAC) were incubated at room temperature with 10 fmol of end-labeled ARE for 20 min. Protein/DNA complexes were resolved on a 7% nondenaturing polyacrylamide gel. Specific complexes were identified by competition with unlabeled specific (hHa-ras) oligonucleotide (data not shown). The arrow denotes specific complexes (C1). Results are representative of two separate experiments.

therefore, analyzed by EMSA for DNA-binding activity to a ^{32}P-labeled hHa-ras ARE oligonucleotide. Proteins eluted at 0.5 M KCl (fraction 5) and crude vSMC nuclear extracts from DMSO- and BaPQ-treated vSMCs were included for comparison. Figure 2B shows that PEI-purified proteins from fraction 6, but not 5, formed a shifted complex with the ARE sequence. The mobility of this complex approximated, but was not identical to, that of complexes resolved from nuclear extracts of DMSO- or BaPQ-treated vSMCs. Treatment with BaPQ increased ARE protein binding over DMSO controls, but this induction was most pronounced for the fastest migrating complex (C2). The specificity of the interaction was

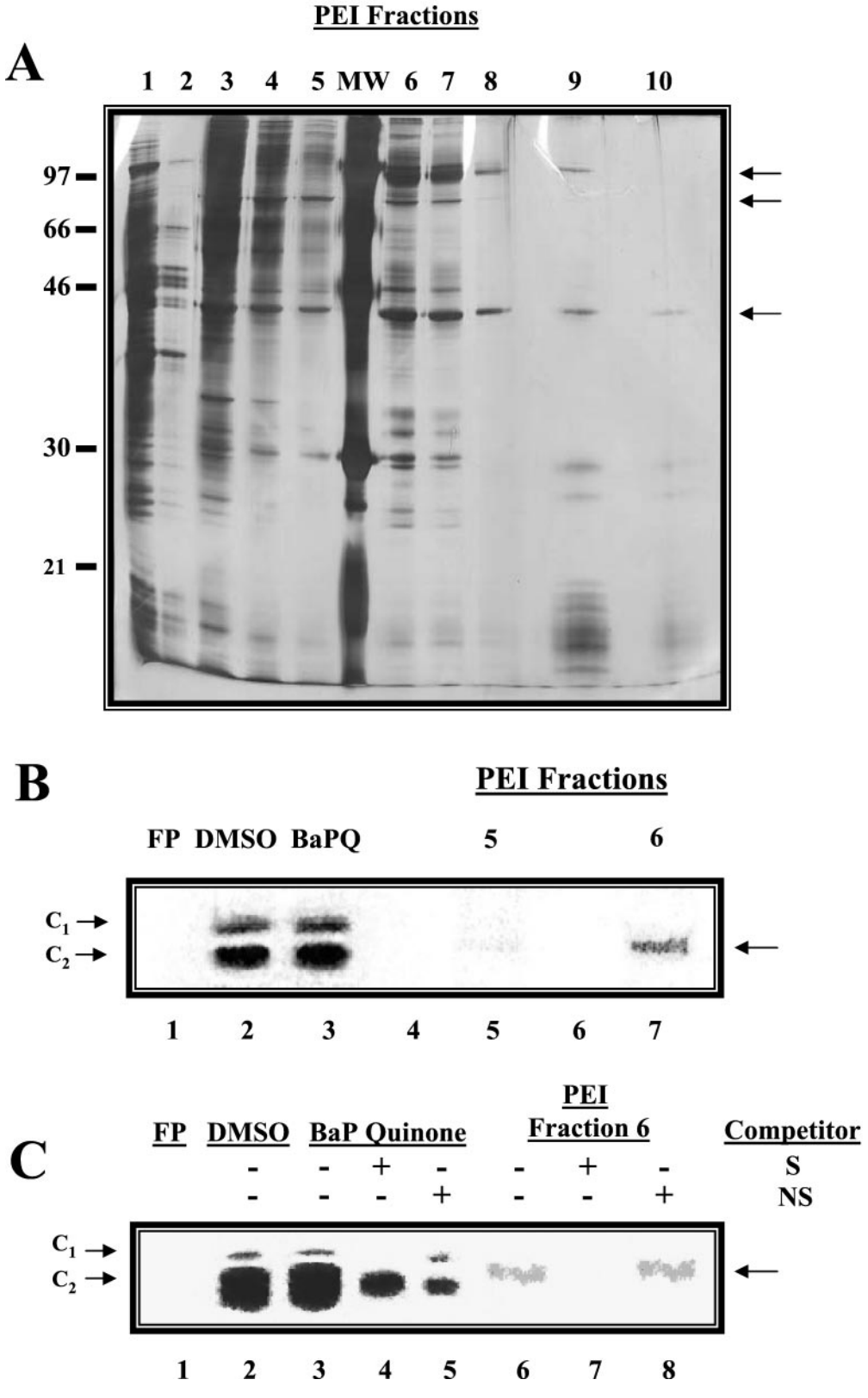

Fig. 2. Reconstitution of EMSA and competition analysis using crude vascular smooth muscle cell nuclear protein and PEI-purified protein. A, fractionation of PEI-purified vSMC nuclear protein by KCl elution and 10% SDS-PAGE. PEI-purified nuclear proteins from BaP-treated vSMCs were eluted from the PEI slurry using various molar concentrations of KCl. Lane 1 represents a wash of PEI resin without KCl, lane 2 nuclear protein from a mild salt wash (0.025 M), and lanes 3 to 5 protein eluted with 0.5 M KCl. Lanes 6 to 8 and lanes 9 to 10 represent 1 and 2 M KCl elutions, respectively. MW, molecular mass standards of indicated masses. B, fraction 6 (1 M KCl) from PEI affinity contains ARE-interacting proteins. Shown in lanes 2 and 3 are two ARE/protein complexes from DMSO- and BaPQ-treated vSMCs. FP, free probe. Treatment with 3 μM BaPQ increases formation of C2 relative to DMSO. Fraction 5 represents protein from 0.5 M KCl elution. Fraction 6 protein (lane 7) from PEI approximates the migration of complexes formed by crude vSMC nuclear protein. Lanes 4 and 6 represent empty lanes. C, PEI fraction 6 protein binds ARE specifically and is competed by excess unlabeled site-specific oligonucleotide (S), but not a mutant oligonucleotide (NS) used to examine nonspecific interactions. EMSA analysis of nuclear extracts from DMSO (0.1% v/v) and BaPQ (3 μM)-treated vSMCs were shown for comparison (lanes 2–5).

confirmed by competition with excess unlabeled hHa-ras wild type oligonucleotide (Fig. 2C). In contrast, a single shifted complex was observed for fraction 6, and was competed by hHa-ras wild-type oligonucleotide (Fig. 2C). Thus, fraction 6 of PEI-purified nuclear extracts contained some of the proteins that specifically interact with the ARE. The slight difference in migration profile between crude nuclear extract and fraction 6 are probably due to differences in salt content, as evidenced by shifting profiles of fractions containing different salt concentrations.

SIDAC EMSA. A SIDAC strategy was also used to purify proteins involved in ARE redox signaling. Approximately 5 mg of nuclear protein was processed through a series of affinity-based chromatographic separations, with protein retained on mGSTA1 ARE-specific resin assayed for ARE-binding activity by EMSA. Figure 3A shows reconstitution of EMSA complexes upon incubation of ARE oligonucleotide with increasing amounts of ARE SIDAC retentate (0–5 μl). Protein from the ARE SIDAC eluate was sufficient for ARE recognition (Fig. 3A) and bound oligonucleotide in a concentration-dependent manner. Competition analysis using increasing amounts of unlabeled ARE oligonucleotide showed that reconstituted EMSA complexes were effectively competed by wild-type mGSTA1 ARE sequence (Fig. 3B). The pattern of mobility retardation for crude vSMC nuclear extract is shown for comparison. This interaction was specific because competition with 200-fold poly(dIdC) did not interfere with protein binding. Collectively, these findings indicate that proteins isolated by PEI and mGSTA1 ARE SIDAC can recognize the ARE binding site.

To further characterize SIDAC-purified fractions, protein was submitted to broad- and narrow-isoelectric point (pI) range two-dimensional electrophoresis. Figure 4A shows the resolution of SIDAC-purified protein over a broad pI range (pH 3–10). Several proteins of 42, 48, 58, and 68 kDa in the pI range of 4 to 5 were observed. The detection of multiple spots within a narrow focus region is suggestive of post-translational modification. Because the 42-kDa protein was also present in PEI-purified protein, additional SIDAC ma-

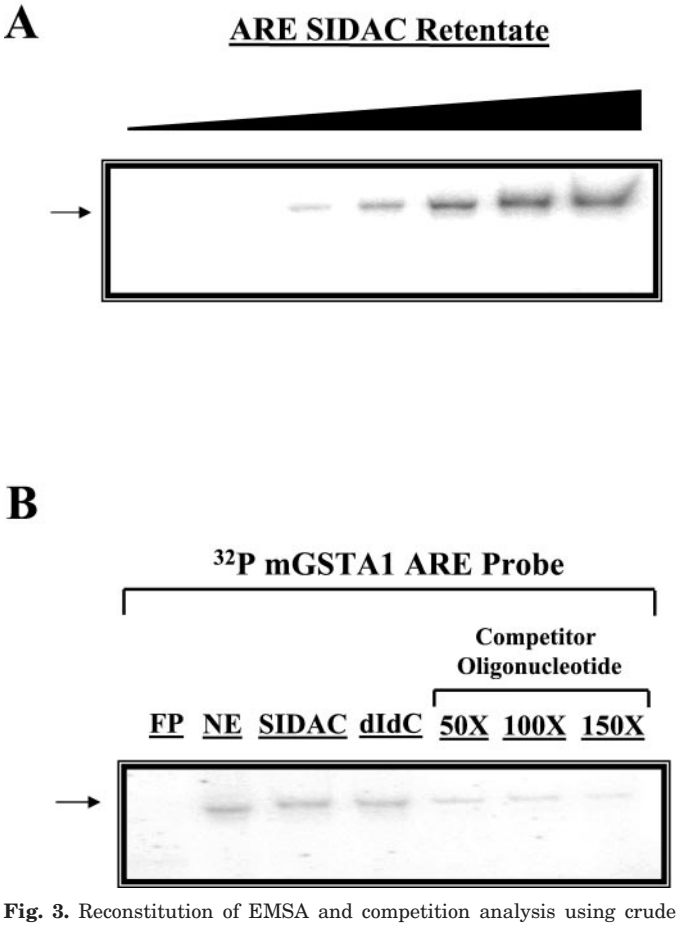

Fig. 3. Reconstitution of EMSA and competition analysis using crude vSMC nuclear protein or SIDAC protein fraction and ARE oligonucleotide from murine GSTA1. A, nuclear protein from BaP-treated vSMCs submitted to ARE SIDAC reconstitutes EMSA in a concentration-dependent manner. Bar indicates the addition of increasing amounts of mGST ARE SIDAC eluate (0–20 μl). B, nuclear protein from BaP-treated vSMCs retained by mGSTA1 ARE SIDAC is competed by unlabeled, site-specific oligonucleotide (5′-GATCTAATGGTGACAAAGC-AACTT-3′), but not by nonspecific sequence [poly(dI/dC)]. Crude nuclear extract from non-treated vSMCs (SMC NE) and mGST ARE SIDAC eluate are shown for comparison (lanes 2 and 3, respectively). Arrows denote specific complexes identified.

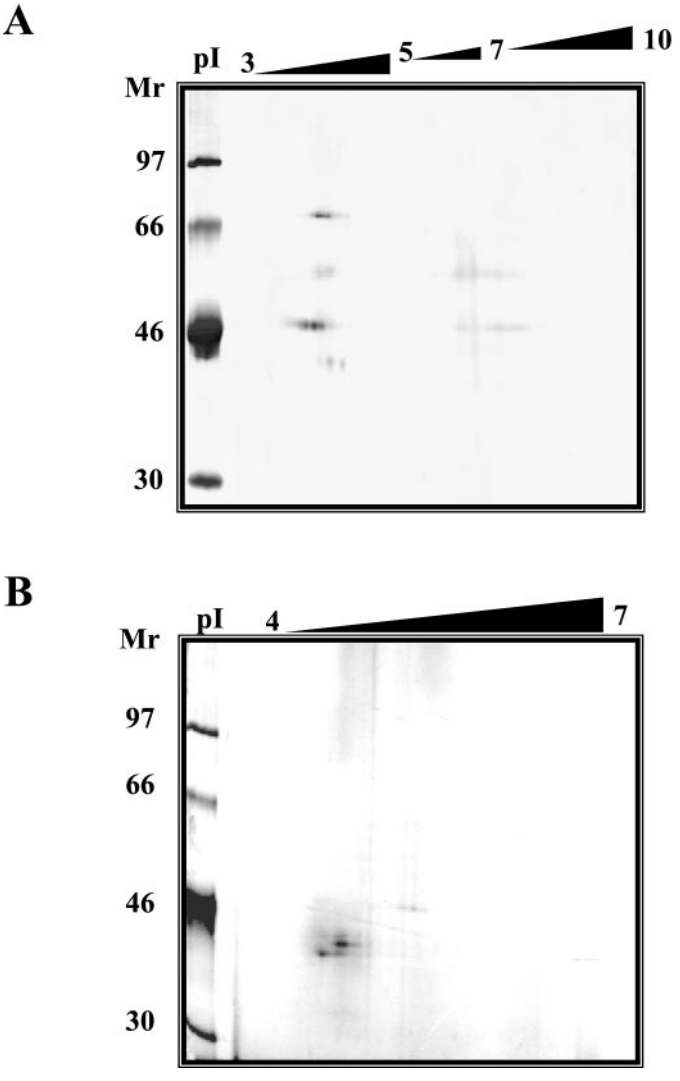

Fig. 4. Two-dimensional electrophoresis of GST-ARE SIDAC protein fraction. A, GST-SIDAC-purified protein from BaP-treated vSMCs resolved by isoelectric point (pH 3–10, nonlinear) and molecular mass. Proteins of 42, 48, 58, and 68 kDa are highly purified and were observed having pIs of 4.0 to 5.0. B, narrow pH range (4–7) shows further resolution of isoelectric properties. The 66- and 97-kDa proteins were not detectable in samples subjected to narrow range analysis due to differences in relative abundance of these proteins.

terial was subjected to narrow-range (pH 4–7) pI focusing for further study of this protein (Fig. 4B). Several of the proteins identified within this region using the broad pI range were further resolved using this approach. The results of two-dimensional electrophoresis show that mGSTA1 SIDAC yielded a highly purified fraction of proteins.

EMSA Western Analysis. To begin to define the composition of protein/DNA complexes in vSMCs, a modified preparative EMSA/Western strategy was used. Mouse GSTA1 ARE DNA-protein complexes from BaP-treated vSMCs were first resolved in a nondenaturing electric field to preserve the multimeric assembly of components within each complex. Individual ARE complexes were excised and subjected to SDS-PAGE for mass resolution, and then transferred to PVDF for immunochemical detection of ARE-interacting proteins. Using this approach, Nrf1 and 2 and JunD were identified in three of the ARE EMSA complexes in vSMCs using the mouse GSTA1 ARE as a template (Fig. 5). Migration of the bands varied slightly due to differences in biological and chemical context. Each of these complexes was specific, as determined by EMSA competition analysis with 50 to 150 times excess mouse GSTA1 oligonucleotide competitor or 200 times poly(dIdC) (data not shown). Nrf1 was present in nuclear extracts from bovine aorta and cultured mouse vSMCs (Fig. 5A, lanes 1 and 2). The protein was highly enriched in low- and high-mobility ARE complexes (Fig. 5A, lanes 3 and 4), but absent or not detected in SIDAC-purified protein fractions (Fig. 5A, lanes 8 and 9). In contrast, expression of Nrf2 was undetectable in crude nuclear extracts of bovine aorta and cultured vSMCs at protein levels comparable with those used for Nrf1 immunodetection, but present in lower mobility EMSA complexes (Fig. 5B, lane 5), and in the 10 μl of SIDAC-purified protein fraction (Fig. 5B, lane 9). Nrf2 immunoreactivity was not detected in higher mobility complexes (Fig. 5B, lane 6), but detectable in crude vSMC extracts at higher protein levels (data not shown). In subsequent experiments, we tested for the ability to discriminate protein signals from EMSA-bound complexes versus protein from background samples where no oligonucleotide was present. Figure 5C shows that JunD is present in complexes 1 and 2. Absence of JunD in the background samples proves that this protein is present in the ARE-protein complex and not detected by comigration of unbound material. These results indicate for the first time that in vSMCs, like in other cell types (Ishii et al., 2000; Jeyapaul and Jaiswal, 2000), the CNC members Nrf1 and Nrf2, as well as JunD, interact with the *cis*-acting ARE sequence.

Edman N-Terminal Protein Sequencing. The proteins isolated by PEI and ARE SIDAC participate in specific recognition of the ARE sequence. The two predominant bands of 80 and 100 kDa from PEI were submitted for Edman N-terminal sequence analysis because proteins of these molecular masses had been detected previously by ARE UV cross-linking (Bral and Ramos, 1997). A 12-amino acid sequence was obtained for the 80-kDa protein NH_2-(X-P-H-X-S-E-I-A-H-R-F-X)-COOH and BLAST analysis of this sequence identified the protein as albumin precursor protein (Table 1). Although albumins are members of a large protein family, some members are known to function as ligand-inducible nuclear proteins, such as vitamin D-binding proteins. Homology searches using the albumin precursor and the 12-amino acid sequence identified Bach2 and several other signaling proteins as homologs. Specifically, three domains of 100% identity exist between albumin precursor protein and Bach2 spanning 11 to 12 nucleotides with one domain at the N-terminal portion of each protein and two domains mapping to the C-terminal portion of the coding region. Eight additional homology domains were identified in this alignment that reside outside the predicted open reading frames of either protein (data not shown). Sequence was also obtained for the 100-kDa protein NH_2-(X-G-E-A-A-T-A-P-R-X-H)-COOH. BLAST analysis showed several homologies to the zinc finger transcription factor AP3 (Table 1). Because a 42-kDa protein was present in active PEI and SIDAC fractions, the purified 42-kDa protein was digested using the protease EndoLys C, and one of the peptide fragments submitted for N-terminal

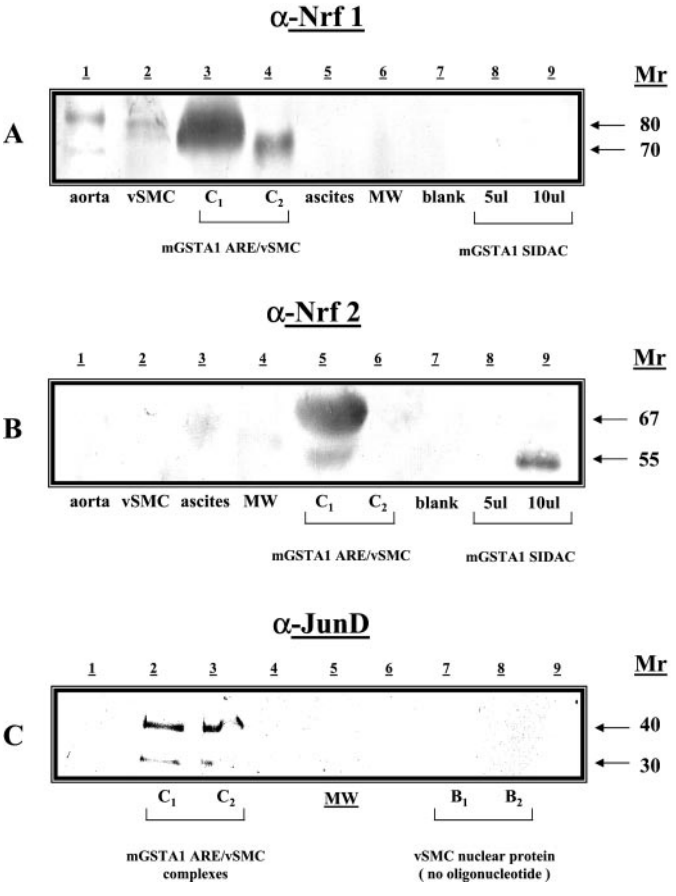

Fig. 5. Cap'n'Collar factors Nrf1 and Nrf2 and JunD are antioxidant response element-interacting proteins in vascular smooth muscle cells. Immunodetection of Nrf1 and Nrf2 and JunD from various protein sources. A, Nrf1 is detected in nuclear protein from different tissue and cellular sources (lanes 1 and 2) and is highly enriched in preparative EMSA complexes 1 and 2 derived from nuclei of untreated vSMCs (lanes 3 and 4, respectively). Nrf1 is not detected in ascites fluid, which serves as a negative control for nonspecific immunoreactivity. Nrf1 was not detected in the 5- or 10-μl aliquots of mGSTA1 SIDAC eluate. Lanes 6 and 7 contain Rainbow (Amersham Biosciences) Molecular Weight ladder and blank sample, respectively. B, Nrf2 is present in the lower mobility (C1) preparative EMSA complex (lane 5), and in mGSTA1 SIDAC-purified protein. Lanes 4 and 7 are Rainbow (Amersham Biosciences) Molecular Weight ladder and blank lane, respectively. C, JunD is detected in preparative mGSTA1 SIDAC complexes C1 and C2 (lanes 2 and 3). Lane 5 is Rainbow (Amersham Biosciences) Molecular Weight ladder and lanes 1, 4, 6, and 9 are blank. No protein is detected in gel samples containing vSMC protein without mGSTA1 oligonucleotide (lanes 7 and 8). All antibodies were purchased from Santa Cruz Biotechnology. Molecular weight determinations for all blots were completed by alignment of immunoreactive bands with Rainbow (Amersham Biosciences) standards.

sequencing. Fourteen amino acids were sequenced from this peptide and the sequence NH_2-(I-W-H-H-S-F-Y-N-E-L-R-V-A-P-E-E-H-P-T)-COOH was determined to be a fragment of mouse aortic α-smooth muscle actin.

Discussion

The results presented herein identify for the first time Nrfs1 and 2, JunD, albumin precursor protein, phi AP3, and aortic α-smooth muscle actin as components of the redox-sensing machinery in vSMCs. Some of these proteins have been characterized as DNA-binding proteins, including Nrf1 and 2, JunD, and phi AP3, whereas others such as albumin precursor and α-smooth muscle actin may be present in the signaling complex as accessory factors. Nrf1 and Nrf2 are known to coordinate with, or antagonize the action of small Maf proteins in ARE signaling (Nguyen et al., 2000). JunD, phi AP3, and members of the albumin and actin family of proteins are emerging as proteins that serve multiple functions. For example, albumin-like proteins, such as the vitamin D-binding protein (DBP), bind sterols to convey transcriptional signals to the nucleus. In fact, DBP translocates to the nucleus upon binding its endogenous ligand, 1,25-dihydroxyvitamin D3 (MacDonald et al., 2001). DBP is positioned on chromosome 4 in proximity to chemokines, such as interleukin 8, and is thought to participate in redox-regulated inflammatory responses (Yamamoto and Naraparaju, 1996). Albumin and DBP contain cysteine residues that predict a characteristic pattern of disulfide bridges and homologous protein folding (Bogaerts et al., 2001). DBP also binds globular actin with high affinity and inhibits actin polymerization by sequestering monomeric G-actin, thereby limiting construction of "signaling roadways" (McLeod et al., 1989). The physicochemical properties of DBP are modified by interaction with actin, an allosteric mechanism that may be significant in the regulation of protein function (Boyer and Peterson, 2000). Furthermore, Meijerman et al. (1999) have shown that cellular stress induces rearrangement of G-actin in the nucleus, and suggested that this may reflect changes in the interaction of G-actin with chromosomes. Interestingly, a 70-kDa albumin-like protein has been described in cornea that is modulated by oxidative stress (Zhu and Crouch, 1992).

Other albumin-like proteins have been identified as components of signaling and/or motor complexes by sequence homology (Table 1). BLAST analysis of the albumin precursor protein showed homology to Bach2, as well as several zinc finger proteins containing homeodomains. Bach2 possesses CNC and Broad-Complex, Tramtrack, and Bric-a-Brac (BTB) domains known be critical for functional interactions with other proteins, ARE sequences, and actin (Jeyapaul and Jaiswal, 2000; Nguyen et al., 2000; MacDonald et al., 2001). The BTB motif is also required for the activation function of Nrfs, and interaction with corepressors such as N-CoR and SMRT (Huynh and Bardwell, 1998). This motif is also found in zinc finger proteins containing a cytoskeleton interaction motif (Collins et al., 2001). Bach2 associates with small Maf factors via the CNC motif to participate in the regulation of transcription through Maf recognition elements and ARE sequences (Igarashi et al., 1998). BTB proteins often contain Kelch domains that mediate interactions with the cytoskeleton, a relationship consistent with previous reports showing Keap-1 interactions with Nrf proteins that inhibit their transcriptional activation potential (Jeyapaul and Jaiswal, 2000). In addition, Hoshino et al. (2000) have suggested that oxidative stress abolishes nuclear export of Bach2, implicating this protein as a central mediator in a redox signaling pathway.

TABLE 1

Protein N-terminal microsequencing and BLAST analysis

Protein microsequencing by Edman identified serum albumin precursor, phi AP3, and α-smooth muscle actin as ARE-interacting proteins. BLAST analysis of the proteins revealed several homologous proteins as indicated. The reported molecular weight for candidate proteins interacting with the ARE is given for comparison.

Edman Sequence	M_r	Sequence Matched	Homolog	Accession No.	%
	kDa				
N-(X-P-H-X-S-E-I-A-H-R-F-X)-C	70	Serum albumin precursor	BTB and CNC homology 2	NP_031547.1	42
			BTB and CNC homology 1	NP_068585.1	42
			Vitamin D-binding protein precursor	P21614	41
			ABINs, A20-binding inhibitor of NF-κB	CAB44239.1	37
			Wnt-8D protein precursor (stimulated by retinoic acid)	Q64527	38
			Left-right dynein	AF183144	41
N-(X-G-E-A-A-T-A-P-R-X-H)-C	100	Phi AP-3 zinc finger transcription factor	E4F transcription factor 1	62065	87
			KRAB zinc-finger protein	AF242378	58
			Zinc-finger protein Gfi-1	A48152	56
			Kruppel-related zinc finger protein	AF211867	58
			RB-associated KRAB repressor	AF226870	56
N-(I-W-H-H-S-F-Y-N-E-L-R-V-A-P-E-E-H-P-T)-C	42	α-smooth muscle actin	Actin-like	NP_038826.1	93
			A-X actin	J04181	95
			Actin-related protein 1A; ARP1	NP_005727.1	73

Thus, oxidative stress in vSMCs may initiate the assembly of protein signaling complexes directed spatially to gather at specific locations on the cytosolic scaffolding to be ferried to the nucleus via cytoskeleton-bound motors. Movement of steroid receptor along microtubular tracks to the nucleus has recently been established (Galigniana et al., 1999).

The presence of albumin precursor protein in ARE complexes suggests that this protein may act as a sensor of oxidative stress within the cell. Cantin et al. (2000) have shown that albumin acts a specific modulator of cellular glutathione levels and predicted a relationship between human serum albumin, cellular glutathione, and nuclear factor-κB activation. Serum albumin increases glutathione levels and protects cells against oxidant-induced cytotoxicity and tumor necrosis factor-α-mediated nuclear factor-κB activation. Furthermore, serum albumin modulates vSMC energy metabolism in carotid artery strips where extracellular albumin is taken up and broken down into by-products that stimulate oxygen consumption and augment glucose oxidation (Barron et al., 2000).

Phi AP3, a murine zinc finger, kruppel-related protein, was also identified as an ARE-interacting protein. Phi AP3 is a murine homolog of E4F-1, a known transcriptional repressor of the adenoviral E4 gene. Phi AP3 is related to the GLI-Kruppel protein and contains six zinc finger domains. The E4F-1 protein competes with ATF proteins for binding to a regulatory sequence within the adenovirus E1A E4 promoter. ATF proteins, however, coordinate with Jun proteins to recognize ARE-like sequences (Falvo et al., 2000). Moreover, the E4F recognition sequence (5′-TGACGTAAC-3′) is strikingly similar to the ARE core sequence (5′-GTGACNNNGC-3′), suggesting that similar complexes may regulate gene expression through these elements. A report describing fragmentation of phi AP3 to generate an active DNA-binding polypeptide via phosphorylation of the parent protein was recently published (Fernandes and Rooney, 1997). Other zinc finger proteins such as estrogen receptors α and β interact with the ARE *cis*-element (Montano et al., 1998). A novel zinc finger protein participates in the regulation of quinone reductase activity, an ARE-regulated gene (Montano et al., 2000). Together, these results suggest that signaling through the ARE sequence involves receptors for sterol compounds that coordinate with structural and signaling intermediates.

Although this is the first report showing actin interactions with proteins that bind a DNA regulatory sequence, other actin-related proteins and actin-binding proteins are known to participate in the regulation of transcription through alterations in high-order chromatin structure. For example, Sung et al. (2001) found that ArpN, a novel actin-related protein, resides in the nucleus, and is part of the human SWI/SNF chromatin remodeling machinery. Moreover, polymerization and binding of actin monomers can be modulated by redox signals, as exemplified by the discovery of a functional interdependence between filamin, an actin-binding protein, and the transcriptional response (Ozanne et al., 2000). Specifically, actin rearrangements have been observed in different cell contexts in response to H_2O_2, and transforming growth factor signals, chemicals that can elicit ARE-mediated transcriptional responses (Milzani et al., 2000; Xu et al., 2001). Interestingly, data from our own laboratory suggest that chromatin-remodeling proteins also associate with AREs and accessory factors (M. T. Holderman and K. S. Ramos, unpublished data).

The current study implicates an albumin-like protein and actin as participants in "architectural signal transduction" in vSMCs through associations with Nrf1 and 2, JunD, and phi AP3. Several reports have recently been published describing cooperation of so-called structural proteins with well characterized signaling proteins. Structural components may also play functional roles in signal transduction through spatial organization of multiprotein complexes or by providing organized transit of macromolecules via molecular motors. These findings suggest a novel signaling mechanism and warrant further investigation.

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