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The characterization and purification of a human transcription factor modulating the glutathione peroxidase gene in response to oxygen tension

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Abstract

An oxygen responsive transcription factor regulating human glutathione peroxidase gene (GPx) through two oxygen responsive elements (ORE1 and ORE2) has been purified and characterized by sequence-specific DNA affinity chromatography. The DNA binding activity, termed Oxygen Responsive Element Binding Protein (OREBP), was partially represented by a 77 kD polypeptide (p70) possessing a blocked N-terminus. The p70 subunit co-eluted with an 86 kD subunit (p80) from affinity columns. N-terminal sequencing analysis of the 86 kD component revealed that this protein represented the larger member of the Ku antigen complex. The identity of the purified 77 kD subunit was determined by Western blot analysis using an antibody directed against the p70 protein. In addition to binding the GPx-ORE, the OREBP was itself regulated by oxygen tension. It was found that the abundance of the ORE binding activity was decreased in cells maintained at low oxygen tension (40 mm Hg). Anti-Ku-antibodies specifically supershifted the OREBP-ORE DNA complex. These observations further add to the numerous nuclear roles of the Ku-transcription factor. (Mol Cell Biochem **229**: 73–83, 2002)

Key words: DNA binding proteins, glutathione peroxidase, hypoxia, Ku-antigen, protein purification, transcription factors

Abbreviations: CBP – CREB-binding protein; CREB – a protein which binds a DNA element called cAMP-regulated enhancer**;** CSPD – disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate; DIG – digoxigenin; DTT – dithiothreitol; EDTA – ethylenediamine-tetraacetic acid; EGTA – ethyleneglycol-bis-(β-aminoethyl ether) N,N,N1,N1-tetraacetic acid; EMSA – electrophoretic mobility shift assay; GPx – the classical human cytosolic selenium-dependent glutathione peroxidase enzyme; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF1 – hypoxia-inducible factor; HPLC – high performance liquid chromatography; NFIV – nuclear factor IV; NRE1 – negative regulatory element 1 in the long terminal repeat of mouse mammary tumor virus; ORE – oxygen-responsive cis-acting DNA element; OREBP – ORE-binding protein; PEG – polyethylene glycol; PMSF – phenylmethylsulfonyl fluoride; PVDF – polyvinylidene difluoride; REF1 – redox factor protein; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Introduction

Cells adapt to changes in oxygen tension by modulating their metabolic activities accordingly. Generally, key enzymes regulating aerobic metabolism are down-regulated as the oxygen tension is lowered while glycolytic enzyme activity is increased [1]. Free radical scavenging enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase are also decreased in response to chronically low arterial oxygen tension [2]. The transcriptional mechanisms regulating such classes of enzymes remain uncharacterized.

The expression of the human GPx gene, encoding the major free radical scavenging enzyme in the heart, is tightly controlled by the level of arterial oxygen tension [3] through the action of two oxygen responsive promoter elements, ORE1 and ORE2 [4]. These elements share the consensus sequence AYCCTC$^{A}/_{T}$RAGAAA and reside at –267 and –1209 bp upstream, respectively, of the GPx translational initiation site. Each element is sufficient to drive the expression of a reporter construct under the context of a minimal promoter, in an orientation independent fashion, thereby conferring enhancer properties to the promoter region. This mode of regulation, in part, explains the increased susceptibility of the cyanotic myocardium to oxyradical injury [5]. Conversely to ORE-elements which activate transcription in response to increasing oxygen tension, hypoxia inducible factor 1 (HIF1) represents a transcription factor whose expression is activated under conditions of low oxygen tension [6, 7]. HIF1 expression acts to stimulate the up-regulation of various mRNAs, for example, those encoding glycolytic and erythropoietin transcripts [6, 8].

Here we report that the ORE-binding protein (OREBP) represents the previously described Ku antigen heterodimer, composed of p70 and p80 subunits, belonging to the leucine-zipper and helix-loop-helix class of DNA binding proteins [9, 10]. The Ku antigen was originally discovered as a DNA-associated nuclear protein found in the sera of some patients with autoimmune diseases [11]. The name 'Ku' is derived from the name of a Japanese patient. We show that the Ku antigen is capable of specifically binding to ORE1 and ORE2 in a sequence specific manner which is distinct from the other non-sequence dependent activities of Ku.

Materials and methods

Isolation of nuclei and nuclear proteins from tissue

Fresh human placentas were immersed into ice, cannulated and washed extensively with ice-cold 0.9% saline. The tissue was cut into 2 cm cubes and homogenized in a tissue blender in a 5-fold excess of ice-cold Buffer A (15 mM HEPES, 0.3 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM EGTA, 2 mM EDTA, 14 mM β-mercaptoethanol, pH 7.5). The homogenate was filtered through cheese cloth and crude nuclei were isolated essentially according to Hahn and Covault [12]. Briefly, nuclei were pelleted by centrifuging at 3000 rpm for 10 min in Beckman JA-20 rotor. The nuclear pellet was resuspended in ice-cold Buffer B (Buffer A containing 0.1 mM EGTA and 0.1 mM EDTA) and Triton X-100 was added to a final concentration of 0.5% (v/v). Percoll (Pharmacia Biotech, Quebec, Canada) was then added to a final concentration of 27% (v/v) and the nuclei were sedimented at 14,000 rpm for 30 min. The nuclear pellet was diluted in 10 times the packed nuclear volume (PNV) in Buffer B, re-centrifuged and used immediately for nuclear protein extraction.

Nuclear proteins were extracted as outlined by Hahn and Covault [12]. Nuclei were resuspended in one-half PNV of low salt extraction buffer (10 mM HEPES, pH 7.9 at 4°C; 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT). With gentle mixing, an equal vol. of high salt buffer (20 mM HEPES, pH 7.9 at 4°C, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) was added and the nuclei were extracted for 30 min at 4°C with gentle inversion. The extracted nuclei were removed by centrifuging at 25,000 × g for 15 min and the crude nuclear proteins were snap frozen in liquid nitrogen and stored at –80°C.

Cell culture and preparation of cellular nuclear extracts

Human heart cells were cultured as described previously [13] at an oxygen tension of 150 mm Hg. Following the third passage, half of the cells were transferred to an oxygen tension of 40 mm Hg. Protein extracts were isolated from the cultured cells using as described by Andrews and Faller [14]. All extract preparations were aliquotted, frozen in liquid nitrogen and stored at –80°C. Protein concentrations were determined according to Bradford [15] using a bovine serum albumin as a standard.

Primers and labeling reactions

Single stranded primers representing the various ORE1, ORE2 or non-consensus sequences are shown below:

ORE1 (top): 5′-AATTCTGTCATCCTCAAAGAAAGTGTATTG-3′
ORE1 (bottom): 3′-GACAGTAGGAGTTTCTTTCACATAACCTAG-5′
ORE2 (top): 5′-AATTCAGGAACCTCTGAGAAAAAACGGAGG-3′
ORE2 (bottom): 3′-GTCCTTGGAGACTCTTTTTTGCCTCCCTAG-5′
ORE2 short (top): 5′-AACCTCTGAGAAAAA-3′
ORE2 short (bottom): 3′-TTGGAGACTCTTTTTT-5′
ORE2 mutant (top): 5′-AATTCAGGAACCTCTAAACGGAGG-3′
ORE2 mutant (bottom): 3′-GTCCTTGGAGATTTGCCTCCCTAG-5′

Each single stranded primer was annealed to its complementary sequence in a final vol. of 25 µl containing: 1 × annealing buffer (10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$ and 1.6 mM DTT) and 2 µg of each single stranded complementary primer. The primer mix was heated to 95°C for 2 min and gradually cooled to room temperature. Annealed primers were labeled using the Klenow fragment of *Escherichia coli* DNA Polymerase I and [α-^{33}P]dATP and purified using a Sephadex G-50 column (Amersham Pharmacia Biotech, Quebec, Canada).

Electrophoretic mobility shift assays (EMSA)

Binding assays were performed at 0°C for 30 min in a buffer containing nuclear extract plus the following components: 20 µg BSA, 20 µg competitor sheared salmon sperm DNA, 1 mM DTT, 20 mM Hepes (pH 7.9 at 4°C), 25% glycerol, and 20 ng ^{33}P-labeled dsORE probes. The KCl concentration of the sample was adjusted to a final concentration of 60 mM.

EMSA were performed in a BioRad Protean II apparatus using 8% acrylamide gels (29:1 acrylamide:bisacrylamide). Fifteen microliters of the above binding reaction were loaded per lane. Electrophoresis was performed at 0°C using ice-cold 0.5 × TBE (1 × TBE: 100 mM Tris base, 100 mM boric acid, 2 mM EDTA (pH 8.3)) as the running buffer. All gels were subjected to electrophoresis for 1 h at 50 mA. Subsequently, the gels were wrapped in plastic wrap and exposed to Kodak BioMax film overnight.

For non-isotopic digoxigenin (DIG) gel shift assays, annealed oligonucleotides were labeled by enzymatic tailing of the 3′-end with digoxigenin-ddUTP and terminal transferase according to the manufacturers instructions (Boehringer Mannheim, Indianapolis, IN, USA) [16]. Labeled oligonucleotides were purified and labeling efficiency was determined by chemiluminescent analysis of dot blots on nylon membranes. The binding reaction (20 µl) contained 10 µg of nuclear protein extract, 0.1 µg poly L-lysine, 1 µg competitor poly (dI-dC) and 0.96 pmol digoxigenin labeled ORE2 oligonucleotide in binding buffer containing 20 mM HEPES, 1 mM DTT, 1 mM EDTA, 10 mM ammonium persulphate, 0.2% Tween-20 and 30 mM KCl. Supershift assays were performed exactly as described above except that 1, 2 or 5 µl of anti-Ku (p70 or p80) (obtained from Cedarlane, Canada), was mixed with the protein extracts and pre-incubated for 30 min on ice before the addition of digoxigenin labeled ORE2. Electrophoresis was performed at 0°C in 0.25 × TBE, pH 8.0. DIG-labeled ORE2 and oligonucleotide-protein complexes were transferred from the polyacrylamide gel to the Hybond-N nylon membranes (Amersham Pharmacia Biotech) immediately after electrophoresis by electroblotting [17] at 50V. The complexes were detected by an enzyme immunoassay using an anti-digoxigenin antibody conjugated to alkaline phosphatase and the chemiluminescent substrate CSPD. Membranes were exposed to Kodak X-OMAT film for 1 h prior to development.

Polyethylene glycol fractionation of crude placental nuclear extracts

Solid PEG_{4000} was added to the placental nuclear extract to a final concentration of 10% (w/v). The sample was incubated on ice for 10 min and centrifuged for 20 min at 14, 000 rpm at 4°C. The supernatant was transferred to a new tube, the PEG concentration was adjusted to 30% (w/v) and the preparation was treated as indicated above. The 10 and 30% pellets were solubilized in Column buffer (CB; 20% glycerol, 20 mM Hepes pH 7.9 at 4°C; 1 mM EDTA) containing 50 mM KCl. An aliquot from each fraction was removed for EMSA and SDS-PAGE analysis and the remainder was snap frozen in liquid nitrogen.

High-Q ion exchange chromatography

The 30% PEG nuclear protein fraction was thawed on ice and the KCl concentration adjusted to 50 mM from a 1 M stock solution. The extract was applied to a 5 ml bed vol. of High-Q ion exchange resin (Pharmacia Biotech, Quebec, Canada) which had been previously washed and equilibrated in CB. The column was washed with 5 column vol. of CB (containing 50 mM KCl) and the proteins were sequentially eluted by increasing the KCl concentration from 50–2000 mM KCl. Fractions (5 ml) were collected across the entire elution range and immediately frozen in liquid nitrogen following the removal of a 10 µl aliquot for analysis.

Heparin-agarose affinity chromatography

The High-Q fractions containing the peak ORE2 binding activities were pooled, the KCl concentration adjusted to 50 mM KCl and this was applied to a 5 ml bed vol. of Heparin-agarose (Pharmacia Biotech, Quebec, Canada). The column was washed with five column vol. of CB and eluted with increasing concentrations of KCl (50–2000 mM). Five milliliter fractions were collected throughout the salt range and these were immediately frozen in liquid nitrogen.

Preparative EMSA

The heparin-agarose fractions exhibiting the peak analytical EMSA activity were concentrated with a Centricon-30 (30,000 mw cut-off) concentrator (Amicon Canada, Ontario, Canada) and added to a EMSA reaction buffer which was scaled up by

approximately 12 times from the analytical conditions described above. The presence of excess competitor DNA at this step ensured the sequence specific binding activity of the preparative fraction. The entire large scale binding reaction was loaded onto a preparative non-denaturing EMSA gel and fractionated as indicated. The shifted complex was excised and layered onto a SDS-PAGE gel, the sample was fractionated by electrophoresis and the protein(s) transferred to PVDF (0.2 μm). The transferred proteins were detected by Coomassie blue staining. To prevent potential interference by acid, the acetic acid was omitted from the staining solutions.

ORE2-affinity chromatography

Biotinylated ORE2 primers were synthesized so as to possess a single biotin group on 3' end of the bottom strand. The biotinylated ORE2 was attached to an avidin-agarose matrix (Sigma Canada, Ontario, Canada) by incubating in Binding buffer (BB; 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) for 1 h at 4°C with inversion. To this were applied the peak fractions from the Heparine-agarose affinity column. Bound proteins were eluted by sequentially increasing the KCl concentration. Aliquots of each fraction were stored for analysis and the remaining samples frozen in liquid nitrogen.

Western blot analysis

Equal quantities of nuclear protein from cultured heart cells grown at oxygen tensions of 150 or 40 mm Hg were fractionated through a 4% stacking/10% running gel and transferred onto a PVDF membrane (Bio-Rad) by electrotransfer. The membranes were blocked with 5% (vol/vol) nonfat dry milk/TTBS (20 mM Tris-HCl, pH7.5/500 mM NaCl/0.1% Tween 20) for 1 h at room temperature, incubated with rabbit anti-Ku (p70) (diluted 1:5000; Cedarlane, Canada) or anti-Ku (p86) (diluted 1:10000; Cedarlane, Canada) in TTBS with 5% nonfat dry milk overnight at 4°C. Subsequently the membranes were washed twice with TTBS. For visualization of the p70 specific polypeptide membranes were incubated with a goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma) (diluted 1:3000) for 1 h at room temperature, washed twice with TTBS and developed with ECL reagents (Amersham Pharmacia Biotech). The p86 specific polypeptide was visualized using a secondary goat anti-mouse antibody coupled with horseradish peroxidase (Bio-Rad) (diluted 1:3000) for chemiluminescent detection.

N-terminal sequencing analysis

Proteins were sequenced from electroblotted samples. Samples eluted from the affinity columns were desalted and concentrated in a Centricon 10 (10,000 mw cut-off; Amicon) and applied to 4–20% gradient SDS-PAGE gels (Novex) under reducing buffer. Gels were run at a constant current of 42 mA per gel for 30 min and transferred onto a PVDF membrane in Tris-glycine buffer containing 20% methanol overnight at 100 mA. Samples were sequenced on an ABI 476A protein sequencer.

Results

Electrophoretic mobility shift assays (EMSA)

EMSA was performed using nuclear proteins prepared from either human heart cells or human placenta. The binding complex was similar in the nuclear proteins extracts derived from either human heart cell cultures or placental tissue (Fig. 1A). Crude placental nuclear extracts consistently showed a shifted complex which was the same for the ORE1 and ORE2 probes (Fig. 1B). A weaker slightly smaller shifted complex was also observed on occasion whose nature and characteristics were not assessed (observed in Fig. 1A). Optimal and stable binding of the major complex was achieved with KCl concentrations of 60–100 mM and with the inclusion of at least 0.5–1 mM DTT. In dilute, crude nuclear preparations, the binding activity was labile and could substantially be prolonged by the addition of a non-specific carrier protein such as BSA [18, 19]. The observed binding was selective and specific as approximately 1000-fold excess non-specific competitor sheared salmon sperm DNA, or competitor poly(dI-dC), could not compete with the binding of the ORE probe.

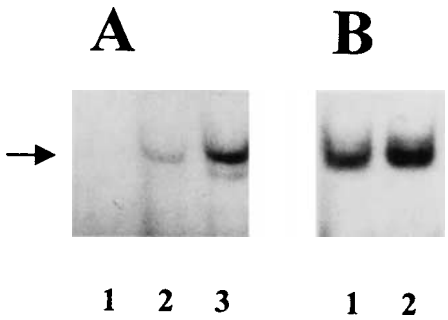

Fig. 1. EMSA analysis using heart cells and placental nuclear proteins extracts. (A) Placental nuclear proteins (5 μg) (lane 2) and heart cells nuclear proteins (10 μg) (lane 3) were incubated in the presence of ^{33}P-labeled ORE2. Lane 1, control (without protein extract). (B) Placental nuclear proteins (20 μg) were incubated in the presence of ^{33}P-labeled ORE1 (lane 1) and ^{33}P-labeled ORE2 (lane 2). The bound complex was subjected to fractionation through an 8% acrylamide gel (50 mA for 1 h at 0°C). The shifted complex was visualized by exposing the gels to X-ray film overnight. The position of OREBP is indicated by arrow.

Selective PEG_{4000} enrichment

PEG_{4000} fractionation was used to partially enrich for the ORE binding activity while simultaneously desalting the sample. Significant binding activity was shown to precipitate at approximately 20% PEG and peak at 25% PEG (data not shown). The bulk of non-specific proteins were precipitated in 10% PEG, while quantitative recovery of the DNA binding activity was found in a 30% PEG fraction (Fig. 2A). This observation permitted considerable enrichment and quantitative recovery of ORE binding activity by simply using two PEG fractionation steps (Fig. 2A). Proteins remaining soluble in 30% PEG did not exhibit any ORE binding activity.

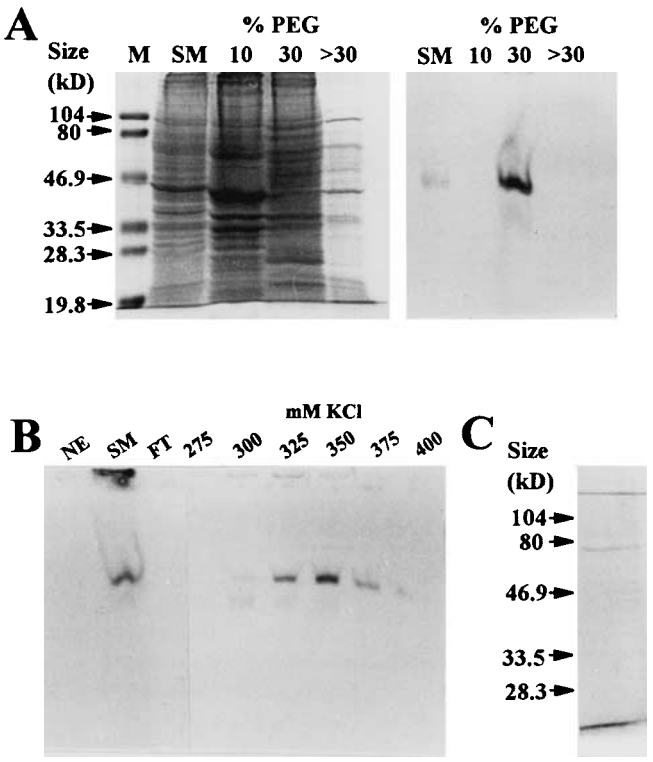

Fig. 2. Purification of OREBP by PEG_{4000} fractionation, chromatography and preparative EMSA. (A) Total placental nuclear proteins were fractionated by sequential addition of PEG_{4000}. Aliquots of each fraction were separated by SDS-PAGE through a 4% stacking/10% running gel (left panel) and tested for ORE-binding activity by EMSA analysis (right panel). M and SM refer to the size marker (Helix Technologies, Ontario, Canada) and starting material, respectively. The PEG fractions used are indicated. (B) Nuclear proteins precipitated by 30% PEG were further fractionated through a High-Q ion exchange column. Nuclear material was loaded onto the column in 50 mM KCl and sequentially eluted by increasing concentrations of KCl. Peak elution occurred in the range shown (325–375 mM KCl). NE, SM and FT refer to no-extract negative control, starting material and flow-through, respectively. (C) Preparative purification of p70 using EMSA gels. Placental nuclear material that had been subjected to PEG fractionation and High-Q and heparin-agarose column chromatography was concentrated and subjected to a large scale EMSA. The bound protein/DNA complex was fractionated through an 8% acrylamide gel and the shifted complex visualized by autoradiography. The radioactive gel slice was excised, layered onto a denaturing SDS-PAGE gel and subjected to electrophoresis. The gel contents were transferred onto a PVDF (0.2 μm) membrane and stained with Coomassie blue. A single band of approximately 77 kD was detected as shown.

High-Q and heparin fractionation

The ORE binding activity was determined to bind quantitatively to a high-Q ion exchange resin and this characteristic was employed in its purification. The binding activity was eluted from the column in the salt range of 300–400 mM KCl (Fig. 2B). The fractions exhibiting the maximum binding activity were combined and concentrated. This indicated that the OREBP exhibited a predominance of exposed negative charge on the surface of the protein at pH 7.9. Since ORE2-BP was much more abundant in purified nuclear extracts than ORE1-BP, only the analysis of the ORE2 shifted pattern was performed. To test the specificity OREBP binding to ORE2, mutant probes (abbreviated ORE2short and ORE2mutant (6 bp were deleted from the middle of ORE2)) were compared. The non-consensus ORE2 mutants did not interact with OREBP (Fig. 3).

On an analytical scale, the OREBP would bind to a heparin-agarose affinity matrix. The bulk of the binding activity was

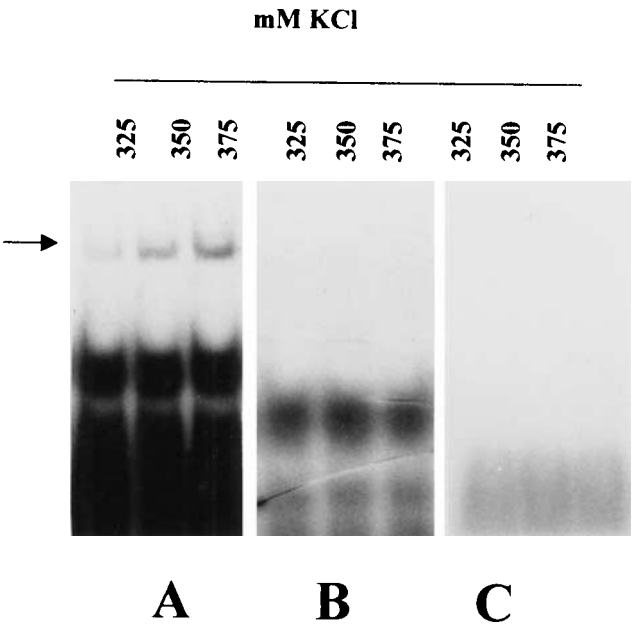

Fig. 3. EMSA analysis using chromatographically purified placental nuclear extract. (A) ORE2; (B) ORE2 short and (C) ORE2 mutant sequences were used. Nuclear protein fractions eluted from a High-Q ion exhange column were used for EMSA. No substantial binding to ^{33}P-labeled probe was detected for ORE2 short (B) and ORE2 mutant2 (C). The position of OREBP is indicated by arrow.

retained on the column until a salt concentration in the range of 275–1000 mM was achieved. Maximal elution occurred between 375 and 450 mM KCl (not shown). Although this fractionation step removed the bulk of the non-desired proteins, the substantial loss of activity that occurred during this fractionation prevented it from being used in a preparative protocol.

Preparative EMSA purification

Concentrated high-Q fractions were used to perform a preparative EMSA. After excising the shifted complex from the gel and subjecting the gel slice to SDS-PAGE and electrotransfer onto PVDF, a single band was observed which migrated with an apparent molecular weight of 77 kD (Fig. 2C). This band was reproducibly obtained over several independent purifications using the same procedure. When subjected to repeated N-terminal sequencing analysis, it was confirmed that the N-terminus was blocked. As insufficient material was available for internal sequence determination a non-gel dependent purification method was employed.

ORE2-affinity purification

Affinity purification of the OREBP was evaluated by using an ORE2-agarose affinity matrix. A nuclear extract which had been subjected to PEG, High-Q fractionation and Heparine-agarose chromatography was applied to the affinity matrix and sequentially eluted with an increasing KCl gradient (150–2000 mM). This purification scheme resulted in the quantitative retention of the OREBP on the affinity matrix. The binding activity was maximally eluted in a salt range of 350–450 mM KCl (Fig. 4). This elution range corresponded in the elution of two major protein bands, one at approximately 77 kD and the second at approximately 86 kD. The increase in the abundance of these bands mirrored the increase in specific ORE-binding activity (Fig. 4) and the profile of the shift was identical among the various EMSA gels used at each step of the purification. The 77 kD band isolated by affinity purification migrated to the same relative position as that seen with the preparative EMSA purification procedure used above. To maintain maximal biological activity and to increase the magnitude of protein recovered, a larger capacity affinity column was generated and a simplified step elution protocol was employed. Nuclear material was PEG fractionated, concentrated and directly applied to the affinity matrix. The matrix was extensively washed with 150 mM KCl buffer to remove non-specific proteins. The bound proteins were eluted with 450 mM KCl buffer which quantitatively recovered the ORE-binding activity from the column. This two-step procedure resulted in a substantial increase in the yield of product which was easily detectable using colloidal Coomassie blue staining (Fig. 5).

Characterization of ORE binding

In the purified state the OREBP would specifically bind to ORE1 and ORE2 sequences yielding an identical shifted pattern. Although the shifted complexes were identical for ORE1 and ORE2 probes, there appeared to be a differential preference for the ORE2 sequence in the purified state as the ORE2 shifted pattern consistently appeared more intense than ORE1 under identical binding conditions (Fig. 6). Accordingly, ORE2 was competed off in the presence of cold ORE2 probe. ORE1 could be partially competed in a quantitative

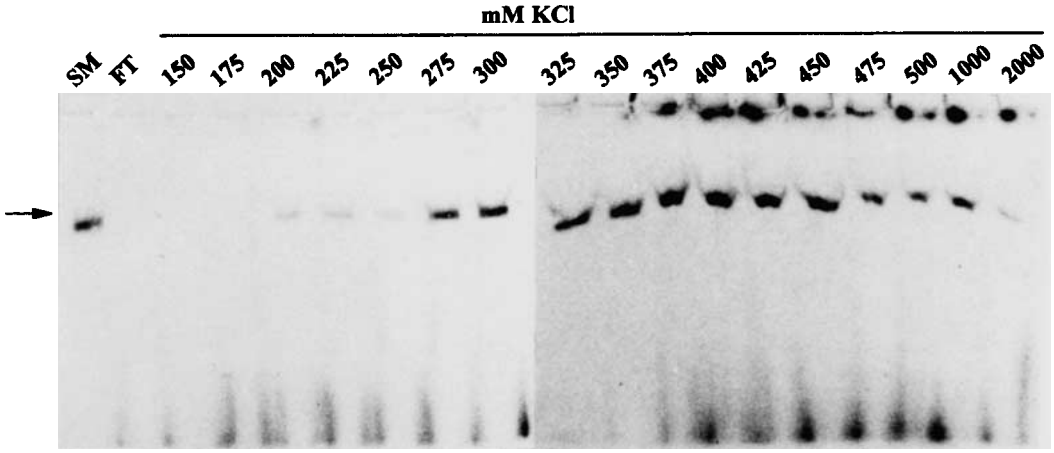

Fig. 4. ORE-2 affinity chromatography purification of OREBP. Placental nuclear extracts, which had been subjected to PEG fractionation and High-Q ion exchange and Heparin-agarose chromatography, were concentrated and applied to an ORE-2 affinity column. The column was eluted by sequentially increasing the KCl concentration. The activity of each fraction was determined by EMSA analysis. The position of the p70 band is indicated. SM and FT refer to starting material and flow-through, respectively.

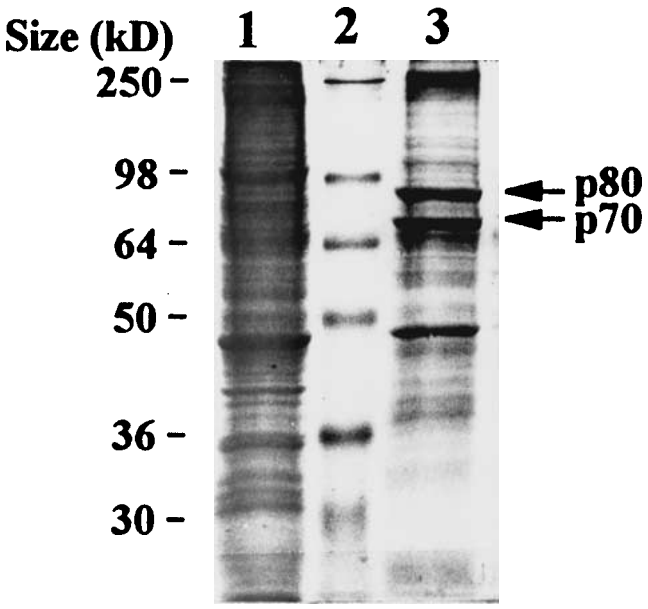

Fig. 5. One-step ORE2-affinity chromatography purification of OREBP. Placental nuclear material which had been PEG fractionated was applied to an ORE2-affinity column. The column was extensively washed with 150 mM KCl column buffer and the bound complex was eluted by increasing the salt concentration to 450 mM. Lane 1, starting material; lane 2, protein size markers (Helix Technologies, Ontario Canada); lane 3, eluted material. The positions of the p70 and p80 polypeptides are indicated. This shortened purification scheme was employed after the migration of the p70 and p80 were fully evaluated using the multistep purification which resulted in essentially a pure p70 polypeptide.

manner in the presence of 10 × or 100 × cold ORE2 probe (Fig. 6). ORE1 was fully competed off in the presence of 100 × unlabeled ORE1 probe (Fig. 6). Accordingly, ORE2 was competed off in the presence of cold ORE2 probe. The competition analysis showed that the interaction of OREBP with ORE was specific as an excess of unlabeled ORE1 or ORE2 prevented binding to the respective labeled probe.

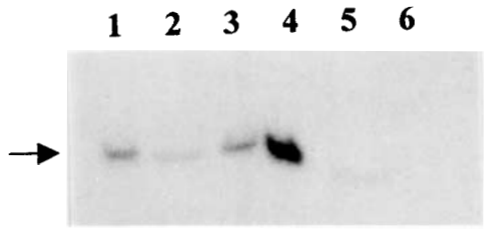

Fig. 6. Competitive binding of purified OREBP to ORE1. Affinity chromatography purified OREBP was subjected to EMSA analysis in the presence of: lane 1, ^{33}P-labelled ORE1; lane 2, ^{33}P-labelled ORE1 + 100 × cold ORE2; lane 3, ^{33}P-labelled ORE1 + 10 × ORE2; lane 4, ^{33}P-labelled ORE2; lane 5, ^{33}P-labeled ORE1 + 100 × cold ORE1; lane 6, transcription factor in the absence of ORE-probe. The position of OREBP is indicated by arrow. All EMSA reactions contained 20 ng of labelled probe and 10 μg of purified OREBP.

Since the order of the addition of the binding reaction components in EMSA could be important [20–22], purified nuclear protein extracts were pre-incubated with competitor DNA before the addition of labeled ORE probe. The EMSA pattern was the same whether the purified nuclear protein extract was added into the reaction before or after the labeled ORE probe.

To test the binding activity of the oxygen responsive elements in protein extracts of human heart cells, cells were incubated at an oxygen tension of 150 mm Hg and at an oxygen tension of 40 mm Hg. The abundance of the OREBP was regulated as a function of oxygen tension. Nuclei from cells maintained at a low oxygen tension for 3 weeks possessed less binding activity than nuclei isolated from cells maintained under normoxic atmospheric conditions (data not shown).

Anti-Ku p70 antibody interaction

A human anti-Ku p70-specific polyclonal antibody generated against the human p70 component of the Ku antigen complex reacted specifically with the 77 kD band on Western blots. The 77 kD band was detected in purified column fractions containing the p70/p80 proteins and in crude nuclear extracts (Fig. 7). When cellular and nuclear extracts were prepared from physiologically hypoxic (40 mm Hg) cells the

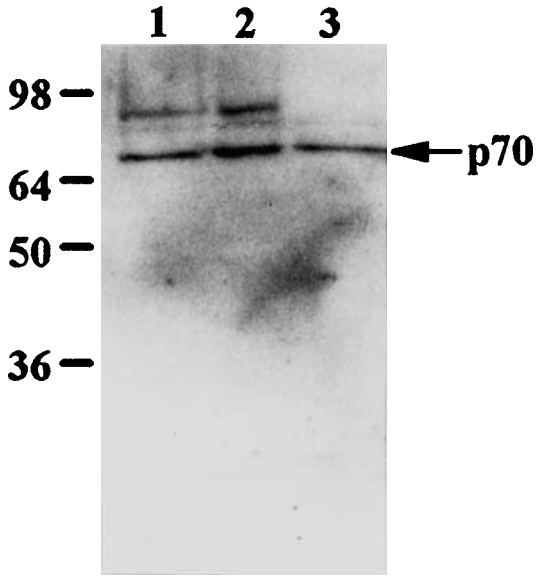

Fig. 7. Western blot analysis using anti-Ku (p70) antisera. Proteins (20 μg) were fractionated through a 4% stacking/10% running gel and electrotransferred onto PVDF. The blot was reacted against a monoclonal antibody directed against the Ku-antigen subunit p70. The p70 specific polypeptide was visualized as indicated in Materials and methods. Lanes 1 and 2, independently isolated placental nuclear extracts; lane 3 contains purified OREBP.

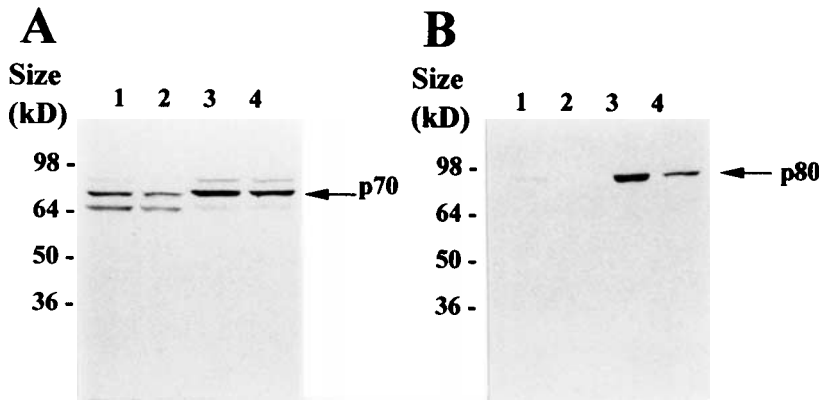

Fig. 8. Western blot analysis following changes to p70 and p80 polypeptides in response to changes in oxygen tension. Nuclear proteins, prepared from normoxic (pO_2 = 150 mm Hg) or hypoxic (pO_2 = 40 mm Hg) cardiomyocytes, were fractionated through a 4% stacking/10% running gel and transferred onto PVDF. (A) p70- and (B) p80-specific Ku polypeptides were detected using subunit specific antisera. Lane 1, cardiomyocyte cytoplasmic extract from cells grown at pO_2 = 150 mm Hg; lane 2, cardiomyocyte cytoplasmic extract from cells grown at pO_2 = 40 mm Hg; lane 3, nuclear extract from cells grown at pO_2 = 150 mm Hg; and lane 4, nuclear extract from cells grown at pO_2 = 40 mm Hg. The positions of the p70 and p80 polypeptides are indicated.

level of p70 was decreased relative to cells maintained at an oxygen tension of 150 mm Hg (normoxic; Fig. 8A), as did the level of p86 (Fig. 8B).

N-terminal sequencing analysis

The ORE-affinity column eluted material was fractionated by reverse phase HPLC and the desired peaks were subjected to N-terminal sequence analysis. The retention time of the desired peaks matched those determined using an analytical scale purification protocol utilizing an additional ion exchange fractionation step in addition to PEG fractionation (data not shown).

The 86 kD protein was unblocked and yielded the N-terminal sequence:

VRSGNKAAVVLCMDVGSTMSN

This sequence matches 20 out of 21 residues (95%) and was homologous to the human 86 kD Ku autoantigen protein which has a phenylalanine rather than a serine at position 17. The 77 kD band possessed a blocked N-terminus and did not yield any sequence, but its size and characteristics correspond to the second subunit of the Ku autoantigen. The blocked N-terminus is consistent with the predictions of Reeves and Sthoeger [10] and Tuteja *et al.* [23] and correlates with the inability to sequence the preparative EMSA purified material.

Supershift assay

To confirm that OREBP activity belonged to autoantigen Ku, the ability of anti-Ku70 and anti-Ku80 to supershift the OREBP complex in DIG-EMSA was tested. The anti-Ku70 specific antibody bound with OREBP resulting in a partial retardation of shifted band in the mobility shift assay (Fig. 9B). DIG-labeled ORE2mutant did not interact with OREBP and only a slight, non-specific, shifted complex was observed under the highest antibody concentration (Fig. 9C). An anti-Ku80 antibody recognized OREBP and retarded the shifted band in a similar manner (Fig. 9A). Control Rabbit IgG did not shift the OREBP complex.

Discussion

To extend observations made previously regarding the nature of the ORE binding activity [4], EMSA experiments using ORE1 and ORE2 probes indicated that a DNA binding activity bound in a consistent and specific manner to each respective element (Fig. 6). Furthermore, purified OREBP would bind to both elements with differing characteristics. This was expected as both elements share the common consensus sequence: AYCCTC$^{A}/_{T}$RAGAAA. Footprinting data indicated that practically all the protein:DNA contacts occurred within this core sequence, although a different methylation interference pattern was observed for each [4]. This was in accordance with EMSA results using purified OREBP, whereby labeled ORE2 exhibited a qualitatively stronger binding signal than ORE1, possibly indicating a greater affinity for the ORE2 sequence. This may indicate that *in vivo* additional factors selectively enhance particular base interactions and fine-tune the regulatory mechanisms of OREBP in response to external stimuli.

The ORE binding activity was enriched from human placenta as this organ possessed a high level of the desired pro-

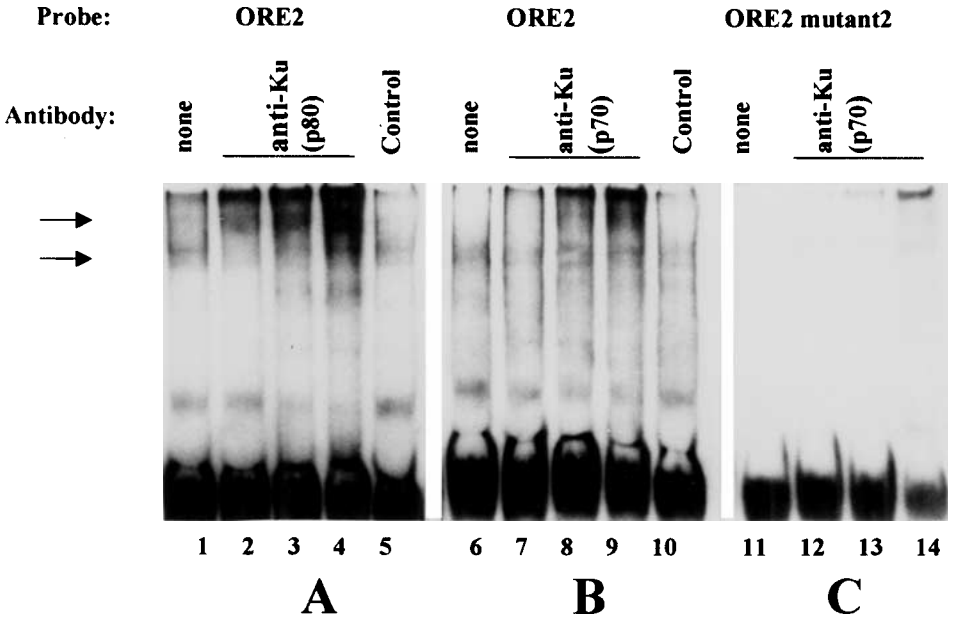

Fig. 9. Supershifting of OREBP-DNA complexes using Anti-Ku (p70) and anti-Ku (p80) antibodies. DIG-EMSA of nuclear proteins was performed either in the absence (lanes 1, 6, and 11) or presence of 1, 2 or 5 μl of anti-Ku (p80) (lanes 2–4) or anti-Ku (p70) antisera (lanes 7–9 and 12–14) for 30 min at 0°C. This was followed by the addition of 1 pmol of DIG-labeled ORE2 probe (lanes 1–10) or ORE2mutant probe (lanes 11–14). An equal quantity of purified normal rabbit IgG served as a negative control (lanes 5 and 10). The supershifted OREBP complex is shown.

tein [24] and represents a readily obtainable source of human tissue.

The ORE binding activity, as identified by preparative EMSA gels, suggested a single polypeptide of approximately 77 kD. This binding activity was selective and specific for ORE sequences as a 1000-fold excess of sheared salmon sperm DNA, or competitor poly(dI-dC), did not interfere with the binding. To distinguish between sequence-specific binding from non-specific DNA-end binding activity, EMSA altered ORE-sequences (ORE2mutant and ORE2short) were used. The non-consensus probes resulted in the loss of sequence specific binding (Fig. 3). Thus OREBP can selectively locate and bind to the consensus ORE-probe in a vast pool of non-specific sequences. Furthermore, unlabeled ORE2 at 20–100-fold excess can effectively compete for the binding with the labeled ORE2.

The apparent size and migration pattern of the 77 kD polypeptide was identical to the nuclear protein purified by affinity ORE-chromatography. Using affinity chromatography, a second 86 kD component was co-eluted from the column which was determined to be the p80, the larger member of the Ku antigen complex. By virtue of its size (approximately 75 kD) [25] and the presence of a blocked N-terminus, the 77 kD band most likely represented the smaller member of the Ku-heterodimer complex [10]. This was consistent with previous reports indicating that p70 confers DNA binding activity to the heterodimeric complex [26–28]. The identity of the p70 subunit in the affinity purified material was confirmed by Western analysis using a p70-specific antibody and by supershift analysis (Fig. 9). As the p80/p70 complex is non-covalently associated, the 86 kD component most likely dissociated under the conditions employed during the preparative EMSA purification procedure. In addition, the presence of a major and minor shifted complex was analogous to the results of Genersch *et al.* [25] who demonstrated a similar phenomenon.

The Ku autoantigen appears to be regulated by a phosphorylation based regulatory mechanism [10] and is involved in a variety of nuclear functions [29, 30]. These include transcriptional regulation, helicase function, the regulation of collagen IV genes [25], telomeric binding [31] and the regulation of the small nuclear RNA U1 promoter [32]. A number of potential sequence-specific Ku binding sites were proposed [29, 33–35]. This suggests the existence of a number of Ku-related proteins that participate in the transcriptional regulation of different genes. It was reported that this protein complex is indistinguishable from NFIV [36], a protein which has also been implicated in numerous nuclear functions including modulation of the HMG-CoA synthase gene in response to fatty acids [37]. Ku and NFIV have been shown to functionally associate with other transcriptional co-activators such as CBP [38] and REF1 [39]. Interaction with an auxiliary protein such as REF1 or kinases such as p350 [28, 40] may enhance not only the ORE-binding specificity but

provide a mechanism to sense the cellular redox state in response to changes in oxygen tension.

Recently, Giffin *et al.* [33] reported the identification of Ku as a transcription factor that binds specifically to NRE1 a DNA sequence element in the long terminal repeat of mouse mammary tumour virus. This NRE1-like sequence motif was absolutely required for sequence specific DNA binding [41]. When comparing ORE2 and NRE1 sequences a partial sequence similarity was evident (GAGAAA) and when this core was removed (ORE2mutant) binding was negated (Fig. 3). It should be noted that nucleotides deleted in ORE2mutant were found by methylation interference to be closely juxtaposed to DNA-binding protein contacts [4]. Although ORE2 short contains the core motif, its flanking sequence length may be too short to support transcription factor binding.

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