Transcriptional Activation of the Human \( \alpha_{12} \) Gene Promoter through Nuclear Factor-\( \kappa \)B and Antioxidant Response Elements

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Very little is known regarding molecular mechanisms underlying transcriptional regulation of any G-protein gene despite the importance of G-protein expression in modulating cellular processes. Here we show that phorbol myristate acetate (PMA) and tert-butylhydroquinone (tBHQ), which induce oxidative stress in cells, up-regulate transcription of \( \alpha_{12} \) in K562 cells. Redox-sensing chemicals abrogated this transcriptional effect. A dominant negative I-\( \kappa \)B double mutant (S32A/S36A) suppressed PMA-induced transcription by 54–62%, suggesting involvement of nuclear factor-\( \kappa \)B (NF-\( \kappa \)B). SN50, a cell-permeable peptide that inhibits nuclear import of stress-responsive transcription factors (such as NF-\( \kappa \)B), inhibited PMA- and tBHQ-induced transcription. Deletion of an NF-\( \kappa \)B-binding motif that maps at +10/+19 in the promoter resulted in 55–60% suppression of PMA-induced transcription, and 81% suppression of tBHQ-induced transcription. Mutation of an antioxidant response element (ARE) that maps at −84/-76 in the promoter resulted in 51 and 86% decrease in PMA- and tBHQ-induced transcription, respectively. In electrophoretic mobility shift assays, this element formed complexes with the transcription factors NF-E2p45 and Nrf2 that are prototypic for binding to the ARE, as well as with c-Fos, which can also interact with the ARE. Chromatin immunoprecipitation analysis demonstrated recruitment of these transcription factors to the promoter. Exogenously transfected Nrf2 transactivated the \( \alpha_{12} \) gene promoter; the cytoskeleton-associated protein, Keap1, abrogated this effect. Taken together, the present studies reveal that transcription factors that bind NF-\( \kappa \)B and/or antioxidant response elements play an activating role in the transcription of the human \( \alpha_{12} \) gene.

Signal transduction through heterotrimeric G-proteins\(^1\) subserves a large variety of cellular and metabolic processes (1–4).

More than a decade ago, Sheth et al. (5) showed that regulation of actin polymerization in differentiating U937 cells correlates with increased levels of \( \alpha_{12} \) in the plasma membrane. There is now growing awareness that changes in the amounts of certain \( \alpha \)-subunits of heterotrimeric G-proteins play important roles in neonatal and embryonic development (3) and in cell differentiation (6–11). For example, in P19 mouse embryonal carcinoma cells that normally differentiate to endodermal phenotype in response to low levels of retinoic acid, stable transfection of constitutively active forms of \( \alpha_{12} \) (Q229L) or \( \alpha_{13} \) (Q226L) results in differentiation even in the absence of retinoic acid (9), indicating a direct effect of these G-proteins on this process. Conversely, suppression of \( \alpha_{12} \) levels with antisense oligonucleotide inhibits butyrate-induced differentiation of K562 cells to erythroblasts, and treatment of these cells with pertussigens to inactivate \( \alpha_{12} \) prevents erythroid differentiation (11), indicating an apparent requirement for \( \alpha_{12} \) in the differentiation of these cells to erythroblasts. In contrast, retinoic acid-induced differentiation of F9 embryonal teratocarcinoma cells to the primitive endoderm is inhibited by expression of \( \alpha_{13} \) (7). Thus, the effect of \( \alpha_{12} \) may be cell-type or cell differentiation inducer specific.

\( \alpha_{12} \) has also been shown to play a role in oncogenesis (12, 13), to enhance insulin signaling, and to trigger the recruitment of glucose transporter, GLUT4, to the plasma membrane through a signaling pathway that mimics insulin action (14). The discovery that \( \alpha_i \) (sub-species undefined) can direct asymmetric cell division in neuroblasts as well as in sensory organ precursor cells in Drosophila in a receptor-independent manner during embryonic development (15) attests to the signaling versatility of this G-protein. Nonetheless, studies on the transcriptional regulation of G-protein genes remain rather sparse (12, 13, 16–19).

We showed previously that the human \( \alpha_{12} \) gene promoter is strongly activated when erythroid differentiation is triggered in K562 cells by the use of histone deacetylase inhibitors; the activation is associated with increased \( \alpha_{12} \) mRNA as well as \( \alpha_{12} \) protein levels, and is mediated by Sp1-binding sites in the promoter (18). With phorbol 12-myristate 13-acetate (PMA), which induces differentiation of K562 cells to megakaryocytes (20–24), we also observed robust activation of transcription from this promoter (18), but the molecular mechanism for the PMA-induced promoter activity is unknown.

Because the generation of oxidative radicals is a hallmark of PMA treatment of various cell types, including K562 cells (25–28), we have addressed, in this report, whether redox-sensitive transcription factors play a role in the transcriptional activation of the \( \alpha_{12} \) gene. Our data demonstrate the potential regulatory role of transcription factors that bind NF-\( \kappa \)B and/or antioxidant response elements.
Experimental Procedures

Cell Cultures and Transfection Studies—K562 cells, obtained from the American Type Culture Collection (Manassas, VA), were maintained in culture as described previously (11). For transfection studies, 1 × 10^6 cells in 1 ml of medium/well (24-well plates) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (50 units of penicillin and 50 μg of streptomycin per milliliter) at 37 °C in 95% air/5% CO2 atmosphere for 24 h before transfection. The cells were then transfected with plasmid DNA containing the Gaα2 promoter construct or mutant (0.5 μg, unless otherwise stated) and 1.5 μg of FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h, followed by addition of PMA or (R/BQ). When used, antioxidants (i.e. CAPE, PDTC, and N-acetylcysteine) were added 30 min or 1 h prior to the addition of inducer (PMA or R/BQ). In co-transfection experiments with dominant negative IκB expression plasmid, PMA was added 2 h after transfection. In all cases, the cells were harvested 24 h after the addition of inducers (unless otherwise stated) by centrifuging (12,000 × g, 45 s) in 1.5-ml microcentrifuge tubes, supplemented with 1 ml of ice-cold phosphate-buffered saline (7.4). The cell pellets were then lysed with 150 μl of 1× Cell Culture Lysis Reagent (Promega, Madison, WI). After 15 min at room temperature, the lysed material was centrifuged for 2 min (12,000 × g) to remove cell debris, and the luciferase activity and protein content (Bradford protein assay method (Bio-Rad)) of the extracts were measured. For luciferase activity, 5 μl of the extract was used to measure the integrated light units in the luciferase assay system (Promega, Madison, WI) and a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany), as recommended by the manufacturers. In collateral experiments using the dual luciferase assay protocol, we noted that PMA robustly induced the luciferase gene in conventional luc-reporter construct (e.g. Promegn’s pRL-TK-luc and pRL-CMV-luc reporter vectors) usually used for normalization in transfection experiments, making it impractical to use such plasmids for this purpose. This problem was encountered with promoter-less constructs (e.g. phRG-B Renilla luciferase reporter vector from Promega). With such constructs, the dual luciferase assay resulted in -fold activation of transcription that was comparable to that obtained by normalizing to the protein content of the samples. Therefore, luciferase activity was normalized to the protein content of each sample, after correcting for basal activity of GL3-basic, and is expressed as -fold stimulation over cells that were not treated with inducers.

Site-directed Mutagenesis—The desired nucleotides in the Gaα2 gene promoter were mutated or deleted by using QuikChange™ mutagenesis kit (Stratagene Inc., La Jolla, CA). DNA sequencing performed by the Molecular Biology Core Facility at Meharry Medical College confirmed the mutations.

Assays—

Western Immunoblotting—Gaα2 protein levels in whole cell lysates and transcription factors in nuclear extracts were measured by Western immunoblotting, using protocols described previously (18). To monitor potential artifacts in loading and transfer among samples in different lanes, the blots for whole cell lysates were stripped and reprobed with antibodies to total ERK2 (Santa Cruz Biotechnology); the blots for nuclear extracts were stripped and reprobed with antibodies to C/EBPβ (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation—ChIP was performed, in principle, according to the protocols recommended by Upstate Biotechnology Inc. (Lake Placid, NY) and by the Farnham Laboratory (www.genomecenter.ucdavis.edu/farnham/). Specifically, K562 cells (1 × 10^6 cells/ml) were incubated with or without PMA (5 or 10 nm) or R/BQ (20 μM) for 2 h or more after transfection with the Gaα2 promoter construct. The cells were cross-linked with 1% formaldehyde, neutralized with glycine (125 mM), and washed twice with ice-cold 1× phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg each of leupeptin, aprotinin, and pepstatin A per milliliter). To isolate crude nuclei (18), the cell pellet was suspended in buffer (10 mM Hapes-KOH (pH 7.9), 140 mM NaCl, 1 mM EDTA, 0.85% Nonidet P-40, 10 mM Dithiothreitol (DTT), 10 μl of protease inhibitor mixture (Sigma) per milliliter), incubated on ice for 10 min, and then centrifuged at 8,500 × g (5 min) at 4 °C. The pellet was resuspended in lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and protease inhibitor mixture (10 μl/ml) (Sigma). After 10 min on ice, the lysate was sonicated to shear the DNA to 200–1,000 bp and then centrifuged at 12,000 × g for 10 min. A small aliquot (10 μl) was saved as “input DNA” for PCR analysis later. Other aliquots of the supernatant solution equivalent to about 1 × 10^6 cells (usually 100 μl) were diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg each of leupeptin, aprotinin, and pepstatin A per milliliter)), and pre-cleared by mild agitation for 1 h at 4 °C, using 40 μl of protein-A/AG PLUS-agarose (Santa Cruz Biotechnology) and 4 μg of sonicated salmon sperm DNA. The agarose beads were pelleted (1,000 × g, 2 min), and the supernatant solution was used for the immunoprecipitation step. Chromatin was immunoprecipitated overnight at 4 °C by mild agitation with 4 μg of various antibodies (see legends to Fig. 10); for negative control, the supernatant solution was incubated with 4 μg of normal rabbit IgG (ab94920, Santa Cruz Biotechnology). Immune complexes were collected by incubation at 4 °C for 2 h with 40 μl of protein-A/AG PLUS-agarose and 4 μg of sonicated salmon sperm DNA, followed by centrifugation at 1,000 × g for 2 min. The agarose beads were then subjected to the five-step washing protocol described in the Upstate Biotechnology ChIP assay kit. The immune complexes were eluted from the beads with a two-step elution protocol, each time using 100 μl of the elution buffer (1% SDS, 0.1 M NaHCO3, 0.5 M NaCl) for 10 min at room temperature. The eluates were combined, mixed with 8 μl of 5 μ NaCl, and incubated at 65 °C overnight, to reverse the protein-DNA cross-links. The saved chromatin input fraction was also processed in the same manner. The samples were then digested with 20 μg of proteinase K (Promega, catalog #V3021) at 45 °C for 1 h. After digestion, 20 μg of glycerogen (Type III, Sigma) was added to the mixture, and the sample was adjusted to 2% SDS with 2 μl of 100 mM NaOH (Sigma) to facilitate adsorption of DNA to the QIAquick columns. The DNA from the samples was purified by using QIAquick PCR purification kit (Qiagen), and eluted from the columns with 50 μl of nuclelease-free water. The DNA samples were stored at −20 °C until used for PCR analysis.

PCR Analysis—To analyze the target region (containing the ARE-binding site at −84/−76 and the NF-κB-binding site at +10/+19 in the Gaα2 gene promoter), the DNA samples were amplified by PCR, using two sets of primers (see legend to Fig. 10) that generated 170- and 288-bp PCR products, respectively. The former bracketed the ARE site; the latter bracketed both the ARE site and the NF-κB-binding site at +10/+19. As negative controls, primers (forward: 5-CSACAGAGA- GCTCATATTTTGC3′; reverse: 5′-TCAGAGGCTACATCCGAG3′) were used to produce a 248-bp PCR product designed to bracket a non-target region in intron 1 of the Gaα2 gene. All primers were synthesized by Integrated DNA Technologies, Inc., Coralville, IA. The PCR cycles were 95 °C for 30 s, 50 °C for 30 s, and 68 °C for 90 s. Following amplification, the PCR products were analyzed by electrophoresis on 2% agarose gels, using 0.5× TBE (45 mM Tris boric, 1 mM EDTA at pH 8.0) buffer, containing ethidium bromide.

Results

PMA Induces Expression of Gaα2—To determine the effect of PMA on the levels of Gaα2 in K562 cells, Western blotting analysis was used to assess Gaα2 protein levels in cells incubated with different concentrations of PMA. In cells cultured in the absence of PMA, Gaα2 was barely detectable in Western blots (11, 18, 19). As shown in Fig. 1A at A and B, PMA caused a pronounced induction of Gaα2 protein expression within 48 h. Pre-treatment of the cells with actinomycin D blocked this induction (Fig. 1C), suggesting transcriptional up-regulation. The treatment with PMA induced robust transcriptional activity of the Gaα2 gene promoter, as assessed by luciferase reporter gene assay; the effect was maximal at 5 nM PMA (Fig. 1D).

Transcription from the Gaα2 Gene Promoter Is Inhibited by Antioxidants—To explore potential transcription factors involved in the observed induction of promoter activity, we considered the fact that generation of oxidative radicals is a hall-
FIG. 1. Phorbol ester induces expression of G\(_{\alpha}\). K562 cells were grown as described under “Experimental Procedures.” In A, Western blotting was used to analyze G\(_{\alpha}\) levels in whole cell lysates from PMA-treated cells, using anti-G\(_{\alpha}\) antibody (L5) (Santa Cruz Biotechnology) and 20 \(\mu\)g of protein per sample in the blotting protocol described previously (18). The same blots were reprobed for ERK2, as loading control. The blot shown is representative of three experiments. In B, the intensity of the G\(_{\alpha}\) band for each lane in A was quantified by densitometric scanning, and normalized to the band intensity for ERK2 in the corresponding loading lane. The results (ratios of G\(_{\alpha}\)/ERK2) are expressed in histogram form by designating the ratio in the absence of PMA treatment as equal to 1.0. Values shown are means \(\pm\) S.E. for triplicate experiments from six different cell cultures.

FIG. 2. Antioxidants, PDTC and CAPE, inhibit PMA-induced transcription from the G\(_{\alpha}\) gene promoter. K562 cells, grown in 24-well plates, were transfected with pG\(_{\alpha}\)(-1214/+115)-luc, as described under “Experimental Procedures.” PDTC or CAPE (various concentrations) was added to the cell cultures 1 h after transfection, followed by the addition of PMA (5 nM) 1 h later. The cells were harvested 24 h after the addition of PMA and processed for luciferase assay as described under “Experimental Procedures.” Values shown are means \(\pm\) S.E. for triplicate assays from four to five different experiments. When not indicated, the S.E. bars were too small for the scale used in the figure. CAPE, caffeic acid phenylethyl ester; PDTC, pyrroldidine dithiocarbamate.
nuclear content of Nrf2, as well as NF-κB (measured by immuno-
blotting for NF-κBp65) and c-Fos (Fig. 4A). Treatment with
PMA also resulted in a time-dependent increase in the nuclear
content of NF-κB (Fig. 4B) and c-Fos. We were unable to detect
changes in the nuclear content of Nrf2, following the treatment
with PMA. We note, however, that Huang et al. (36) previously
demonstrated PMA-induced nuclear accumulation of Nrf2 in
HepG2 cells, using much higher concentration (100 nM) of
PMA. Perhaps the lack of effect of PMA in our study is a
reflection of the very low concentration of PMA used in this
study (10 nM), or a reflection of the cell type used.

Involvement of NF-κB in PMA- and tBHQ-induced Trascrip-
tion from the Gα2 Gene Promoter—To test the involve-
ment of NF-κB, we used three different experimental appro-
aches: first, we blocked activation of NF-κB by using dominant
negative I-κB (S92A/S36A); second, we blocked nuclear trans-
location of activated NF-κB components by using a cell-perme-
able inhibitor peptide (SN50) that inhibits nuclear import of
NF-κB as well as other stress-responsive transcription factors
(35, 38–40); third, we mutated the putative NF-κB-binding sites
in the Gα2 gene promoter. Western blotting was performed with 7
representative of three experiments. B, PDTC, N-acetylcysteine (NAC), and
CAPE inhibit tBHQ-induced transcription from the Gα2 gene promoter.
PDTC (10 μM), NAC (0.5 mM), or CAPE (1 μM) was added to the cell
cultures 1 h after transfection, followed by the addition of tBHQ (10 or
20 μM) 1 h later. The inhibitors were tested only in cultures to which
tBHQ was added at 10 μM. The cells were harvested 24 h after the
addition of tBHQ and processed for luciferase assay as described under
“Experimental Procedures.” Values shown are means ± S.E. for dupli-
cate assays from four to five different experiments.

Overexpression of dominant negative I-κB (S92A/S36A) se-
questers NF-κB components in their inactive state, in the cyto-
plasm, thus blocking their ability to induce transcription (41, 42).
As seen in Fig. 5A, co-transfection of pGα2(-1214/+115)-luc
with S92A/S36A blocked PMA-induced transcription by 54
and 62%, respectively, at 0.25 and 0.5 μg of the mutant I-κB
cDNA/ml of culture. These data mimic the blocking effect of
antioxidants (see Fig. 2).

In the second approach in which we used SN50 to block
nuclear import of NF-κB (35, 38–40), induction of Gα2 pro-
moter activity was drastically curtailed (~71%) (Fig. 5B), and
the expression of Gα2, as judged from Western blots, was
 correspondingly inhibited (Fig. 5C). In these experiments, we
verified, by Western blotting, that PMA-induced nuclear im-
port of NF-κB was indeed blocked by the treatment with SN50
(Fig. 5D, compare lane 3 with lane 2). As controls, the mutant
form of SN50 (SN50M) that permeates cells but does not inhibit
nuclear translocation of NF-κB (35, 38–40) had no effect on the
parameters measured (Fig. 5, B–D). Because nuclear accumu-
lation of NF-κB increased after tBHQ treatment (see Fig. 4A),
we tested whether tBHQ-induced transcription from the Gα2
gene promoter would also be sensitive to the inhibitory effects
of SN50. As seen in Fig. 5E, SN50 almost completely quenched
(~90%) tBHQ-induced transcription. Interestingly, the treat-
ment with SN50 also decreased the nuclear content of Nrf2
(Fig. 5F), suggesting that Nrf2 may be translocated into the
nucleus through the same mechanism as NF-κB.

In the third approach, we assessed the impact of mutating
NF-κB-binding sites on the promoter, on both PMA- and tBHQ-
duced transcription. The human Gα2 gene promoter contains
two putative NF-κB-binding sites (MatInspector Professional®,
Genomatix), the locations of which are illustrated schemati-
cally in Fig. 6A. To ascertain whether these putative NF-κB-
binding sites actually bind NF-κB, we verified by electro-
phoretic mobility shift assays that nuclear extracts from K562
cells bind to oligonucleotide probes (S-GTCGCTCGAACTGC-
CGACCGAGTGCC-3' and 5'-GTGGGAACCCACCCTATGCG-
CTTTCTCC-3') corresponding to the regions in the Gα2 gene
promoter containing the putative NF-κB sequences (under-
lined) at positions 1 and 2, respectively (Fig. 6). The binding
activity was competitively abolished by an NF-κB consensus
oligonucleotide but not by a mutated NF-κB oligonucleotide.
of DNA in the wells. Thus, the wells used in the experiments for the left panel are means to the addition of PMA. The mutant peptide (SN50M) was used as control. The cells were harvested for luciferase assay 20 h later. Values shown for G from the G was measured by using SN50 (Biomol) to inhibit nuclear translocation of NF-

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transcription by only about 16% (Fig. 6).

we found that deleting the site 1 locus inhibited PMA-induced transcription by about 52%, whereas deleting the site 2 locus inhibited transcription by only about 16% (Fig. 6E). With a promoter construct mutated at both loci, the loss of promoter activity was essentially similar to that observed with only site 1 promoter mutant, indicating that the sequence at site 1 was crucial for the observed PMA-induced transcription from the Gαi2 gene promoter. The sequence at site 1 was also crucial for rBHQ-induced transcription from this promoter (Fig. 6F). The mutation at this site did not affect the core transcriptional machinery, because with sodium butyrate, which induces transcription from this promoter through a different transcription machinery, because with sodium butyrate, which induces transcription from this promoter through a different transcription factor (18), transcriptional activation was essentially the same with this mutant as with the wild-type promoter (data not shown). We interpret the data in Fig. 6 to mean that both sites
Fig. 6. Effect of deletion of putative NF-κB-binding sites on PMA- and tBHQ-induced transcription from the Go12 gene promoter. A, locations of putative NF-κB-binding motifs and ARE in the Go12 gene promoter. Panels B–D, electrophoretic mobility shift assays (EMSAs) of NF-κB binding sites in the Go12 gene promoter. EMSA was performed using nuclear extracts from PMA-treated K92 cells. The annealed oligonucleotide was labeled with [α-32P]dCTP, by using the Klenow fill-in reaction, and purified as described previously (18). B, consensus NF-κB oligonucleotide competes against nuclear extract binding to DNA probe(s). In the upper panel, binding of nuclear extracts to DNA probes corresponding to location 1 was measured with a probe (5′-GTCGCTGGAACCTGCCGACCCGAGTGC-3′) corresponding to a sequence in the Go12 gene promoter containing a putative NF-κB binding site (underlined, designated as site 1). This assay was carried out with 5 μg of nuclear extract protein. The probe (5′-GTTCGAGCCACCCCTAATGCTTTTCTCC-3′) used in the lower panel corresponds to a sequence in the Go12 gene promoter containing a putative NF-κB binding site (underlined, designated as site 2). 2 μg of nuclear extract protein/lane was used in this assay. For competition assays, corresponding unlabeled double-stranded oligonucleotides (10-fold excess) were used in lane 5; consensus NF-κB oligonucleotide (125-fold excess for site 2 probe, 50-fold excess for site 1 probe) and mutant thereof (200-fold excess for site 2 probe, 80-fold excess for site 1 probe) were used in lanes 3 and 4, respectively. Lane 1 contained no nuclear extract (NE). C, time course of binding of nuclear extract (NE) to the DNA probe. D, treatment of cells with PDTC decreased binding to DNA probes. The cells were incubated with 10 μM PDTC for 1 h prior to the addition of PMA (5 nM). Cells were harvested 24 h after addition of PMA, and nuclear extracts were prepared as described previously (18). EMSA results shown were carried out with the DNA probe corresponding to location 1 and are representative of three experiments. Panels E and F, promoter activity of wild type and mutant Go12 gene promoters in PMA (E)- and tBHQ (F)-treated cells. The results are means ± S.E. of 4–7 separate transfection experiments, expressed as fold stimulation, relative to cells that were not treated with PMA (5 nM) or tBHQ (20 μM). WT, wild-type Go12 gene promoter pGo12; −1214/+115-luc; NF-κB mut 1 and mut 2, mutant Go12 gene promoter constructs lacking NF-κB-binding sites 1 and 2 (see schematic in A), respectively. In the double mutant, both sequences were deleted. *+, statistically significant (p < 0.05) compared with WT. For each data set, the value for corresponding control (Con = no PMA or tBHQ) was set at 1.0.

1 and 2 bind NF-κB, but only the site 1 locus is functionally relevant to the transcription-inducing effect of PMA and tBHQ. Together with the results of experiments using the other two approaches, these data are consistent with the involvement of NF-κB in PMA- and tBHQ-induced expression of Go12.

Nrf2 Induces Transcriptional Up-regulation of Go12 Gene Promoter—In the cytoplasm, Nrf2 is normally complexed with the cytoskeleton-associated protein, Keap1 (43). Following activation, Nrf2 is subsequently translocated to the nucleus, where, in partnership with small Maf proteins or other accessory proteins, it binds to ARE to effect transcription of target genes (33, 36, 44–47). To determine whether Nrf2 can trans-activate the Go12 gene promoter, we co-transfected pGo12(−1214/+115)-luc with a plasmid containing the cDNA for Nrf2, and monitored transcription in the absence of any inducer. Fig. 7A shows that Nrf2 increased basal transcription in a dose-dependent manner. Co-transfection with an expression plasmid containing cDNA for Keap1 repressed this transactivation (Fig. 7B). With or without exogenous Keap1, the electrophile tBHQ robustly enhanced Nrf2-induced promoter activity (Fig. 7B). Hematopoietic cells contain high levels of Keap1 (43); this might explain the low level of promoter activity in the presence of Nrf2 alone (Fig. 7A). The booster effect of tBHQ is consistent with the finding that electrophilic agents antagonize Keap1 inhibition of Nrf2 activity, liberating Nrf2 from Keap1, and allowing it to translocate to the nucleus to potentiate ARE response (43).

Mutation of the ARE in the Go12 Gene Promoter Diminishes Transcription from This Promoter—The sequence of the human Go12 gene promoter (48–50) contains an ARE motif (5′-TGACTGGGC-3′) that maps at −84/−76 (see schematic in Fig. 6A). This sequence is identical to the ARE core sequence established
to be the minimal sequence necessary for both basal and inducible activity (33, 51). To investigate whether this ARE motif was involved in transcription from the \( \text{Go}_{\alpha} \) gene promoter, we carried out transfection assays with promoter construct mutated at that site (\( \text{NF}^\text{B绑} \). In some experiments, 0.1 \( \mu \)g of an expression plasmid harboring cDNA for Keap1 (pCMV-Keap1 (43)) was also transfected. The total amount of plasmid DNA in each well was adjusted to 0.6 \( \mu \)g, if necessary, with pCI-Neo or pcDNA3. 

**Fig. 7.** 

**A:** 

Nrf2 increases transcription from the \( \text{Go}_{\alpha} \) gene promoter. K562 cells were co-transfected with 0.2 \( \mu \)g of \( \text{Go}_{\alpha} \) gene promoter (pG\( \alpha^\text{B} \)-1214+115-luc) and up to 0.4 \( \mu \)g of the control (empty) plasmid (pCI-Neo) or the expression plasmid containing the cDNA for Nrf2 (pCI-Nrf2 (37)). In some experiments, 0.1 \( \mu \)g of an expression plasmid harboring cDNA for Keap1 (pCMV-Keap1 (43)) was also transfected. The total amount of plasmid DNA in each well was adjusted to 0.6 \( \mu \)g, if necessary, with pCI-Neo or pcDNA3. Here, the promoter activity was measured 20 h later, as described under “Experimental Procedures.” A, dose-dependent transcriptional effect of Nrf2. B, repression of Nrf2- induced transactivation by Keap1. The electrophile \( \text{tBHQ} \) antagonizes Keap1 repression of Nrf2 activity and robustly enhances transactivation by Nrf2. Values shown are means \( \pm \) S.E. for duplicate assays from four different experiments.

**Fig. 8.** 

Mutations at NF-\( \kappa \text{B} \) and ARE elements inhibit Nrf2-, PMA-, and \( \text{tBHQ} \)-induced transcription from the \( \text{Go}_{\alpha} \) gene promoter. QuikChange \textsuperscript{TM} mutagenesis kit (Stratagene, La Jolla, CA) was used to delete the NF-\( \kappa \text{B} \)-binding sites (at +10/+19 and −229/−220) or to introduce substitution mutations at the ARE-binding site (−84/−76) in the \( \text{Go}_{\alpha} \) gene promoter. The ARE-binding site \((5'-\text{TGACTGGGC-3'}) \) was mutated to \( 5'-\text{GACAAGGGC-3'} \). Mutated bases are indicated in **bold** and are *italicized*. In A, K562 cells were co-transfected with 0.2 \( \mu \)g of \( \text{Go}_{\alpha} \) gene promoter (pG\( \alpha^\text{B} \)-1214+115-luc) and 0.3 \( \mu \)g of the control (empty) plasmid (pCI-Neo) or the expression plasmid containing the cDNA for Nrf2 (pCI-Nrf2 (37)), and luciferase activity was measured 20 h later. In B and C, 0.5 \( \mu \)g of promoter construct was transfected 1 h before the addition of PMA (5 nM) \((B) \) or \( \text{tBHQ} \) (10 \( \mu \)M) \((C) \), and the cells were harvested 24 h later, and processed for luciferase assay as described under “Experimental Procedures.” Values shown are means \( \pm \) S.E. for duplicate assays from four different experiments.
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Binding Activity of the ARE in the Gα2 Gene Promoter—The binding activity of the ARE was examined with an electro-photographic mobility shift assay (EMSA) as well as with a chromatin immunoprecipitation (ChIP) assay. When EMSA was performed with a DNA probe containing this ARE sequence, well-defined stimulus-induced protein-DNA complexes were evident with nuclear extracts from both tBHQ- and PMA-treated cells (Fig. 9A). In both cases, an unlabeled probe abolished the binding of nuclear proteins to the labeled DNA (Fig. 9B, lanes 3 and 9), whereas mutation of four critical bases (see figure legend) in the ARE sequence contained in this probe resulted in little effect on the binding (Fig. 9B, lanes 4 and 10). An unrelated oligonucleotide (Sp1 consensus oligonucleotide) had no effect (Fig. 9B, lanes 6 and 12) on this binding, whereas an NF-κB consensus oligonucleotide decreased the binding in extracts from PMA-treated cells (Fig. 9B, lane 11) but had much less effect on the binding by extracts from tBHQ-treated cells (Fig. 9B, lane 5). In the presence of antibodies to selected redox-sensitive transcription factors (Nrf2, NF-E2p45, c-Fos, and NF-κBp65), the intensity of the protein-DNA complex was decreased in all cases in tBHQ-treated cells (Fig. 9C), suggesting that binding of these antibodies prevents association with the labeled probe. The decreases in band intensity suggest the presence of these transcription factors in the complex. In PMA-treated cells, however, the decrease in band intensity was evident for NF-κBp65 and c-Fos, but not for Nrf2 and NF-E2 (Fig. 9C, compare lanes 8 and 9 with lanes 10 and 11). Consistent with the data in Fig. 4, this result suggests that activation of Nrf2 (or NF-E2) was much less in PMA-treated cells than in tBHQ-treated cells.

ChIP Analysis—We used ChIP analysis to assess association of these transcription factors with the Gα2 gene promoter, in the context of an intact cell. For the PCR analysis of the target region, we used two sets of primers, one that brackets a 170-bp fragment containing the ARE site, and another that brackets a 288-bp fragment containing both the NF-κB site 1 and the ARE site. Because the size of the sheared DNA is 200–1000 bp long, most of the sheared DNA would be expected to contain both the NF-κB site 1 and the ARE, which are only 84-bp apart. Therefore, it is not possible to entirely discriminate between these sites, with ChIP analysis. Given this caveat, it is not surprising that the results obtained with the two sets of primers were essentially the same. Therefore, only one series of data, using the same primer sets, is displayed here. As shown in Fig 10A, PCR analysis of the target region indicated association of c-Fos, NF-κB, NF-E2, and Nrf2 with this promoter when the cells were treated with PMA. The analysis also detected association of one or more small Maf proteins, which are known to be heterodimer partners of Nrf2 on nuclear targets (33, 46, 47, 52). No association was detected in the non-target region (negative control) (Fig. 10B), or in samples treated with normal IgG

50-fold excess of the following oligonucleotides: unlabeled oligonucleotide identical (in sequence) to the labeled oligonucleotide probe (lanes 3 and 9), mutated oligonucleotide (lanes 4 and 10), NF-κB consensus oligonucleotide (lanes 5 and 11), and Sp1 consensus oligonucleotide (lanes 6 and 12). The EMSA was performed with a labeled double-stranded DNA probe 5'-GCCGGCGGCCGGCGCCAGCTCAACAGCTTGTTCC-3', which contains the canonical ARE (underlined) that maps at −84/−76 in the Gα2 gene promoter. The sequence of the mutated oligonucleotide was 5'-GCCGGCGGCCGGCGCCAGCTCAACACCTTGTTCC-3' (mutated bases are in bold and italic); Consensus NF-κB and Sp1 oligonucleotides were from Santa Cruz Biotechnology, Inc. In C, the nuclear extract was incubated with the labeled probe for 20 min at 25°C prior to addition of 1–2 μg of each antibody (Santa Cruz Biotechnology, Inc.) and then incubated for additional 30 min, followed by electrophoresis. Lane 1 contained no nuclear extract. EMSA results shown are representative of two experiments. NE, nuclear extract.

![Fig. 9. Binding of nuclear extract proteins to DNA probe containing ARE element. EMSA was performed using nuclear extracts from control, PMA-, and tBHQ-treated K562 cells, using the protocol described in detail elsewhere (18). Nuclear extracts were prepared from cells incubated with or without 5 nM PMA or 20 μM tBHQ. The EMSA reactions were carried out with 2 μg of nuclear extract protein for each lane. In A, stimulus-induced protein-DNA complexes (see arrow) were measured in nuclear extracts from cells stimulated with tBHQ or PMA for up to 120 min. In B, competition experiments were carried out with](image-url)
Traditionally, phorbol ester-induced gene transcription has been deduced to occur through the 2-O-tetradecanoylphorbol-13-acetate-response element (53–56), which is identical to the canonical AP-1 sequence (TGAC/G/G/TG/C) (57) that classically binds Fos-Jun dimer. An AP-1 half-site (TGAC) is embedded in the canonical ARE (TGACNNNGC), a sequence that is an excellent binding site for members of the NF-E2 family of transcription factors (a family of so-called cap ‘n’ collar basic region-leucine zipper proteins), typified by Nrf2 (33). Other bZIP transcription factors are also reputed to be involved in forming binding complexes with the ARE (58–61). The human Goα2 gene promoter studied here does not contain the classic AP-1 response element (48–50), but it contains ARE as well as two NF-xB-binding motifs. Our study demonstrates that one of these NF-xB-binding sites, and the ARE-binding site are involved in the transcriptional response of the Goα2 gene, to PMA and tBHQ, which are known to induce oxidative stress in cells. In both cases, the transcriptional effect was sensitive to the antioxidants, PDTC, CAPE, and N-acetylcysteine. A variety of experimental approaches indicated that PMA and tBHQ activated multiple redox-sensitive transcription factors, which include NF-xB, c-Fos, and Nrf2. Use of ChIP analyses revealed association of these transcription factors with this gene promoter. The analyses also detected association of one or more small Maf proteins, which are known to be heterodimer partners of Nrf2 on the ARE on nuclear targets (33, 46, 47, 52), as well as NF-E2, which, like Nrf2, also binds the ARE (33). Collectively, these data indicate that both NF-xB and antioxidant response elements are involved in redox-regulated transcription from the Goα2 gene promoter. The area amplified in the ChIP analysis also contains two Sp1-binding sites (−50 or −92) that we identified previously to mediate sodium butyrate-induced transcription from this promoter (18). Collateral experiments indicated that these Sp1 sites did not mediate PMA- or tBHQ-induced activation of transcription from the Goα2 gene promoter.

Nrf2 is emerging as a key physiological regulator of the cellular adaptive response to oxidants and xenobiotics (33, 36, 37, 43, 62). When complexed in the cytoplasm with the cytoskeleton-associated protein, Keap1, Nrf2 is inactive. Electrophilic agents or reactive oxygen species induce release of Nrf2 from Keap1, allowing Nrf2 to translocate to the nuclear compartment, to effect the ARE response (43). The ARE has been documented as playing a major role in binding to Nrf2, and in the response to oxidative stress in cells, by genes encoding enzymes catalyzing phase II reactions of drug metabolism (33). Our study is the first report describing activating effect of NF-xB, Nrf2, and the ARE sequence, on transcription of a G-protein gene, and adds an additional dimension to the importance of the ARE in controlling gene expression. Although translocation of Nrf2 into the nucleus, following its release from Keap1 repression in the cytoplasm, is understood as being integral to its transcriptional effect, the exact mechanism by which Nrf2 is transported to the nucleus has not been characterized (33, 43, 62). Our experiments with SN50 (Fig. 5F) suggest that the mechanism of importation of Nrf2 into the nucleus may be similar to that of NF-xB and other stress-responsive transcription factors that are translocated into the nucleus through the importin α karyopherin 2 nuclear import system (35).

Generation of reactive oxygen species is a hallmark of PMA treatment of various cell types, including K562 cells (25–28). Reactive oxygen species have been reported to activate Gx1 and Gx2, in a receptor-independent manner in neonatal rat myocytes.
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(63), presumably by modifying key cysteine residues that influence binding of GTP (64). Until now, there has been no previous demonstration that generation of reactive oxygen species is accompanied by induction of G_{\alpha}2, or any G-protein gene. What role G proteins may play in cellular adaptation to oxidative stress needs to be explored in future studies. Because G_{\alpha}2 is also expressed at high levels during erythroidic differentiation in K562 cells (11, 18), the significance of the increased transcription of the G_{\alpha}2 gene to the overall process of megakaryocytic cell differentiation, induced in K562 cells by PMA, poses a challenge for further investigation. Presently, we speculate that this increase could be part of an overall genetic program that sustains G_{\alpha}2 levels adequate to maintain related communication networks that subserve cellular differentiation.

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