Alterations in intracellular oxidative status activate several signal transduction pathways resulting in distinct patterns of gene expression. Treatment of colorectal cancer cells with antioxidants can lead to apoptosis by induction of p21 through a mechanism involving CCAAT/enhancer-binding protein β (C/EBPβ). Herein, we demonstrate that the antioxidant pyrroldinodithiocarbamate activates cAMP-dependent protein kinase (PKA) in a colorectal cancer cell line DKO-1. Activation of PKA phosphorylates Ser299 within C/EBPβ, which is essential for protein translocation to the nucleus. Pharmacological inhibition of PKA and mutation of Ser299 to alanine blocks C/EBPβ nuclear translocation and induction of p21. Our results indicate that a cAMP-dependent phosphorylation of C/EBPβ at Ser299 is critical for nuclear translocation of this protein and its subsequent transactivation of genes in response to antioxidant treatment.

Antioxidants have been associated with a diminished risk of cancer at various anatomical sites, including the colon (1, 2). The primary mechanism of chemoprevention by antioxidants is through the reduction of DNA-damaging free radicals (3). We have reported that two antioxidants, pyrroldinodithiocarbamate (PDTC) and vitamin E, induce G1 cell cycle arrest and apoptosis in various human cancer lines including breast, colon, and lung (4). These cell cycle perturbations were mediated by induction of p21, a powerful inhibitor of the cell cycle, through a mechanism involving activation and binding of C/EBPβ to the p21 promoter.

C/EBPβ is a member of a diverse group of nuclear transcription factors that contain a leucine zipper motif required for dimer formation and a basic DNA-binding domain that facilitates interactions between these factors and the regulatory domains of promoters and/or enhancers of target genes (5–11).

C/EBPβ activates several acute phase protein genes through the NF-IL6 responsive elements (8, 12) and also has been implicated in adipocyte differentiation and inflammatory and immune responses (13). Thus, C/EBPβ is a pleiotropic transcriptional activator involved in a myriad of signal transduction and cell differentiation events.

Control of C/EBPβ expression and activity is complex and poorly understood. It is known that C/EBPβ gene is transcriptionally activated by IL-1 and lipopolysaccharide (5), whereas in other instances its binding to cognate DNA sequences is enhanced by cytokines (5, 11). Additionally, C/EBPβ can be a target for post-translational modification. Various kinases, including cAMP-dependent protein kinase (PKA) (14), protein kinase C (PKC) (15), a Ras-dependent MAP kinase (16), and a calcium calmodulin-dependent kinase (17) have been shown to phosphorylate C/EBPβ in vitro. These phosphorylation events modulate DNA binding and transcriptional activity of C/EBPβ. However, it is unknown if the phosphorylation status of C/EBPβ influences its subcellular compartmentalization. Elevation of cAMP levels in PC-12 cells or activation of tumor necrosis factor receptors in hepatocytes leads to a redistribution of C/EBPβ from the cytosolic to nuclear compartment (9, 18). As a first step toward understanding the antioxidant-mediated increase in C/EBPβ DNA binding activities, we evaluated the effect of the antioxidant PDTC on the post-translational modification and subcellular localization of C/EBPβ in DKO-1 cells, a human colorectal cancer cell line. Our results indicate that PDTC induces a rapid and sustained translocation of C/EBPβ protein from the cytoplasm to the nucleus, resulting in induction of p21. Moreover, we demonstrate that these antioxidant-mediated events are regulated by PKA-mediated phosphorylation of Ser299 in C/EBPβ.

EXPERIMENTAL PROCEDURES

Materials—DKO-1 cells were obtained from Dr. Takehiko Sasazuki (Department of Genetics, Kyushu University, Fukuoka, Japan). FLAG antibodies were purchased from Amersham, and antibodies raised against p21 and the C/EBP proteins α (14AA), β (C-19), and δ (C-22) were obtained from Santa Cruz (Santa Cruz, CA). C/EBPβ and p21 cDNAs were provided by Dr. Linda Sealy (Vanderbilt University, Nashville, TN) and Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD), respectively. Myristoylated PKI (mPKI) was purchased from Biomol (Plymouth Meeting, PA) and tamofoxifen citrate and KN-93 from Calbiochem (San Diego, CA). Radiosotopes were purchased from NEN Life Science Products. All other chemicals were from Sigma or J. T. Baker Inc. (Phillipsburg, NJ).

Cell Culture—DKO-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with high glucose and supplemented with 10% heat-inactivated fetal bovine serum, 1-glutamine, and penicillin (100 unit/ml) and streptomycin (100 μg/ml) (Life Technologies, Inc.) at 37 °C in 5% CO2 in air. When cells reached about 80% confluence (time 0), cultures were treated with the appropriate agents for the indicated periods of time (5 min to 24 h).
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**Fig. 1.** PDTC induces C/EBPβ DNA binding activity via a post-translational modification. A, DKO-1 cells were treated with 70 μM PDTC for the indicated times, and nuclear extracts were prepared with a γ-32P-labeled p21-NF-IL6 oligonucleotide (lanes 1–9). Specificity assays were as follows. Lanes 10–12, competition controls were performed on a nuclear extract derived from cells treated with PDTC for 3 h (lane 10), with excess unlabeled wild type (lane 11) and mutant (lane 12) oligonucleotide. F represents free probe. Lanes 13–15, supershift analyses using C/EBPα (lane 13), β (lane 14), or γ (lane 15) antibodies as described under “Experimental Procedures.” B, DKO-1 cells were treated with PDTC for the indicated times, and C/EBPβ mRNA (3 μg) and protein (100 μg of protein) levels were evaluated by Northern and Western blot analyses, respectively. IB15 is shown as a control for equivalent mRNA loading and transfer. C, DKO-1 cells were treated with PDTC in the presence of [32P]orthophosphate and, at the indicated times, C/EBPβ protein purified from the cytosol and nucleus by immunoprecipitation. Treatment-related variations in C/EBPβ localization were analyzed by SDS-PAGE followed by autoradiography. D, DKO-1 cells were grown in the presence of PDTC for 3 h and then processed for immunofluorescence (see “Experimental Procedures”) to detect differences in the compartmentalization of endogenous C/EBPβ protein (red). Cellular nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Pink represents co-localization of red and blue signals. In all experiments, parallel cultures treated with anti-C/EBP antisera that had been preincubated with in vitro translated C/EBPβ protein demonstrated no fluorescent (red) signal (left panel).

**Fig. 2.** PDTC induces a rapid and sustained elevation in cAMP levels and PKA activity. DKO-1 cells were treated with 70 μM PDTC for the indicated times. Cell lysates were prepared and assayed for endogenous cAMP levels (A) or PKA activity (B) (see “Experimental Procedures”). The values are expressed as the mean pmol/μg protein ± S.E. and are representative of three experiments carried out in quadruplicate.

RNA Isolation and Northern Blot Analysis—RNA was extracted by the method of Schwalb et al. (19). Poly (A)+ mRNA was separated by electrophoresis through 1% (w/v) agarose-formaldehyde gels, and Northern blotting was performed as described previously (20). A human p21 cDNA probe was labeled with [α32P]dCTP by the random primer extension method. Hybridization and post-hybridization washes were carried out at 43 °C. IB15 was used as a control for equivalent loading and transfer (21).

Immunoprecipitations and Western Blot Analysis—For immunoprecipitations, cells were washed twice in ice-cold phosphate-buffered saline containing 1 mM Na2VO4 and 100 μg/ml phenylmethylsulfonil fluoride and lysed in RIPA buffer (50 mM Tris-CI, pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.5% SDS, 0.5 mM phenylmethylsulfonil fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 2 μg/ml leupeptin). Alternatively, nuclear proteins were isolated as described (22). Resulting extracts were precleared with protein A-Sepharose (Fierce) for 15 min at 4 °C. Following addition of the agarose-conjugated anti-FLAG (M2), anti-C/EBP antisera, and immunoreactive proteins were visualized by chemiluminescence (Amersham).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (22). Each 20-μl reaction mixture contained 5 μg of nuclear protein plus a γ-32P-labeled 20-base pair oligonucleotide probe containing the C/EBPβ-binding site in the p21 promoter (5′-GTACTTTAAGAAATTGGAAT-3′). The reaction mixture was incubated at room temperature for 10 min and loaded directly onto a 6.5% polyacrylamide (49:0.6 acrylamide/bisacrylamide) gel in a buffer of 25 mM Tris borate, pH 8.0, 0.25 mM EDTA. In some experiments, antiserum specific for unique C/EBP isoforms or a mutant C/EBP oligonucleotide (5′-GTACAAAAGAAATTGGAAT-3′) was added to reaction mixtures.
PKA Phosphorylation of C/EBPβ Mediates Nuclear Translocation

C/EBPβ is a member of a diverse group of nuclear transcription factors that contain a leucine zipper motif and a basic DNA-binding domain (5). We previously have shown that p21 expression is induced by antioxidants through a mechanism involving activation of C/EBPβ (4). To determine the mechanism by which this occurs, we performed EMSA with a 32P-labeled oligonucleotide containing the p21-NF-IL6 cis element and nuclear extracts from the colorectal cell line DKO-1, treated over a 24-h period with PDTC. As shown in Fig. 1A (lanes 1–9), DNA binding activity was increased 5-fold in cells treated with PDTC for 30 min. Shifted complexes were competed by a 50-fold molar excess of an unlabeled oligonucleotide containing a consensus NF-IL6 sequence (lane 11) but not by an oligonucleotide containing a mutated NF-IL6 consensus sequence (lane 12), indicating that the induced complex was specific for the NF-IL6 cis element. Supershift experiments confirmed that this was predominately due to increased C/
EBPβ (lane 14) but not C/EBPα (lane 13) or C/EBPδ (lane 15) binding activity.

We next sought to determine if the observed PDTC-induced increase in C/EBPβ DNA binding activities was due to increased C/EBPβ expression. As shown in Fig. 1B, both C/EBPβ mRNA and whole cell protein concentrations remained relatively constant over the 24-h treatment with PDTC. These results suggest that the increased DNA binding activities are unlikely to be mediated by increased synthesis of C/EBPβ.

However, in vivo [32P]orthophosphate-labeling of C/EBPβ revealed the rapid appearance and sustained elevation of a phosphorylated form of C/EBPβ in nuclear extracts following PDTC treatment (Fig. 1C). Immunocytochemical analysis confirmed that under these conditions, PDTC induces a rapid redistribution of C/EBPβ to the nucleus (Fig. 1D), suggesting a potential mechanism for the observed increase in DNA binding activity. These findings implicate protein phosphorylation of C/EBPβ as a possible mechanism for its nuclear translocation. Previous studies have shown that C/EBPβ translocates to the nucleus following elevation of cAMP levels in PC-12 cells (9) or activation of tumor necrosis factor receptors in hepatocytes (18). PDTC does not, however, induce nuclear translocation or increase DNA binding activity of the transcription factor NF-κB in DKO-1 cells (data not shown).

A reduction in H2O2 levels can activate adenylyl cyclase and hence elevate cAMP levels (27). Because PDTC has been shown to alter the cellular oxidative status, in part by decreasing endogenous H2O2 levels (4), increased cAMP levels may initiate the observed post-translational effects of this antioxidant. Therefore, we measured the effect of forskolin (a known elevator of intracellular cAMP) or PDTC on cAMP levels and PKA activity in DKO-1 cells over a 24-h period. As shown in Fig. 2 (A and B), both PDTC and forskolin were able to rapidly increase intracellular cAMP levels and PKA activity within 5 min. Interestingly, cAMP levels and PKA activity returned to baseline following 24 h of treatment with forskolin, whereas PDTC induced a sustained elevation in these parameters. Cholera toxin was able to inhibit PDTC-induced PKA activity (data not shown), suggesting that PKA is activated via a cAMP-dependent pathway through Gs stimulation of adenylyl cyclase. The sustained elevation in cAMP and PKA activity in response to PDTC and not in response to forskolin may be a result of modulation of phosphodiesterase activity by the antioxidant. In addition, a PDTC-induced alteration in phosphatase activity also may contribute to the observed persistence in PKA activity. We are presently exploring these potential mechanisms.

We utilized DKO-1 cells constitutively expressing epitope-

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(A) DKO-1 cells stably transfected with epitope-tagged WT C/EBPβ were grown in the presence or absence of PDTC or PDTC and mPKI for 3 h. Cells were fixed with paraformaldehyde and FLAG-tagged C/EBPβ visualized by immunofluorescence. Parental DKO-1 cells were treated as above and immunostained for endogenous C/EBPβ. Treatment of parental DKO-1 cells and/or stable transfectants with mPKI alone failed to induce nuclear translocation of C/EBPβ (data not shown). B, parental DKO-1 cells or cells stably transfected with FLAG-tagged WT C/EBPβ or the Ala299 mutant were treated with PDTC (0 or 70 μM) in the presence or the absence of mPKI (1 μM) for 3 h. Treatment-related variations in p21 mRNA were evaluated by Northern blot analysis. IB15 is shown as a control for equivalent loading and transfer. C, DKO-1 cells were transfected with either a p21 reporter plasmid and a cytomegalovirus expression plasmid containing WT C/EBPβ (0.5 μg each) alone or with the indicated amounts of pCMV-Ala299 C/EBPβ. Luciferase activity was measured in relative light units after 24 h and normalized to chloramphenicol acetyltransferase activity, and results were reported as fold activation above basal levels. Values represent mean ± S.E. of three transfections carried out in triplicate.
tagged C/EBPβ protein to further investigate whether post-
translational modification (phosphorylation) of C/EBPβ is re-
sponsible for the observed increase in C/EBPβ activity. In vivo
labeling with $^{32}$Porthophosphate followed by immunoprecipita-
tion revealed a 6-fold increase in epitope-tagged C/EBPβ
phosphorylation in response to PDTC or forskolin (3 h) with no
change in protein levels (data not shown). Truncated versions
of C/EBPβ that contained only the 160 or 200 C-terminal amino
acids were poor substrates for PDTC-induced phosphorylation,
whereas mutant C/EBPβ that contained the 305 C-terminal
amino acids was phosphorylated by PDTC as efficiently as the
full-length C/EBPβ (data not shown). Closer inspection of the
primary amino acid sequence between 236 and 305 revealed
that this region contained a consensus PKA phosphorylation
site (Arg-Xaa-Ser299-Xaa).

Comparison of the tryptic phosphopeptide maps of epitope-
tagged C/EBPβ from PDTC- or forskolin-treated and untreated
stable transfectants showed most of the inducible phosphoryl-
ation to occur on one distinct phosphopeptide (X$_2$; Fig. 3A).
To confirm that both endogenous and FLAG-tagged C/EBPβ are
subject to similar changes in their phosphorylation state in a
different cell type, we repeated these experiments in differenti-
tated 3T3 cells stably expressing PKA that additionally ex-
pressed high levels of C/EBPβ. As observed in DKO-1 cells, PDTC
stimulated site-specific phosphorylation of both endogenous
and expressed C/EBPβ (data not shown). Although the level of
several phosphopeptides was increased after PDTC treatment,
the only change common to both cell types was a higher level of
the phosphopeptide, X$_3$ (Fig. 3A and data not shown).

The migration position of phosphopeptide X$_3$ appeared iden-
tical to that of a phosphopeptide that contains Ser$_{277}$ and
Ser$_{299}$ as its phosphoacceptors (data not shown), sites previ-
ously reported to be weakly phosphorylated by PKA in vitro
(15). To determine whether Ser$_{277}$ and/or Ser$_{299}$ were indeed
PDTC-responsive phosphorylation site(s), we substituted ei-
either amino acid with alanine. Vectors expressing FLAG-tagged
wild type (WT) C/EBPβ or C/EBPβ (Ala$_{277}$ or Ala$_{299}$) were
stably transfected into DKO-1 cells, and the resultant proteins
were isolated after in vivo labeling with $^{32}$Porthophosphate.
As shown in Fig. 3 (B and C), substitution of Ser$_{299}$ but not
Ser$_{277}$ led to selective loss of PDTC-inducible phosphorylation
and nuclear translocation of C/EBPβ.

Interestingly, the low levels of Ala$_{299}$ C/EBPβ detected by
$^{32}$Porthophosphate labeling appeared as a doublet similar to the
phosphorylated forms observed with endogenous protein (Fig.
1C and 3B). Furthermore, substitution of Ser$_{299}$ with alanine
failed to alter the rate of C/EBPβ migration during SDS-PAGE (Fig.
3B). These data suggest that these different C/EBPβ species do not represent phosphorylation at Ser$_{299}$.

The appearance of this doublet form in the nucleus of DKO-1
but not HCT 116 or HCT 15 colon cancer cells (4) may represent
additional post-translational modifications, independent of
the PDTC-translocation signal.

Previous studies by our group have shown that over expres-
sion of C/EBPβ leads to increased p21 expression (4). Induction
of p21 by DNA damage frequently relies on the tumor suppres-
sor protein p53 (28), presumably through interaction with p53-
binding sites present in the p21 promoter region. However,
many inducers of p21, including cytokines, prostaglandins, and
genotoxic agents (29), operate to a large degree through poorly
characterized p53-independent mechanisms. Therefore, we
investigated the effect of PDTC-mediated phosphorylation on
C/EBPβ activity by comparing the ability of wild type C/EBPβ
and the Ala$_{299}$ mutant in stably transfected DKO-1 cells to
activate p21 gene expression following nuclear translocation.
As shown in Fig. 4 (A and B), wild type C/EBPβ (endogenous
and epitope-tagged WT) was able to translocate to the nucleus
and induce p21 expression following PDTC treatment (Fig. 4, A
and B). PDTC treatment of these cells in the presence of a
highly specific PKA inhibitor, mPKI, completely abolished
these cellular changes (Fig. 4B). In contrast, co-administration
of PDTC with the selective kinase inhibitors, tamoxifen citrate
(PKC) or KN-93 (calcium-calmodulin-dependent), failed to
inhibit nuclear translocation of C/EBPβ or its ability to induce
p21 expression (data not shown). As predicted, Ala$_{299}$ C/EBPβ
failed to translocate to the nucleus following PDTC treatment
(Fig. 4A). Surprisingly, expression of this mutant acted as a
dominant-negative in these cells, preventing the ability of
dependent protein to induce p21 expression (Fig. 4B). The
dominant-negative effect of this mutation was confirmed by tran-
sient transfection analysis (Fig. 4C). These results demonstrate
that translocation of C/EBPβ to the nucleus is directly en-
hanced by the PKA-induced phosphorylation of Ser$_{299}$, an effect
inhibited by the presence of mPKI.

Previous reports have demonstrated that C/EBPβ is phos-
phorylated by PKC (Ser$_{105}$ in vitro or Ser$_{248}$, Ser$_{277}$, and
Ser$_{299}$ in vitro) (14, 15), PKA (Ser$_{277}$ and Ser$_{299}$ in vitro) (15), a
Ras-dependent MAP kinase (Thr$_{235}$ in vitro) (16), and a calci-
um-calmodulin-dependent kinase (Ser$_{276}$ in vivo and in vitro)
(17), leading to alterations in DNA binding activities. Although
these reports provide insights into the mechanisms by which
the activity of C/EBPβ could be potentiated by selected agents
to affect activation of genes, specific biological end points
were not explored. For example, transactivation of C/EBPβ by
PKC in hepatocytes may affect activation of acute phase protein
synthesis, whereas phosphorylation of Thr$_{235}$ by MAP kinase
may lead to cell growth or inhibition. We envision that differ-
ential phosphorylation of C/EBPβ may contribute, at least in
part, to the diverse signals mediated by this transcription
factor. Collectively, our results demonstrate that in DKO-1
cells phosphorylation of C/EBPβ at Ser$_{299}$ following activa-
tion of PKA is critical for its nuclear translocation and sub-
sequent transactivation of genes in response to altered oxida-

deative states.

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REFERENCES

1. Bussey, H. J. (1982) Cancer 50, 1434–1448
2. Morsen, B. C., Bussey, H. J. R., Day, H. J. R. & Hill, M. J. (1983) Cancer Surv.
2, 451–465
3. Greenland, P. (1995) Cancer Epidemiol. Biomark. Prev. 4, 691–705
4. Chinery, R., Brockman, J. A., Peeler, M. O., Shyr, Y., Beauchamp, R. D. &
Coffey, R. J. (1997) Nat. Med. 3, 1233–1241
5. Akira, S., Ishikii, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y.,
Nakajima, T., Hirano, T. & Kishimoto, T. (1990) EMBO J. 9, 1897–1906
6. Chang, C. J., Chen, T. T., Lee, H. Y., Chen, D. S. & Lee, S. C. (1990) Mol.
Cell. Biol. 10, 6642–6653
7. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. & Schiller, U. (1990)
Genes Dev. 4, 1541–1553
8. Pal, V., Manetti, F. C. & Gertocco, R. (1990) Cell 63, 643–653
9. Metz, R. & Ziff, E. (1991) Genes Dev. 5, 1754–1766
10. Cao, Z., Uemek, R. M. & McKnight, S. L. (1991) Genes Dev. 5, 1538–1552
11. Williams, S. C., Cantwell, S. C. & Johnson, P. F. (1991) Genes Dev.
5, 1553–1567
12. Miyazima, A., Kitamura, T., Harada, N., Yokota, T. & Arai, K. I. (1992) Ann.
Rev. Immunol. 10, 295–331
13. Akira, S. & Kishimoto, T. (1992) Immunol. Rev. 127, 25–50
14. Transtwaiw, C., Caelles, C., Van der Greer, P., Hunter, T., Karin, M. & Chojkier,
M. (1993) Nature 364, 544–547
15. Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C. & Huang, K. P.
(1992) J. Biol. Chem. 267, 19296–19403
16. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto,
T. & Akira, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2207–2211
17. Wegner, M., Cano, Z. & Rosenberg, M. G. (1992) Science 256, 370–373
18. Yin, M., Yang, S. Q., Lin, H. Z., Lane, M. D., Chatterjee, S. & Diehl, A. M.
(1997) J. Biol. Chem. 272, 17974–17978
19. Schwab, M., Altalaloo, K., Varmus, H. E. & Bishop, J. M. (1983) Nature
363, 497–499
20. Coffey, R. J., Goutain, A. S., Soderquist, A. M., Shipley, G. D., Wolfshohl, J.,
Carpenter, G. & Moses, H. L. (1987) Cancer Res. 47, 4590–4594
21. Danielson, P., Forss-Petter, S., Brow, M., Calavetta, L., Douglass, J., Milner,
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