Suppression of Nerve Growth Factor-induced Neuronal Differentiation of PC12 Cells

*N-ACETYLCYSTEINE UNCOUPLES THE SIGNAL TRANSDUCTION FROM Ras TO THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE*

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The cellular redox state is thought to play an important role in a wide variety of cellular signaling pathways. Here, we investigated the involvement of redox regulation in the nerve growth factor (NGF) signaling pathway and neuronal differentiation in PC12 cells. N-acetyl-L-cysteine (NAC), which acts as a reductant in cells both by its direct reducing activity and by increasing the synthesis of the cellular antioxidant glutathione, inhibited neuronal differentiation induced by NGF or by the expression of oncogenic ras in PC12 cells. NAC suppressed NGF-induced c-fos gene expression and AP-1 activation. These results suggest that neuronal differentiation and NGF signaling are subject to regulation by the cellular redox state. NAC also suppressed the NGF-induced activation of mitogen-activated protein kinases (MAPKs) and decreased the amount of tyrosine phosphorylation of MAPKs. The suppression of MAPK by NAC was independent of glutathione synthesis. In parallel with the suppression of MAPK, the activation of MAPK kinase activity was also suppressed in the presence of NAC. In contrast, NGF-induced activation of Ras was not inhibited by NAC. The inhibitory effect of NAC on the MAPK cascade was independent of transcription and translation. Thus, NAC suppresses NGF-induced neuronal differentiation by uncoupling the signal transduction from Ras to the MAPK cascade in PC12 cells.

Reactive oxygen intermediates (ROIs), such as H2O2, superoxide (O2•−), and hydrogen peroxide radicals (OH•), are generated in cells by several pathways, including electron transfer reactions, xanthine oxygenase, NADPH oxidase, and γ-ray and UV light irradiation. The increased level of ROIs, referred to as oxidative radical stress, is generally cytotoxic, and hence cells possess several antioxidant systems, such as the antioxidant glutathione (GSH), thioredoxin/adult T cell leukemia-derived factor, and antioxidant enzymes, such as catalase, which operate to eliminate ROIs. These regulatory systems of the cellular redox state are thus essential for cell survival. When either excessive oxidative radical stress occurs or cellular antioxidant enzymes, such as superoxide dismutase, are down-regulated, the cells die by necrosis or apoptosis. In neuronal systems, oxidative radical stress is postulated to be the cause of several neuronal degenerative diseases, such as Parkinson’s disease (1, 2) and a familial form of amyotrophic lateral sclerosis (3–5).

Several lines of evidence have indicated that cellular redox plays an essential role not only in cell survival but also in cell signaling systems and cell growth. First, thioredoxin/adult T cell leukemia-derived factor has been shown to promote interleukin 2 receptor expression in T cells (6). Second, the activity of the transcription factors, NFκB and AP-1, is regulated by the redox state, i.e. redox regulation. AP-1 is subjected to redox regulation through its conserved cysteine residue (7) and is activated by antioxidants (8, 9). The activity of NFκB is enhanced by antioxidants in vitro (10, 11) but is potently and rapidly activated from its inactive cytoplasmic form by treatment of cells with H2O2 (8, 12). In addition, the antioxidants N-acetyl-L-cysteine (NAC) and thioredoxin prevent the activation of NFκB both by H2O2 and by several other extracellular stimuli, such as tumor necrosis factor α, phorbol ester, interleukin 1, and lipopolysaccharide (8, 9, 12). Furthermore, oxidative stress induces the expression of WAF1/CIP1, a cyclin-dependent kinase inhibitor, and arrests cell cycle progression (13). Thus, it is plausible that redox widely regulates many cellular functions.

The rat pheochromocytoma PC12 cell line is one of the most widely used cell lines in the study of the cellular signaling system, and these studies have revealed a common pathway linked to many growth factor stimuli, namely, the mitogen-activated protein kinase (MAPK) cascade (for review, see Refs. 14–17). The striking feature of the PC12 cell line is its ability to differentiate into neuronal cells in response to nerve growth factor (NGF). The binding of NGF to its receptor TrkA on the plasma membrane triggers the activation of Ras, the GDP-bound form of which changes to the GTP-bound form. The activated Ras then stimulates the signaling pathway known as the MAPK cascade, which consists of three kinases forming a chain reaction in a linear alignment. In the MAPK cascade, MAP kinase kinase kinases (MAPKKKs) are directly activated by Ras, which in turn control the dual-specificity kinases, MAP kinase kinases (MAPKKs). Finally, MAPKs are activated by MAPKK and then activate several transcription factors to induce neuronal differentiation. The importance of this cascade has been verified by the fact that the activation of Ras and MAPK is sufficient and necessary for neuronal differentiation.
of PC12 cells (18–21). Furthermore, this cascade has been revealed to be linked not only to many receptor tyrosine kinases but also to trimeric G proteins, which transduce signals from seven-helix transmembrane receptors (for review, see Ref. 22).

Although the importance of both the signaling system involving the MAPK cascade and the cellular redox regulation system has been established, the relationship between them so far remains unclear. However, several studies have suggested the existence of an intimate cross-talk between cellular redox and the signal transduction of NGF. First, NGF increases the level of cellular GSH or the activity of the antioxidant enzymes, GSH peroxidase and catalase, in PC12 cells (22–25). Second, NGF effectively protects cells from injury by oxidative radical stress (26), and the activation of Ras is also effective in suppression of cell death caused by H₂O₂ (25). Third, the expression of a protein phosphatase CI100, which is capable of dephosphorylating MAPK, is potently induced by oxidative stress (27), and the essential signaling molecule Ras is reported to be regulated by redox stress (28). Finally, exogenously added H₂O₂ or other oxidizing agents induce the activation of MAPK in several cell types (29, 30).

In this study, we investigated the involvement of the cellular redox state in neuronal differentiation and the cellular signaling pathway of NGF in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) and 5,5-dithiothreitol (DTT) were obtained from Wako Chemical Industries (Osaka, Japan). β-Thionine (S,R)-sulfoximine and myelin basic protein were obtained from Sigma Chemical Co. (St. Louis, MO). L-Lysine (L-lysine) was purchased from Wako Chemical Industries (Osaka, Japan). GSH-oxidizing agents induce the activation of MAPK in several cell types (29, 30).

**Cell Cultures and Cell Lines**—PC12 cells and PC1MTras21 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 50 μg/ml kanamycin. PC1MTras21 (25) was cloned from PC12 cells transfected with the plasmid pMTIDrasneo, which was constructed by insertion of the neomycin-resistant gene into the plasmid pMTID-ras encoding the c-Ha-ras oncogene (25). 20 μg of RNA was electrophoresed through 1% agarose containing 17% formaldehyde, and the gels were then stained with 0.05 μg/ml ethidium bromide. The fractionated RNA was transferred to a nylon filter (Hybond-N+, Amersham Corp.) following the manufacturer’s instructions.

**Northern Blot Analysis**—Total RNA was prepared from the cells by RNAzol (Cinnaconics, Toronto, ON) following the manufacturer’s instructions. 10 μg of RNA was electrophoresed through 1% agarose gel and transferred to a nylon filter (Hybond-N+, Amersham Corp.) following the manufacturer’s instructions.

**Chloramphenicol Acetyltransferase (CAT) Assay**—For transfection with plasmids, PC12 cells (1 × 10⁶) on a 6-cm dish were incubated with 20 μl of Lipofectin (Life Technologies, Inc.), together with 2 μg of plasmid (pAPCAT) in 2 ml of Opti-MEM medium (Life Technologies, Inc.). pAPCAT encodes the triple AP-1 binding sites between the HindIII and SalI sites upstream of the CAT gene of the plasmid pBLCAT5 (34). After transfection, the culture was divided equally between four dishes and cultured further in 4 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and 5% fetal bovine serum for 2 days. The cells were collected and disrupted by three freeze-thaw cycles in 100 μl of 25% Tris-Cl, pH 7.4, and then centrifuged at 15,000 rpm for 15 min. 90 μl of supernatant was incubated with 0.1 μCi [³⁵S]chloramphenicol and 1 μm acetyl-CoA at 37 °C overnight. The samples were developed by thin-layer chromatography (TLC) using a silica gel in chloroform/methanol (114:6 by volume). CAT activity was normalized by the protein content in each sample.

**Kinase Assay for MAPK and MAPKKK**—For the MAPK kinase assay, PC12 cells (2 × 10⁹/10 cm dish) were solubilized in 0.5 ml of extraction buffer A (20 μg/ml Tris-Cl, pH 7.4, 5 mM EDTA, 0.5% Triton X-100, 50 mM β-glycerophosphate, 1 mM amionophenylmethylasulfonfluoride, 50 units/ml aprotinin, 2 mM DTT, 1 mM Na₃VO₄, and 0.1 mM NaF) and then centrifuged at 15,000 g for 10 min. MAPK was immunoprecipitated using anti-MAPK antibody, and the immune complexes were then incubated with 12 μl of reaction buffer containing 20 μg/ml Tris-Cl, pH 7.4, 25 μM MgCl₂, 300 μg/ml myelin basic protein, and 0.5 μCi [γ-³²P]ATP for 15 min at 20 °C.

**Mono-Q Column Chromatography**—Fractionation of proteins by column chromatography was performed using the Smart System (Pharmacia Biotech Inc.) for the MAPKKK assay. The recovered GST-MAPKK bound to the glutathione Sepharose 4B column was eluted with 20 μl of bacterially expressed glutathione S-transferase-MAPK kinase fusion protein (GST-MAPKK), 100 μM ATP, and 10 mM MgCl₂ at 30 °C for 1 h. The resulting mixture was further incubated with 15 μl of GSH Sepharose at 4 °C for 30 min and then centrifuged. The pellet was then washed by the buffer, and then the recovered GST-MAPKK bound to GSH Sepharose was incubated with kinase-negative MAPK (KN-MAPK) and 1 μCi [γ-³²P]ATP in the presence of 10 mM MgCl₂ at 30 °C.

**Electrophoretic Mobility Shift Assays**—Nuclear extract was prepared as described by Dignam et al. (33). A ³²P-labeled DNA probe of the AP-1 binding site (5′-GTGCATGACTCAGCAATGTCGTAGTCAAGAGTCATCAA and labeling it with Klenow fragments and [³²P]dCTP. Nuclear extracts (10 μg) were mixed with the ³²P-labeled DNA probe (1 × 10⁶ cpm) in a reaction buffer containing 10 μg Tris-Cl, pH 7.6, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol (DTT), 10 mg/ml BSA, and 100 μg/ml poly(dI-dC) and then were incubated at room temperature for 30 min. Following the incubation, the samples were fractionated through 8% acrylamide gel in a buffer containing 40 mM Tris acetate, pH 8.5, 1 mM EDTA.

**Western Blotting**—Following SDS-polyacrylamide gel electrophoresis, the proteins were transferred to an ECL membrane (Amersham Corp.) using electroblotting apparatus (AE-6675P, ATTO). The filter was stained with the antibody using an ECL Western blotting system (Amersham Corp.) following the manufacturer’s instructions.

**Analysis of GTP/GDP Bound Forms of Ras**—GTP/GDP binding to Ras was analyzed as described by Muroya et al. (36). Briefly, 1 × 10⁶ cells on a 6-cm dish were incubated with 50 μM/ml [³²P]phosphorus in phosphate-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 1% horse serum and 0.5% fetal bovine serum.
The cells were washed with ice-cold buffer containing 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 1 mM Na$_3$VO$_4$ and then solubilized in 1 ml of buffer containing 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 20 mM MgCl$_2$, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 mM Na$_3$VO$_4$. The cell extract was incubated with 1 μl of anti-Ras antibody, 2 μl of anti-Rat IgG antibody, and 2 μl of protein A-Sepharose at 4°C for 1 h, and then the immune complex was washed with 1 ml of ice-cold buffer four times. Guanine nucleotides bound to Ras were analyzed by TLC using a polyethylene imine sheet (Marchery-Nagel) in 0.8M KH$_2$PO$_4$ (pH 3.4).

**RESULTS**

Treatment with NGF for 3 days induced neuronal differentiation of PC12 cells with the appearance of neurite extensions (Fig. 1A). This NGF-induced morphological change in PC12 cells was completely abolished in the presence of NAC in concentrations higher than 10 mM.

Ras is an essential signal mediator of NGF (18, 19), and its activation mimics the effects of NGF in inducing neuronal differentiation in PC12 cells (37, 38). The PCMTras21 cell line, which carries an oncogenic Ha-ras gene under the control of the metallothionein promoter, is induced to differentiate, with extension of neurite processes, by ZnCl$_2$. We found that the neuronal differentiation of PCMTras21 cells triggered by ZnCl$_2$ was also completely inhibited by NAC (Fig. 1A). Thus, NAC is suspected to abolish NGF-induced neuronal differentiation by suppressing signal transduction downstream of Ras activation.

To elucidate the mechanism of the action of NAC in suppressing the differentiation, Northern blot analysis was performed (Fig. 2). Neuronal differentiation by NGF is thought to be mediated by the expression of several genes, such as c-fos, expression of which is regulated by several transcription factors linked to the cellular signaling pathway of NGF (for review, see Ref. 39). Northern blot analysis revealed that NGF rapidly induced c-fos expression in PC12 cells as reported by Greenberg et al. (40). NGF-induced expression of the c-fos gene was completely suppressed when PC12 cells were cultured in the presence of NAC. Furthermore, we investigated the activation of AP-1, which is one of the first transcription factors activated by NGF (for review, see Ref. 39) using the electrophoretic mobility shift assay method (Fig. 3A). AP-1 has been reported previously to be subjected to redox regulation (7) in which NAC increases its binding activity by an increase in AP-1 expression (8). Consistent with this, electrophoretic mobility shift assay showed a slight increase in the DNA binding activity of AP-1 in cells treated with NAC. However, the stimulatory effect of NAC on AP-1 was minimal, and it was not possible to detect an NAC-induced increase in AP-1 activity.
when using the CAT assay with a reporter plasmid encoding AP-1 binding sites (Fig. 3B). In contrast, the most striking effect of NAC on AP-1 was the suppression of its activation induced by NGF. The DNA binding activity of AP-1 increased rapidly within 30 min after NGF stimulation and reached maximal levels after 60 min in control cells. This AP-1 activation was apparently suppressed in cells treated with NAC (Fig. 3A). The inhibitory effect of NAC on NGF-induced AP-1 activation was confirmed by CAT assay (Fig. 3B). These results indicate that the step(s) inhibited by NAC occurs upstream of the activation of the transcription factors in the NGF signaling pathway.

Activation of MAPK is thought to be occur upstream of the activation of transcription factors in the nucleus in response to stimulation by growth factors (for review, see Ref. 15). MAPK was rapidly activated within 5 min after NGF stimulation, and the activated levels were sustained for a long period in PC12 cells (Fig. 4A), consistent with the previous study (41). MAPK activation was markedly suppressed in cells treated with NAC. Western blotting analysis revealed that the treatment with NAC did not alter the quantity of MAPKs with molecular masses of 41 and 43 kDa in PC12 cells (Fig. 4B, left), whereas the electrophoretically retarded bands of MAPKs (activated MAPKs) induced by NGF were markedly reduced by NAC, indicating that NAC inhibited NGF-induced phosphorylation of MAPKs. The reduction of phosphorylation of MAPKs by NAC was confirmed by blot analysis using anti-phosphotyrosine antibody (Fig. 4B, right). Thus, a step(s) upstream of MAPK in the NGF signaling pathway was thought to be suppressed by NAC. NAC is a unique compound that acts as an antioxidant both
Experimental Procedures.

Increased cellular GSH more than 10-fold, whereas 100 \textmu M buthionine sulfoximine completely abolished the increase of GSH level in normal cells. In these GSH-depleted cells, NAC inhibited below the basal level when the cells were treated with NAC above 10 mM. These results, taken together, suggest that NAC inhibits NGF-induced neuronal differentiation by suppressing the kinases comprising the MAPK cascade.

NAC was able to suppress both MAPK and MAPKKK activity, even in the presence of actinomycin-D or cycloheximide, indicating that the inhibitory effects of NAC on the MAPK cascade were independent of both transcription and translation (Fig. 6). In fact, the action of NAC was so rapid that NGF-induced MAPK activation was apparently decreased in cells incubated with NAC for only 10 min, and treatment with NAC for 1 h was sufficient to develop the full inhibitory effects on MAPK (Fig. 7).

In PC12 cells, three kinases, including Raf-1, B-Raf, and MEKK, are known to act as MAPKKKs (42–44). Among these MAPKKK species, B-Raf is refractory to inhibition by cAMP, whereas Raf-1 is effectively inhibited in PC12 cells cultured in serum-containing medium, suggesting a difference in the regulation of enzyme activity in these kinases (45). This prompted us to analyze the effects of NAC on each of these MAPKKK species. For this purpose, we attempted to separate the three kinases by fractionation of cell lysates using Mono-Q column chromatography (Fig. 8). Western blotting revealed that these MAPKKK species eluted separately from the column: B-Raf, with a molecular mass of about 97 kDa, was enriched in fractions 2–11; Raf-1, about 70 kDa, was in fractions 2–4 and 7–13; and MEKks of about 66 and 97 kDa were in fractions 8–9 and 10–11, respectively. Faint bands between 97 and 66 kDa on the MEKK immunoblot were nonspecific. Kinase assay revealed that NAC enhanced the MAPKKK activity to twice basal levels in these Mono-Q fractions. This activation was completely suppressed by NAC in all of the fractions, suggesting that Raf-1, B-Raf, and MEKK were equally subject to suppression by NAC.

Transcription- and translation-independent inhibition of NGF-induced MAPKKK and MAPK activation by NAC. Cells were incubated with either 1 \mu g/ml actinomycin-D (ACT) or 10 \mu g/ml cycloheximide (CHX) for 3 h and then incubated for 2 h with or without 30 mM NAC. Lysates were prepared from cells left untreated or treated with NGF for 10 min. The kinase activity of MAPKKK (top) and MAPK (bottom) was estimated as described under “Experimental Procedures.”

Fig. 5. Dose-dependent inhibitory effects of NAC on NGF-induced activation of kinases comprising the MAPK cascade. PC12 cells were incubated with various concentrations of NAC for 4 h, and then the cell lysates were prepared either 10 min after NGF addition or without NGF stimulation. The kinase activity of MAPKKK (upper panel) and MAPK (lower panel) was estimated as described under “Experimental Procedures.”

Fig. 6. Transcription- and translation-independent inhibition of NGF-induced MAPKKK and MAPK activation by NAC. Cells were incubated with either 1 \mu g/ml actinomycin-D (ACT) or 10 \mu g/ml cycloheximide (CHX) for 3 h and then incubated for 2 h with or without 30 mM NAC. Lysates were prepared from cells left untreated or treated with NGF for 10 min. The kinase activity of MAPKKK (top) and MAPK (bottom) was estimated as described under “Experimental Procedures.”

2 S. Matsuda, unpublished observations.
the precise mechanism is unknown, the exchange of the GDP-bound form of Ras to the GTP-bound form (i.e., Ras activation) triggers the activation of these MAPKKKs in the NGF signaling pathway. Therefore, we analyzed GTP/GDP binding to Ras in cell lysates labeled with [32P]phosphorus by using thin-layer chromatography (Fig. 10). In control cells, the amount of GTP binding was less than 5% of the total guanine nucleotides bound to Ras. NGF rapidly activates Ras; the GTP-bound form increased to 20% within 5 min. NAC had no effect on Ras activation by NGF. Furthermore, we could not detect any effect of NAC on the activation of Ras 30 min after NGF stimulation. In accordance with this, NAC inhibited neuronal differentiation as described above and shown in Fig. 1A. Taken together, these results may indicate that the signal transduction from Ras to MAPKKK is specifically inhibited by NAC.

DISCUSSION

An antioxidant, NAC, was reported to modulate the activity of several redox-sensitive cellular signal transduction components by its direct reducing activity via a thiol base on the molecule and/or by an increase in cellular GSH levels. In addition, we have demonstrated in this report that NAC suppresses NGF-induced activation of AP-1 and the kinases of the MAPK cascade in accordance with the suppression of neuronal differentiation. We could not rule out the possibility that a pharmacological effect of NAC unrelated to its reducing activity, if any, would be most plausible that NAC inhibits MAPK activation by its direct reducing activity. This suggests that NGF signaling and neuronal differentiation of PC12 cells is subject to redox regulation. In fact, high concentrations of β-mercaptoethanol and DTT also significantly suppress MAPK activation, although less prominently than NAC. However, the reducing reagents, only NAC is effective in suppressing differentiation in PC12 cells; the other reductants are cytotoxic when used over a long period.
The mechanism by which NAC suppresses NGF signaling is unclear, but there are some possible explanations. One is that NGF stimulates the production of ROIs, which may act as a signal mediator, and the effects of these ROIs are suppressed by NAC acting as an antioxidant or free radical scavenger. Recently, several lines of evidence have been reported showing that ROIs are generated by extracellular stimuli, such as TGF-β (46), tumor necrosis factor α, βFGF (47), CD25 (48), and platelet-derived growth factor (30). In these cases, the blockage of the shift in cellular redox state induced by reducing agents or antioxidant enzymes results in the attenuation of cellular responses to these stimuli. In the case of vascular smooth muscle cells, H$_2$O$_2$ is generated by platelet-derived growth factor stimulation, and both NAC and catalase block the protein phosphorylation and the activation of MAPK elicited by platelet-derived growth factor (30). In addition, it was reported recently that another radical molecule, nitric oxide (NO) triggers a switch to growth arrest during neuronal differentiation in PC12 cells (49). Thus, it can be speculated that the production of ROIs plays an essential role in NGF signaling. To clarify this, we assessed the cellular redox state using a redox-sensitive fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate, which is oxidized to fluorescent 2′,7′-dichlorofluorescein, as described by Ohba et al. (46). We observed the shifts in cellular redox to the reduced state by 12 h of incubation with NAC, but so far we have been unable to detect any significant changes in redox state after NGF stimulation (data not shown). Thus, the inhibitory effects of NAC in PC12 cells are unlikely to be due to the suppression of ROIs.

NAC partially activates the transcription factor AP-1 (Ref. 8 and see Fig. 3A). Furthermore, the survival-promoting effects of NAC in suppression of apoptosis in PC12 cells are dependent on specific gene expression (50). This leads to another possible explanation that NAC causes the expression of specific gene(s), resulting in the suppression of NGF signaling and neuronal differentiation. However, this possibility is ruled out by the result that the inhibition of MAPK by NAC is not blocked by either cycloheximide or actinomycin-D.

Thus, it is most plausible that the NGF signaling pathway consists of a redox-sensitive step(s) that may be regulated by NAC. The results that NAC suppressed MAPKKKs without affecting Ras activation indicate that the signal transduction from Ras to MAPKKK is the primary step sensitive to NAC. This idea is supported by the observation that NAC causes the suppression of neuronal differentiation induced by the expression of oncogenic Ras in PC12 cells (see Fig. 1). The uncoupling of all types of MAPKKK species ( Raf-1, B-Raf, and MEKK) from Ras may result in suppression of the downstream events in the NGF signaling system.

It has been elucidated that Ras activates Raf, but it still remains to be elucidated how MAPKKK molecules are activated by Ras (for review, see Refs. 51 and 52). So far, it has been revealed that Raf kinase binds to activated Ras (53–55), whereas it is fully activated by reduction (10, 11). However, several reports have revealed that antioxidants (including NAC) suppress the activation of NFκB elicited by extracellular stimuli, such as tumor necrosis factor α, phorbol ester, interleukin 1, and lipopolysaccharide (8, 9, 12), suggesting that NAC suppresses an unknown redox-sensitive kinase(s) upstream of NFκB (61–64). This suggests that NFκB signaling may be subject to a dual redox regulation in which the reductant(s) directly activates NFκB while it suppresses upstream kinase(s). Thus, the signaling pathways to AP-1 and NFκB are both modulated by a dual redox regulation system. Interestingly, many transcription factors, such as Myb (65), are also reported to be regulated by redox. It might be that most signaling pathways linked to transcription factors are subject to dual redox regulation.

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