The repression of nuclear factor I/CCAAT transcription factor (NFI/CTF) transactivating domain by oxidative stress is mediated by a critical cysteine (Cys-427)

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INTRODUCTION

The NFI (nuclear factor 1) proteins are a family of ubiquitous transcription factors encoded by four different genes, NFI-A, NFI-B, NFI-C/CTF (or NFI/CTF, where CTF is CCAAT transcription factor) and NFI-X[1,2]. All isoforms share a highly conserved DNA-binding domain (DBD) that recognizes the TCCGN6GCCA sequence or a single half site (GCCA). It contains 220 amino-acids and is not related to any well-characterized class of DBDs. NFI isoforms differ in their C-terminal sequences and can either activate or repress the initiation of transcription. They were originally described as proteins involved in the transcription and replication of viral DNA [3]. They form homo- and heterodimers [2] that regulate a wide range of gene promoters such as collagen, albumin, vitellogenin, aspartate aminotransferase and cytochrome P450 1A1 (CYP1A1; see [4]).

The human isoform NFI/CTF contains several functional domains (see Figure 3A, below). The N-terminus is required for DNA binding, dimerization and adenovirus DNA replication. The 100 C-terminal residues containing a characteristic proline-rich region constitute the transcription-activating domain (TAD) [5].

Several conditions modulate NFI activity. In hepatoma cell lines, we have shown that the DNA binding of NFI proteins is specifically impaired by H$_2$O$_2$ treatment (millimolar concentrations) or glutathione depletion [4]. NFI/CTF, the most abundant isoform, is particularly sensitive to these treatments. In experiments in vitro, critical cysteines located in the DBD have been identified as the likely targets of this regulation [6]. In cell cultures, the modulation of the NFI transactivating function by various compounds was reported. It is activated by transforming growth factor β (TGFβ; thus mediating the induction of the collagen gene) and repressed by tumour necrosis factor α and oxidative stress (thus mediating the CYP1A1 gene inhibition) [4,7,8]. The last 13 residues of NFI/CTF were identified as the TGFβ-responsive domain (TRD) [9]. In fibroblasts, the TGFβ effect is associated with an increase in cytosolic calcium [7]. In addition, overexpression of the small GTPase Ras or of the Raf-1 kinase represses NFI/CTF transcriptional activity through a mechanism that does not involve the phosphorylation of the TRD [8].

The molecular mechanisms controlling NFI repression by reactive oxygen species (ROS) were still obscure despite the studies which focused on the modulation of its DNA-binding activity [4,6]. Indeed, the repression of several NFI-driven promoters by micromolar H$_2$O$_2$ concentrations that do not alter the DNA-binding activity raised the important question of the regulation of the TAD by ROS. Recently, we have shown that H$_2$O$_2$, as produced by CYP1A1 activity within the cell, could repress the transactivating function of NFI/CTF [10]. In this study, we show that the TAD of NFI/CTF is particularly sensitive to oxidative stress compared with the TAD of other general transcription factors. We have identified Cys-427 as the amino-acid target required for the regulation by H$_2$O$_2$. The study shows that, in addition to the well-characterized oxidative repression of DBDs [11], transactivating domains are also sensitive to oxidative stress.

MATERIALS AND METHODS

Chemicals and cell culture

H$_2$O$_2$ was used from a 30% stock solution obtained from Merck (Darmstadt, Germany). N-ethylmaleimide (NEM) was obtained from Sigma (Saint-Quentin Fallavier, France) and oligonucleotides were from Genset (Paris, France).

Abbreviations used: DBD, DNA-binding domain; NEM, N-ethylmaleimide; NFI, nuclear factor 1; CTF, CCAAT transcription factor; TAD, transcription-activating domain; TGFβ, transforming growth factor β; TRD, TGFβ-responsive domain; ROS, reactive oxygen species; CYP1A1, cytochrome P450 1A1; ERK, extracellular signal-regulated kinase.

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The human hepatoma cell line HepG2 was maintained as described elsewhere [12]. These cells were used because the NFI-driven gene promoter was regulated by H$_2$O$_2$ [4] and because of their good transfection efficiency [10].

Plasmids and site-directed mutagenesis

The firefly and Renilla luciferase expression plasmids pG5-FL and pG5glob-RL have been already described in [10]. The latter, which is not sensitive to submillimolar H$_2$O$_2$ concentrations [4], was used as an internal control of the transfection efficiency. The activity of this promoter was not sensitive to the NEM concentrations used in this study (mean 4% increase for 2.5 μM NEM, results not shown). pRSV.Gal.CTF, pRSV.Gal.Sp1 and pRSV.Gal.Oct expressed fusion proteins containing the Gal4 DBD fused to the TAD of the human NFI/CTF, Sp1 and Oct2 transcription factors respectively. They were derived from the pGal[399-499], pGalOct2 and pGalSp1 plasmids (described in [9]) in which the simian virus 40 (SV40) promoters have been replaced by Rous sarcoma virus (RSV) promoters because the SV40 promoter is very sensitive to oxidative stress whereas that of RSV is not [4]. Amino acid point mutations in the TAD of NFI/CTF were produced using a double PCR technique with mutated oligonucleotides [13].

Transfection experiments

Transfection experiments were performed in HepG2 cells as described previously [12]. Briefly, 1 day prior to the transfection, cells (0.5 × 10^6 cells/6-cm dish) were seeded into the usual culture medium. The vector expressing the fusion protein (2.5 μg), and firefly and Renilla luciferase expression vectors (2 and 1 μg respectively), were introduced into the cells by the calcium phosphate co-precipitation technique followed 4 h later by a 2-min glycerol shock. Cells were treated 5 h later by adding H$_2$O$_2$ or NEM to the culture medium. After an overnight incubation, cells were homogenized for enzymic assays. Dual luciferase assay (firefly and Renilla) was performed with a Promega kit according to the manufacturer’s instructions. Blanks were obtained by assaying luciferase activity in mock-transfected cells. Luciferase assays were performed in duplicate or triplicates from at least three independent transfection experiments.

Cellular extracts

Cells were transfected with pRSV.Gal.CTF (5 μg) as described above and grown for 48 h. Cells were treated or not with 50 μM H$_2$O$_2$ 1 h before harvest. Confluent 6-cm dishes were then washed with PBS and harvested by scraping. All subsequent steps were performed at 4°C. Cells were centrifuged and then resuspended in an extraction buffer (20 mM Tris, pH 7.5, 20% glycerol, 500 mM KCl, 1 mM dithiothreitol and 1 mM PMSF). Cells were lysed by three freeze–thaw cycles (each cycle consisting of freezing in liquid nitrogen followed by thawing on ice). After centrifugation (13000 g, 5 min), the supernatant was aliquoted and stored at −80°C. The protein concentration was measured according to the method of Bradford.

Electrophoretic mobility shift assay

The Gal4 probe used here contained a Gal4-binding site (5’-CGGGTGCGGATCTGGTCCTCCGAC-3’, with the Gal4-binding site underlined). Strands were annealed and labelled using the Klenow fragment of DNA polymerase I (Promega) and [α-32P]dCTP (Amersham). The NFI probe (5’-TATTCTTTGGATTGAGCCAATGATA-3’, with the NFI/CTF-binding site underlined) was used in competition experiments. Binding reactions were carried out as described previously [4]. Samples were run in an 8% polyacrylamide gel in 0.5 × Tris/borate/EDTA buffer for 100–120 min (260 V, 13 cm) at 4°C. The gels were then dried and autoradiographed. A PhosphorImager (Storm, Molecular Dynamics) was used to quantify the ratio (bound probe/free probe) relative to the formation of the Gal4-CTF fusion protein–DNA-probe complex.

Statistics

Student’s two-tailed t tests were performed using Statview software (Abacus Concepts).

RESULTS

Dose-dependent repression of the TAD of NFI/CTF by H$_2$O$_2$

Since several gene promoters in which NFI plays a critical role were down-regulated by H$_2$O$_2$ at concentrations that do not affect NFI DNA binding [4], we asked whether the TAD of NFI/CTF could mediate this effect. In this study, we used the human hepatoma cell line HepG2 and the pG5-FL vector, which contains a luciferase reporter gene driven by Gal4 binding sites. We co-transfected a vector expressing a fusion protein consisting of the DBD of Gal4 fused to the TADs of various human transcription factors. This method allows a specific assay of TAD activity (see below). In a previous study, we showed that 50 μM H$_2$O$_2$ repressed the TAD of NFI/CTF [10]. As can be seen in Figure 1, the TAD of NFI/CTF was repressed by micromolar concentrations of H$_2$O$_2$, in a dose-dependent manner. Half-maximal repression was observed at 10 μM H$_2$O$_2$.

The effect of oxidative stress on the TAD of other transcription factors was also tested. The TAD of Sp1 was repressed by H$_2$O$_2$ but at higher concentrations. In fact, it has already been shown that several Sp1-driven promoters were down-regulated by oxidative stress.

![Figure 1](image.png)

**Figure 1** Specificity and sensitivity of NFI/CTF transactivating function regulation by oxidative stress

Cells were co-transfected with pG5-FL as a reporter vector and pG5glob-RL as an internal control. Cell cultures were treated with various concentrations of H$_2$O$_2$ and harvested 16 h later. Firefly and Renilla luciferases were assayed as described in the Materials and methods section.

Results were expressed as the firefly luciferase activity/Renilla luciferase activity ratio (mean ± S.E.M., n = 6). Effect of increasing H$_2$O$_2$ concentrations on cells transfected with pRSV.Gal.CTF (□), pRSV.Gal.Sp1 (●) or pRSV.Gal.Oct2 (▲). For each TAD, 100% corresponds to the firefly luciferase activity/Renilla luciferase activity (F.Luc/R.Luc) ratio in control cells. The basal activities of Sp1 and Oct2 TADs were 102 ± 9% and 41 ± 4% that of NFI/CTF respectively (see also Figure 5B).
Oxidative repression of nuclear factor I/CCAAT transcription factor

Figure 2 DNA binding of the Gal4/CTF fusion protein

Electrophoretic mobility shift assays were performed with 4 μg of cellular extracts from either non-transfected (NT, lane 1) or pRSV.Gal.CTF-transfected HepG2 cells (lanes 2–5) and the Gal4 probe. In addition, cells were left untreated (lanes 1–4) or treated with 50 μM H2O2 for 1 h before harvest (lane 5). For competition experiments, a 60-fold excess of either unlabelled ‘NFI’ or ‘Gal4’ oligonucleotide was used (lanes 2 and 3 respectively). The arrow indicates a specific fusion-protein–DNA complex.

oxidative stress [14]. This was explained by a decrease of the DNA-binding activity of Sp1, but our data suggest that the regulation of the TAD of Sp1 could also be an important mechanism. The TAD of the transcription factor Oct2 was not regulated by H2O2 at the concentrations used in this study, showing that the regulation is not a general phenomenon. Thus the sensitivity of the TADs to oxidative stress displayed the following order: NFI/CTF > Sp1 > Oct2.

In our experiments, no endogenous HepG2 protein was able to bind the Gal4 DNA sequence (see Figure 2, lane 1). On the contrary, in cells transfected with the pRSV.Gal.CTF expression plasmid, we observed the formation of one specific complex. In competition experiments, this complex was displaced by an excess of unlabelled probe (Figure 2, lane 3) but not by an excess of an unrelated oligonucleotide containing an NFI-binding site (Figure 2, lane 2). In addition, we have observed that the DNA binding of the Gal4 fusion protein was only slightly modified by 50 μM H2O2 (see Figure 2, lanes 4 and 5). The amount of complex formed in H2O2-treated cells was 9% lower than in control cells (mean from three independent experiments). This limited decrease can not account for the drastic repressive effect of 50 μM H2O2 on the activity of the TAD of NFI/CTF as assessed in transfection assays and is in agreement with the lack of regulation of the TAD of the Oct transcription factor.

The N-terminal part of the TAD is required for the regulation by H2O2

To localize the subregion of the TAD that mediates the effect of H2O2, various deletions were performed (as shown in Figure 3). The transcriptional activity of these truncated TADs and their responsiveness to H2O2 were investigated. The C-terminal part of the TAD (amino acids 486–499) displays transcriptional activity but it is less active than the whole TAD. This region has been described previously as a TRD [9]. It is noticeable that it is not sensitive to H2O2 (compared with the intact TAD). Surprisingly, the 438–499 fragment did not display transcriptional activity in our experiments. This suggests that fragment 438–486, which comprises the proline-rich region, has an inhibitory effect on the activity of the TRD. This inhibitory effect seems to be relieved by the adjunction of the N-terminal part of the TAD, which accounts for the high activity of the whole TAD. The N-terminal region (399–438) alone displays only a weak activity just above the background level. Under these conditions, it is difficult to assess the effect of the H2O2 treatment on this region.

This experiment shows that the C-terminal TRD domain of the TAD is active on its own and is not sensitive to oxidative stress. However, within the context of the whole TAD, it requires the N-terminal region to reach maximal activity and this syn-
A Figure 4  Effect of amino acid mutations in the TAD of NFI/CTF on the H$_2$O$_2$ effect

Cells were co-transfected with pG5-FL as a reporter vector, various Gal4-fusion expression vectors and pAglob-RL as an internal control. Cell cultures were treated (grey bars) or not (white bars) with 50 μM H$_2$O$_2$ and harvested 16 h later. Firefly and Renilla luciferases were assayed as described in the Materials and methods section. Results were expressed as firefly luciferase activity/Renilla luciferase activity (F.Luc/R.Luc; mean ± S.E.M., n = 8). 100% corresponds to the F.Luc/R.Luc ratio in control cells transfected with pRSV.Gal.CTF (containing the wild-type TAD). For each fusion protein, statistically significant differences between cells treated with H$_2$O$_2$ and the corresponding untreated controls are shown by * (P < 0.01) or ** (P < 0.0001). (A) Effect of the mutation of the cysteine residues. As a control, cells were not transfected with any fusion-protein expression vector (two left-most bars). Cells were transfected with either pRSV.Gal.CTF carrying the wild-type (wt) TAD or a TAD containing Cys → Ser mutations as indicated. (B) Effect of the mutation of serine residues. Cells were transfected with either pRSV.Gal.CTF carrying the wild-type TAD or a TAD containing a Ser → Ala mutation as indicated. (C) Effect of the mutation of the tyrosine residues of the TRD domain. Cells were transfected with either pRSV.Gal.CTF carrying the wild-type TAD or a TAD containing a Tyr mutation as indicated.

Cell signalling by oxidative stress is mediated through either direct redox modification or a kinase/phosphatase cascade, we chose to evaluate the contribution of the oxidation-sensitive cysteines and of the residues potentially targeted by kinases or phosphatases. The TAD of NFI/CTF contains two cysteines located at positions 405 and 427. Both were mutated into serines (i.e. a thiol moiety was replaced by a hydroxy moiety). The mutation of Cys-405 decreased the reporter-gene activity by half, suggesting that this residue is important for the transactivating mechanism (Figure 4A). This mutation did not affect the reprogramming is oxidative-stress-sensitive. A possible mechanism accounting for this observation is that an oxidative-stress-driven modification of the N-terminal part of the TAD affects the global conformation of the whole TAD and prevents the interactions of the C-terminal part with other transactivating proteins.

Cys-427 is required to mediate the repression by H$_2$O$_2$

To further characterize the amino acid targeted by H$_2$O$_2$, we tested the activities of TADs carrying point mutations. Since

Figure 5  Effect of NEM on NFI/CTF transactivating function

Cells were co-transfected with pG5-FL as a reporter vector, pAglob-RL as an internal control and various fusion-protein expression plasmids. Cell cultures were treated or not with the indicated concentrations of NEM and harvested 16 h later. Firefly and Renilla luciferases were assayed as described in the Materials and methods section. Results were expressed as firefly luciferase activity/Renilla luciferase activity (F.Luc/R.Luc; mean ± S.E.M., n = 6). 100% corresponds to the F.Luc/R.Luc ratio in untreated control cells transfected with pRSV.Gal.CTF. For each fusion protein, statistically significant differences between cells treated with NEM and the control are indicated by * (P < 0.05) or ** (P < 0.01). (A) Cells were transfected with pRSV.Gal.CTF carrying the wild-type TAD (white bars) or with pRSV.Gal.CTF carrying the Cys → Ser mutation at position 427 within the TAD (grey bars). (B) Cells were transfected with either pRSV.Gal.CTF (white bars), pRSV.Gal.Oct (black bars) or pRSV.Gal.Sp1 (hatched bars).
repression by $H_2O_2$. Conversely, the mutation of Cys-427 did not modify the reporter-gene expression. When both Cys-427 and Cys-405 were mutated, the transcriptional activity was slightly more decreased than in the case of the Cys-405 → Ser mutation alone. The most important observation was that the Cys-427 → Ser mutation totally abolished the repressive effect of $H_2O_2$ (alone or in addition to the Cys-405 → Ser mutation). We conclude that Cys-427 is required for the repressive effect of $H_2O_2$.

We also investigated the possible role of phosphorylation-sensitive residues. Indeed, it has been shown that kinases such as mitogen-activated protein kinase are activated by $H_2O_2$ [15]. An SPXSP amino acid sequence present in the TAD of NFI/CTF (amino acids 461-465) is similar to the consensus sequence recognized by p38 kinase, as in the CHOP/Gadd153 protein [16]. It is also similar to the consensus amino acid target sequence PXSP of several other kinases, including extracellular signal-regulated kinases (ERKs) [17]. We thus mutated Ser-461 and Ser-464 in the TAD. Both mutations increased the basal levels of regulated kinases (ERKs) [17]. We thus mutated Ser-461 and Ser-464 in the TAD. Both mutations increased the basal levels of the reporter-gene expression by approx. 50 % (Figure 4B). However, they had no effect on the negative regulation by $H_2O_2$. We also examined the influence of the mutation of the tyrosine residues located within the transcriptionally active TRD region (Figure 4C). The mutation of Tyr-491 almost completely abolished the transcriptional activity of the TAD, whereas that of Tyr-497 slightly increased its basal level without affecting the negative regulation by $H_2O_2$.

Cys-427 is sensitive to NEM

In order to assess if Cys-427 could be sensitive to thiol-modifying reagents other than $H_2O_2$, we used the thiol-alkylating compound NEM. Cells were exposed to various concentrations of NEM, under the same conditions and in some cases were confirmed in cell-culture systems. However, cysteine residues are present in other functional domains of transcription factors which could also be targeted by oxidative conditions. For example, a cysteine located in the nuclear-localization signal of the glucocorticoid receptor has been shown recently to mediate the inhibition of its nuclear

translocation by oxidative stress [20]. We show that the TAD of NFI/CTF is repressed by oxidative stress and this repression involves the critical redox-sensitive Cys-427. Therefore, the role of cysteine residues within TADs should also be evaluated when the redox regulation of a transcription factor is investigated, all the more so as TADs can display a much greater sensitivity to oxidative stress than DBDs. Indeed, in the case of NFI/CTF, our data suggest that there is a 100-fold difference in sensitivity between the DBD and the TAD. Thus TADs may have a crucial contribution to the redox regulation of gene transcription within a physiological context.

There are several signalling mechanisms that could account for the effects of oxidative stress. In addition to the oxidation of redox-sensitive residues, oxidative conditions can also activate kinase/phosphatase cascades and modify the phosphorylation status of target amino acids. In the present case, several observations show that the likely mechanism involves the oxidation of Cys-427. First, the Cys-427 → Ser mutant is sensitive to neither $H_2O_2$ nor NEM. Thus Cys-405 alone cannot be the target of the repression. Secondly, additional investigations showed that the potentially phosphorylatable residues Ser-461 and Ser-464 located in the TAD are not involved. These residues are located in a sequence [which is similar to the C-terminal domain (CTD) sequence of RNA polymerase II] that was shown to be required for the activity of the TAD [21]. This polypeptide is also a consensus phosphorylation site for p38 kinase and various other kinases that can be activated by $H_2O_2$ [16,17]. Finally, the use of specific kinase inhibitors targeting either the p38 kinase or the ERK pathways (SB 203580 and PD 960598) did not affect the $H_2O_2$ effect on the TAD (results not shown).

Further studies are required to investigate whether the thiol moiety of Cys-427 undergoes an oxidation with a gain of oxygen atoms or if it forms an intra- or intermolecular disulphide bridge. However, the conserved $H_2O_2$ sensitivity of the Cys-405 → Ser mutant suggests that the formation of an intramolecular disulphide bridge within the TAD is not the mechanism most likely to be triggering the $H_2O_2$ effect. The oxidation of Cys-427 could affect the conformation of the TAD and disrupt protein–protein interactions required for the activation of transcription. This cysteine, contrary to Cys-405, seems to be easily modified by either oxidizing or alkylating reagents, suggesting a different exposure of these residues within the TAD protein sequence. Moreover, this TAD has been shown to interact directly with several proteins involved in the control of transcription, such as the TATA-box-binding protein TBP [22], the co-activator CBP/p300 [23] and the histones H1 and H3 [9,24]. This TAD was also shown to enhance synergistically the activation of transcription mediated by the oestrogen receptor [25] and the aryl hydrocarbon receptor (AhR) [10] pathways.

We have already reported that the activity of NFI-driven genes was repressed not only by exogenous $H_2O_2$, but also by glutathione depletion, tumour necrosis factor α or intracellular $H_2O_2$ production [4,10]. Thus physiological ROS concentrations can repress the transactivating function of NFI. We have shown here that Cys-427 is very sensitive to oxidative stress and can therefore mediate the repressive effect of $H_2O_2$. Since the oxidation of a cysteine is rapid and reversible, the oxidation of Cys-427 could be a biological mechanism common to a wide range of conditions that cause an intracellular production of $H_2O_2$, including physiological or exogenous stimuli (reviewed in [11]).

The repression of NFI in case of intracellular oxidative stress could be involved in an adaptive response. Indeed, the cellular-defence mechanisms during oxidative stress comprise the induction of antioxidant enzymes [26] and also the repression of endogenous ROS-producing systems. In this respect, NFI regu-
lation contributes to the latter mechanism by mediating the oxidative repression of expression of the CYP1A1 gene product, an H₂O₂-producing enzyme [10].

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REFERENCES


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