TCF11/Nrf1 overexpression increases the intracellular glutathione level and can transactivate the \(\gamma\)-glutamylcysteine synthetase (GCS) heavy subunit promoter

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Abstract

\(\gamma\)-Glutamylcysteinylglycine or glutathione (GSH) performs important protective functions in the cell through maintenance of the intracellular redox balance and elimination of xenobiotics and free radicals. The production of GSH involves a number of enzymes and enzyme subunits offering multiple opportunities for regulation. Two members of the CNC subfamily of bZIP transcription factors (TCF11/Nrf1 and Nrf2) have been implicated in the regulation of detoxification enzymes and the oxidative stress response. Here we investigate the potential role of one of these factors, TCF11/Nrf1, in the regulation of GSH levels in the cell and particularly its influence on the expression of one of the enzymatic components necessary for the synthesis of GSH, the heavy subunit of \(\gamma\)-glutamylcysteinylglycine synthetase (GCS\(_h\)). Using overexpression of the transcription factor in COS-1 cells we show that TCF11/Nrf1 stimulates GSH accumulation. Using co-transfection with reporter constructs where reporter expression is driven through the GCS\(_h\) promoter we show that this increase may be mediated in part by induced expression of the GCS\(_h\) gene by TCF11/Nrf1. We further show that a distal portion of the promoter including two antioxidant-response elements (AREs) predominantly mediates the TCF11/Nrf1 transactivation and an electromobility shift assay showed that just one of these AREs specifically binds TCF11/Nrf1 as heterodimers with small Maf proteins. We suggest that TCF11/Nrf1 can operate through a subset of AREs to modulate the expression of GCS\(_h\) together with other components of the pathway and in this way play a role in regulating cellular glutathione levels. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The tripeptide \(\gamma\)-glutamylcysteinylglycine or glutathione (GSH) is a ubiquitous cellular non-protein sulphhydryl. GSH has two important roles in the cell: maintaining the intracellular redox balance and eliminating xenobiotics and free radicals [1]. GSH is synthesised from amino acids in two enzymatic reactions catalysed by \(\gamma\)-glutamylcysteine synthetase (GCS) and glutathione synthetase [2]. The reaction catalysed by GCS is the rate-limiting step in the de novo synthesis of GSH, so that regulation of GCS levels is likely to be an important aspect in the regulation of GSH synthesis [3]. GCS consists of two subunits, the heavy subunit (GCS\(_h\)) with catalytic activity and the light subunit (GCS\(_l\)) with regulatory activity [4-6]. Regulation of enzyme activity occurs through multiple mechanisms affecting one or both subunits and the heavy subunit in particular is regulated by both transcriptional and post-transcriptional mechanisms [7].

Transcriptional control of GCS\(_h\) is mediated by a region spanning approx. 5 kb of the 5’ flanking sequence of the GCS\(_h\) gene [8]. Analysis of this region by Mulcahy et al. revealed the presence of response elements including several AP-1 sites, antioxidant-response elements (AREs; also known as the electrophile-response element EpRE) and one NF-κB site. Similar ARE motifs are also found in promoters of other genes that participate in the defence against free radicals and toxic insults, and this has led to the definition of a consensus core ARE motif (A/GTGAC/GnnnGCA/G). Among such genes are NADPH:quinone oxidoreductase [9], glutathione S-transferase [10] and UDP-glucuronosyltransferase [11]. Many of these genes
are induced through AREs in response to chemical or oxidative stress [12].

The transcription factor TCF11/Nrf1 (also known as LCR-F1) is a member of the CNC subfamily of bZIP transcription factors, most closely related to Nrf2, Nrf3 and p45-NF-E2 [10,13–16]. Like the other members of this group, TCF11/Nrf1 can heterodimerise with small Maf transcription factors to more efficiently bind DNA target sequences [17]. We originally showed that TCF11/Nrf1 can bind to the same target sequence as p45 NF-E2 in vitro (the NF-E2 site), both alone and in the presence of small Maf proteins [17]. We have further demonstrated transactivation of a reporter gene through this site when TCF11/Nrf1 is transfected alone while the transactivation is inhibited when MafG is co-transfected in COS-1 cells [18]. In order to investigate differences in specificity between the closely related CNC DNA binding factors we used a selection approach to identify the optimal binding sites for TCF11/Nrf1. We found a clear consensus binding site in the presence of small Maf, which matches both the NF-E2 site and the ARE motif. We therefore postulated that genes stimulated in response to oxidative and other forms of stress through AREs might be targets for TCF11/Nrf1 transcriptional regulation [18]. In fact, more recent evidence suggests that both TCF11/Nrf1 and the closely related CNC factor Nrf2 are involved in the regulation of expression of a number of phase II detoxifying enzymes. Both genes were found to positively regulate the expression of the NAD(P)H:quinone oxidoreductase gene [19]. Interaction between Nrf1 or Nrf2 and Jun proteins has also been described and appears to coordinately induce at least two genes involved in detoxification of xenobiotics, with Nrf2 having a significantly stronger effect [20]. Recently it has also been shown that Nrf2 is a positive regulator of ARE dependent GCSb and GCSi subunit gene expression in cells exposed to β-naphthoﬂavone and pyrrolidinedithiocarbamate [21]. More direct evidence of a role for these two factors in regulating stress response comes from the genetic manipulation of the tcf11/nrf1 and nrf2 genes in mice: null mutants for Nrf2 respond poorly in phenolic antioxidant induced expression of NAD(P)H:quinone oxidoreductase [22] and tcf11/nrf2 mutants show reduced protection against the effects of oxidants, reduced levels of glutathione and reduced induction of the GCSi gene following exposure to paraquat [23].

To investigate whether TCF11/Nrf1 may regulate the cellular glutathione level and in particular expression of the GCSb gene, we performed a series of transfection experiments transiently expressing TCF11/Nrf1 in COS-1 cells. We found that such expression led to a signiﬁcant increase in cellular glutathione. When expressed in the presence of reporter constructs, we showed that TCF11/Nrf1 transactivates the GCSb promoter and that this transactivation is predominantly mediated through the distal part of the GCSb promoter. Our data further implicate TCF11/Nrf1 in the regulation of antioxidant and detoxification enzymes and show for the ﬁrst time that its inﬂuence may be exerted in part through GCSb gene regulation.

2. Materials and methods

2.1. Cell culture

COS-1 cells, purchased from the American Type Culture collection, were grown in Dulbecco’s modiﬁed Eagle’s medium (DMEM) supplemented with 2 mM l-glutamine, 5 units/ml penicillin, 50 μg/ml streptomycin and 10% foetal calf serum (COS-1 medium). The cell cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C.

2.2. Plasmid constructs

The complete GCSb-luc reporter construct (−3802/GCSb-luc) was kindly provided by Mulcahy et al. [8]. Briefly, the construct was obtained by cloning a 4.2 kb fragment from the 5’ ﬂanking region of the human GCSb gene into HindIII-NeoI sites of the pGL3 basic vector (Promega).

The distal GCSb-luc construct (−3258−2946/GCSb-luc) was obtained by ampliﬁcation of a 312 bp fragment containing ARE 3 and ARE 4 of GCSb from human genomic DNA by PCR using primers 1 and 2 (1: 5′-ACT GCG GCA ATC CTA GCA GC-3′ and 2: 5′-AAG CTT CTG GAC CGT GGA GAT CC-3′). The PCR product was cloned into the pCR 2.1-TOPO vector (Invitrogen, USA). This plasmid was digested with HindIII and the resulting fragment was ligated into the HindIII site of a TATA luc vector [24].

The proximal GCSb-luc construct (−2752/GCSb-luc) was obtained by digestion of the recombinant −3802/GCSb-luc plasmid with SacI. The resulting fragment was isolated and religated.

The expression construct was obtained by cloning a full-length TCF11/Nrf1 cDNA (clone pZeEA20, up to the EcoRV site in the 3’ non-coding sequence at bp 3550) into the expression vector pcDNA3 (Invitrogen).

MBP-TCF11/Nrf1-α, a fusion protein between Escherichia coli maltose binding protein (MBP) and the 300 C-terminal amino acids of TCF11/Nrf1, and the two MBP-Maf fusions, in which MBP was fused to chicken MafG or MafK, were prepared as previously described [17]. pRSVCAT is also described previously [17].

2.3. Transient transfection of COS-1 cells

The cells were plated in 35 mm tissue culture wells the day before transfection at a density of approx. 60% confluence and transfected using a standard dextran-chloroquine method as described previously [25] with DNA.
concentrations as indicated in the results. Two hours after transfection the cells were subjected to a DMSO shock (Sigma, St. Louis, MO, USA) (10% in PBS), after which they were incubated overnight in COS-1 medium. COS-1 cells were also transfected using a standard calcium phosphate method and a total amount of 10 μg DNA as previously described [18], including CAT as a control plasmid for transfection efficiency.

2.4. Luciferase activity measurements

Luciferase activity was measured in the cell lysates according to the manufacturer’s protocol (Promega). Briefly, the cells were washed in PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup> prior to the addition of 300 μl lysis buffer (Promega). The cells were incubated at room temperature for 20 min before removal by scraping and subsequent vigorous vortexing. The lysate was then centrifuged to remove cell debris. Luciferase activity was measured by adding 100 μl luciferase assay solution (Promega) to 20 μl of the lysate and the luminescence was detected in a TD 20/20 luminometer (Turner Design, Sunnyvale, CA, USA). Protein concentration was measured in the cell lysate using Coomassie brilliant blue reagent from Bio-Rad (CA, USA).

2.5. Electrophoretic mobility shift assay

Bacterially expressed MBP-TCF11/Nrf1-A (25 ng) alone or together with either MBP-MafG (2 ng) or MBP-MafK (2 ng) were used in an electrophoretic mobility shift assay (EMSA) essentially as described previously [18], with the exception that <sup>32</sup>P-labelled DNA probes were purified using a spin column (Bio-Rad).

2.6. GSH measurements

Cells were harvested in a phosphate buffer (10 mM, pH 6) containing 10 μM EDTA and thereafter lysed by several freeze-thaw cycles. Proteins were precipitated with approx. 2% TFA and the resulting supernatant was neutralised to pH 2 with 85% phosphoric acid and acetonitrile (Rathburn, Walkerburn, UK) in a ratio of 49:1. GSH (Sigma) stock solution (1 mg/ml) was made weekly in the mobile phase containing 1 mM EDTA (Sigma). Standard curves were made daily in the range of 30–650 μM in the same buffer used for analyses of cell lysates. The injection volume was 10 μl for all analyses. Data were collected and analysed with HP Chem Station software.

3. Results

3.1. TCF11/Nrf1 increases the glutathione level in transfected cells

To investigate the potential role of TCF11/Nrf1 in regulating GSH production, we measured the level of GSH in COS-1 cells following transfection of the TCF11/Nrf1 expression construct. Thirty-six hours post transfection the cells were harvested and the reduced glutathione level was measured by HPLC. We found that when 1 μg of Nrf1 expression plasmid was transfected per 35 mm culture well the glutathione level was significantly increased approx. 24% compared to empty expression vector pcDNA3 (Table 1). This shows that TCF11/Nrf1 can activate the GSH synthesis pathway. The effect, however, could be mediated by influencing the expression of one or more of several components of the pathway including GCS<sub>a</sub>, GCS<sub>b</sub> and glutathione S-transferase. In fact both GCS<sub>a</sub> and glutathione S-transferase were shown to be downregulated in Nrf1−/− fibroblasts [23]. We wished to further explore the possibility that TCF11/Nrf1 can also regulate the GCS<sub>b</sub> gene.

3.2. TCF11/Nrf1 transactivates a GCS<sub>b</sub> driven reporter

TCF11/Nrf1 has been shown to bind to sequences often found in the promoter region of genes involved in cellular stress response [17,19,20,26]. To test the possibility that expression of GCS<sub>b</sub> is regulated by TCF11/Nrf1, COS-1 cells were co-transfected with a luciferase reporter construct including 4.2 kb of the 5′ flanking region of the

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<td>Increased glutathione level in COS-1 cells expressing TCF11/Nrf1</td>
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<tr>
<td>Amount DNA (μg)</td>
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<tr>
<td>pcDNA3 vector (control)</td>
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<tr>
<td>TCF11/Nrf1 expression vector</td>
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GSH is expressed as μmol/g protein. Data are presented as mean ± S.D. of three experiments each performed in duplicate. * indicates that there is a statistically significant difference between empty control vector and Nrf1 expression vector (P<0.05), as determined using a two-tailed Student’s t-test.
GCSh gene (Fig. 1) and a TCF11/Nrf1 expression plasmid. The cells were transfected with a constant amount of the complete GCSh-luc reporter (0.5 μg) together with 0.1 μg or 0.5 μg TCF11/Nrf1 expression plasmid or the same amount of the empty expression vector pcDNA3 per 35 mm culture well. Thirty-six to 40 h post transfection the luciferase activity in cell lysates was measured. A 2.7-fold increase in luciferase activity was observed at the highest concentration of the TCF11/Nrf1 expression plasmid used (Fig. 2). The same results were obtained when cells were transfected using calcium phosphate and including CAT as internal control for transfection efficiency. TCF11/Nrf1 was also able to activate transcription through the complete GCSh promoter in HepG2 cells although the effect was lower in HepG2 cells compared to COS-1 cells (data not shown).

As outlined in Fig. 1, the 4.2 kb GCSh promoter construct contains several response elements that could mediate the transactivation observed in Fig. 2. These include four ARE motifs, two located at the distal end of the promoter (ARE3 and ARE4), and two at the more proximal end (ARE1 and ARE2). ARE3 and ARE4 completely match the consensus ARE sequence while ARE1 and ARE2 show differences in the first and last positions, respectively. None of the AREs perfectly match the consensus described for TCF11/Maf [18], but ARE4 differs only in the flanking base pairs whereas all the others show differences in the core sequences. To further define the part of the GCSh promoter through which TCF11/Nrf1 exerts its effect, we produced two reporter constructs where luciferase is driven either from the proximal part of the promoter containing ARE1 and ARE2 (proximal GCSh-luc, Fig. 1) or from the distal promoter containing AREs 3 and 4 (distal GCSh-luc, Fig. 1). As shown in Fig. 3, we found that the construct containing the distal AREs was induced 2.3-fold following transfection with 0.1 μg TCF11/Nrf1 expression construct whereas the proximal promoter was not clearly induced. Taken
together, our results show that Nrf1 induces the GCS<sub>n</sub> promoter and that this induction may be mediated predominantly through the distal part of the promoter.

### 3.3. TCF11/Nrf1/MafG heterodimers bind specifically to ARE4

The luciferase induction we observed through the distal region of the promoter (Fig. 3) together with a previous report indicating that ARE4 is particularly important in GCS<sub>n</sub> regulation, prompted us to measure the binding of TCF11/Nrf1 to the distal ARE motifs. TCF11/Nrf1 heterodimerises with small Maf bZIP proteins and such dimers are known to bind to DNA more efficiently than TCF11/Nrf1 alone [18]. EMSA was therefore used to measure the binding of TCF11/Nrf1 to ARE3 and ARE4 both in the absence and presence of the small Maf proteins, MafG and MafK.

Using a 66 bp oligonucleotide (pARE34, Table 2) containing both ARE3 and ARE4 as a probe, neither MBP-TCF11/Nrf1 nor MBP-Maf fusions show any binding as homodimers in EMSA (Fig. 4A, lanes 2–4). Such inability to bind a probe in vitro has previously been shown with a...
single NF-E2 site [17]. To test whether TCF11/Nrf1-small Maf heterodimerisation would enhance protein binding to the different ARE sites we performed EMSA with bacterially synthesised TCF11/Nrf1 and Maf fusion proteins. As heterodimers, both the combinations of TCF11/Nrf1/MafG and TCF11/Nrf1/MafK show enhanced protein binding to the pARE34 probe (Fig. 4A, lanes 5 and 6).

To further determine whether there were any differences between the binding affinities of the TCF11/Nrf1/MafG heterodimer to the different ARE elements, two 33 bp oligonucleotides each containing one ARE element (named pARE3 and pARE4, Table 2) were used as competitors in EMSA. By including an excess of unlabelled pARE3 or pARE4 (Fig. 4B, lanes 3 and 4) it is shown that only the probe with the response element ARE4 is able to compete out the shift formed by heterodimerisation of TCF11/Nrf1 and MafG to the pARE34 probe. Unlabelled pARE3 used as a competitor does not seem to have any effect on the shift. To show that it was the ARE4 element that was responsible for competing out the shift, a new 33 bp oligonucleotide with a mutation in the TCF11/Nrf1 half-site of the ARE4 response element (named pARE4m, Table 2) was used as a competitor (Fig. 4B, lane 5). This oligonucleotide was not able to interfere with the shift.

This indicates that the TCF11/Nrf1/MafG heterodimer has a higher affinity for ARE4 when both ARE3 and ARE4 are present. To further confirm this, probes with only a single ARE element were labelled. Using pARE3 as the labelled probe, no detection of heterodimeric binding was observed (Fig. 4C, lane 2). Using pARE4 as the probe, a shift with the TCF11/Nrf1/MafG heterodimer was obtained (Fig. 4C, lane 4). This shift was competed out when an excess of unlabelled pARE4 was added (Fig. 4C, lane 6). On the contrary, adding an excess of either unlabelled pARE3 or pARE4m did not interfere with the shift (Fig. 4C, lanes 5 and 7). Using pARE4m as the labelled probe, no heterodimeric binding was observed, strongly suggesting that this single base pair mutation is enough to completely abolish TCF11/Nrf1/MafG binding to the response element. These results establish that TCF11/Nrf1/Maf binds specifically to the distal element ARE4 in the GCS1 promoter and that heterodimeric binding to ARE3 does not occur in vitro. Mulcahy et al. have
shown that both constitutive and β-naphthoflavone-induced GCSₙ expression was dependent upon the same distal ARE4 site in the promoter [8].

4. Discussion

Expression of TCF11/Nrf1 in COS-1 cells led to an increase in the intracellular glutathione level. Recently, it was reported that fibroblasts from Nrf1 knockout mice showed a decreased level of glutathione and enhanced sensitivity to oxidants as compared to cells from wild type mice [23]. In the Nrf1 knockout fibroblasts the GCSₙ mRNA level was reduced, whereas the GCSₙ mRNA level was not noticeably changed. Our data suggest that TCF11/Nrf1 can also transactivate the GCSₙ promoter since we show a clear but low level transactivation of the GCSₙ promoter in transfection assays. Kwong et al. did not find a detectable difference in GCSₙ levels by Northern blot analysis in cells from Nrf1 null mice whereas GCSₙ levels were induced to a lesser extent as compared to wild type cells following stress induction. These observations may be reconciled if TCF11/Nrf1 has a relatively moderate influence on the GCSₙ locus. Alternatively, Nrf2 or other transcription factors may be able to compensate for the loss of TCF11/Nrf1 at the GCSₙ locus whereas it/they cannot do so at the GCSₗ locus.

The transactivating effect of TCF11/Nrf1 on the GCSₙ promoter was largely mediated through the distal portion, where two response elements (ARE3 and ARE4) that perfectly match the ARE consensus sequence, are located. Yet only ARE4 was able to bind TCF11/Nrf1/MafG heterodimers in EMSA analyses. Therefore the presence of consensus ARE motifs in the GCSₙ promoter, or elsewhere, does not necessarily constitute a binding site for TCF11/Nrf1. This is in accordance with the findings of Johnsen et al. [18] where the optimal TCF11/Maf binding site was found to be TGCTgaGTCAt, with GTCAT representing the TCF11/Nrf1 half-site. This is a more limited definition of a response element than the ARE sequence and a better indication of a possible site for TCF11/Nrf1 binding. It further emphasises that not all AREs are qualitatively equal; TCF11/Nrf1 can bind to a subclass of ARE elements while other potential AREs, if functional, may be regulated by other factors or other combinations of factors. A single base mutation in the ARE4 core to TGCTgaGTCCₗ totally abolished the binding of Nrf1/MafG in our EMSA analyses. The same single base mutation in the ARE4 sequence diminished both constitutive and β-naphthoflavone induced expression of GCSₙ [8]. The ARE4 element may therefore represent a key regulatory element in the regulation of GCSₙ.

The GCSₙ promoter contains four ARE motifs in addition to two AP-1 sites and one NF-xB site [8]. This indicates that a tight regulation of the GCSₙ level, including positive and negative influences, may be important. The limited response to TCF11/Nrf1 we observe may be due to this type of complex regulation. The GCSₗ promoter has so far only one identified ARE element [27], which again might indicate that a tight regulation through ARE elements is of less importance for this gene. Indeed, it has been shown that the individual levels of mRNA for the two subunits vary between different tissues [28,29].

Nrf2 can bind to and transactivate through ARE sites in both the heavy and the light chain GCS promoters [21,30]. Nrf1 was found to be present (or detected) in nuclear extracts capable of binding the ARE element in the GCSₙ promoter, although Nrf2 was more prominent [30]. Both small Mafs and JunD are also implicated in the regulation of the GCS genes [21,30]. The relatively large number of possible bZIP dimer combinations may therefore contribute to a flexible but tightly regulated response system of the GCS genes. We propose here that the low, but significant level of transactivation by TCF11/Nrf1 through the GCSₙ promoter is important to achieve the right level of the GCS protein complex in the cells.

A common function of the CNC bZIP factors TCF11/Nrf1 and Nrf2 may be the regulation of the cellular response to chemical and oxidative stress mediated via ARE motifs. Yet, it is clear that the two transcription factors do not have entirely redundant functions, as they cannot compensate for each other in knockout models [22,23,31], both mutants suffering from reduced protection against oxidative stress. Based on our findings we propose that TCF11/Nrf1 plays a role in the regulation of GSH synthesis. GSH is, however, the end point in a complex metabolic pathway involving numerous proteins, including GCSₙ and glutathione synthetase as well as GCSₗ. We therefore suggest that TCF11/Nrf1 has the ability to activate transcription of several genes including GCSₙ, and that regulation of the cellular antioxidant defence system may be mediated, at least in part, through regulation of TCF11/Nrf1 activity.

References


