GASTROINTESTINAL CANCER

Gastroprotective peptide trefoil factor family 2 gene is activated by upstream stimulating factor but not by c-Myc in gastrointestinal cancer cells

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Background: Damage to the gastrointestinal mucosa results in the acute up-regulation of the trefoil factor family peptides TFF1, TFF2, and TFF3. They possess protective, healing, and tumour suppressive functions. Little is known about the regulation of *TFF* gene expression. The promoters of all three *TFF* genes contain binding sites (E box) for upstream stimulating factor (USF) and Myc/Max/Mad network proteins.

Aims: To determine the nature and function of transcription factors that bind to these E boxes and to understand their role for *TFF* gene expression.

Methods: TFF promoter activities were determined by reporter gene assays. DNA binding was monitored by electromobility shift assays and by chromatin immunoprecipitation analyses. Expression of endogenous *TFF* was determined by multiplex RT-PCR.

Results: It was observed that the *TFF2* promoter is specifically and efficiently activated by USF transcription factors but not by c-Myc. USF displayed comparable binding to a high affinity Myc/Max binding site compared with the three *TFF* E boxes, while c-Myc exhibited lower affinity to the *TFF* E boxes. In contrast, pronounced binding differences were observed in cells with a strong preference for USF to interact specifically with the *TFF2* E box, while Myc was not above background. Exogenous expression of USF was sufficient to activate the chromosomal *TFF2* and to a lesser extent, the *TFF1* gene.

Conclusion: These findings define USF factors as regulators of the *TFF2* gene and suggest that promoter specific effects are important for a pronounced gene activation of this cytoprotective peptide.

The three members of the trefoil factor gene family (*TFF1*, *TFF2*, and *TFF3*) encode secreted peptides (formerly designated pS2, SP, and ITF) characterised by three loop trefoil domains and expressed in specific epithelial cells of the gastrointestinal tract.¹ In vitro and in vivo studies showed that TFFs protect epithelia against experimentally induced mucosal damage.² In addition, TFFs stimulate cellular motility, promote mucosal defence and wound healing, and inhibit tumour cell proliferation.^{1,3} TFF2 is expressed early in response to experimentally induced ulceration in animals and has been proposed to be the principal cytoprotective trefoil peptide.^{4,5} The findings summarised above are supported by the analysis of the corresponding knock-out animals. *Tff1*^{-/-} mice show aberrant gastric mucosa and develop gastric carcinoma⁶ whereas *Tff3*^{-/-} mice exhibit impaired intestinal defence.⁷ Recently *Tff2*^{-/-} mice were demonstrated to possess a decreased thickness and proliferation rate of the gastric mucosa and an increased sensitivity to indomethacin induced ulceration.⁸

Human TFFs are up-regulated near sites of damaged mucosa in ulcerative conditions and in a variety of gut cancers.^{4,9} Interestingly the expression of *TFF1* and *TFF2* is down-regulated in intestinal metaplasia and many gastric carcinoma.¹⁰ A role in anti-proliferation of TFF1 and TFF3 has also been supported by genetic analyses.^{3,6} In addition the three human *TFF* genes are clustered in a region on chromosome 21q22.3 that is frequently deleted in gastric cancer.^{9,11} This suggests that TFFs may have tumour suppressor activity.

The regulation of *TFF* gene expression is of considerable interest in respect to the above described functions of TFF peptides. We recently demonstrated activation of the three human *TFF* genes by the transcription factor HNF-3 and

Figure 1 Schematic representation of the promoters of the three *TFF* genes. The locations and sequences (deviation from the consensus are underlined) of E box elements in relation to the core promoters are given. Binding sites for C/EBP factors (C), HNF-3 (H), and GATA-6 (G) are indicated. The TATA box in TFF3 is CATAAA.

GATA-6,^{12,13} conserved factors known to potentiate the competence of gastrointestinal differentiation and development.¹⁴ In addition we noticed E box elements in all three *TFF* promoters⁹ (fig 1). These are recognised by several different transcriptional regulators, including members of the Myc/Max/Mad network and upstream stimulating factor (USF) that belong to the family of basic region/helix-loop-helix/leucine zipper factors.¹⁵ A considerable amount of data links

Abbreviations: USF, upstream stimulating factor; TFF, trefoil factor family; ChIP, chromatin crosslinking and immunoprecipitation; EMSA, electrophoretic mobility shift assay

Figure 2 USF factors activate a *TFF2-luc* reporter gene construct. (A) The gastrointestinal cell line MKN45 was cotransfected with the *TFF2-luc* reporter gene construct and indicated amounts of expression plasmids encoding USF1, USF2, USF1/USF2, or c-Myc. (B) As in (A) but the cell line LS174T was used. (C) MKN45 cells were cotransfected with wild type (wt) or E box mutated (mut) *TFF2-luc*, with (+) or without (-) expression plasmids encoding USF1 or a mutant USF protein that cannot bind DNA (m). (D) As in (A) but the M4-mintk-luc reporter gene construct with four high affinity Myc/Max binding sites was used. (E) As in B but with the M4-mintk-luc reporter gene.

the Myc/Max/Mad network to the control of cell behaviour.¹⁶ In particular Myc proteins are strongly associated with proliferation and with tumour development both in humans and in animal model systems.¹⁷ The ubiquitously expressed USF1 and USF2 have also been implicated in the control of cellular proliferation.^{18, 19} However unlike Myc, USF proteins have been linked to growth inhibition. In particular transformation of primary cells by c-Myc and an activated Ha-Ras is repressed by both USF1 and USF2.²⁰ Furthermore partial or complete loss of USF transcriptional activity is a common event in breast cancer cell lines.²¹ Thus Myc/Max and USF/USF complexes may antagonise each other at least in part by competing for the same DNA binding elements.

Our aim was to determine whether the expression of *TFF* genes is regulated through their E box DNA elements by Myc or USF proteins. We observed that USF, but not c-Myc, activated the *TFF2* gene in gastrointestinal cells and bound to the *TFF2* promoter in cells.

METHODS

Cell lines and reporter plasmids

The gastric adenocarcinoma cell line MKN45, the colon adenocarcinoma cell lines LS174T and HT-29, and COS-7 cells were cultivated as described.^{22, 23} Reporter gene constructs were generated by cloning the promoter regions of human *TFF1* (position -1100 to +38), *TFF2* (position -821 to +61), and *TFF3* (position -867 to +63) in front of luciferase genes.²² Site directed mutagenesis eliminated the *TFF2-luc*¹³ E box (from 5'-TGCACTGGC to 5'-TGCA-G-GGC) resulting in mE-TFF2-luc. The *M4-mintk-luc* reporter gene has been described previously.²⁴ A renilla luciferase reporter gene (pRL-CMV, Promega) was used to standardise for transfection efficiency.

Figure 3 Effects of USF1 on *TFF1-luc* and *TFF3-luc*. MKN45, LS174T, and HT-29 cells were cotransfected with the indicated TFF reporter gene constructs in the presence or absence of USF1. The fold induction by USF1 is shown.

Transient transfection assays

Transient transfections were performed as described before.²² For cotransfection experiments, 320 ng of reporter plasmid and the indicated amounts of the following expression plasmids were used: pUHD-USF1 and pUHD-USF1mutbr²⁵; psvUSF2 was kindly provided by M Sawadogo.²⁰ Expression

plasmids for c-Myc (pCMV-hu-c-myc), Mad1 (pCMV-mad1) and Maxp22 (pSP-maxp22) have been described previously.²³ For the analysis of endogenous *TFF* expression, cells were seeded on 24 well plates, transfected with 1 µg of plasmids encoding USF1 and USF2 and total RNA was prepared 48 hours later. Multiplex PCR with *TFF1*, *TFF2*, *TFF3*, and GAPDH primer pools were performed as described.^{13, 26} Transient transfection of COS-7 cells were performed as described.²³

Chromatin crosslinking and immunoprecipitation (ChIP)

ChIP assays were performed as described previously.^{27, 28} The following antibodies were used: anti-c-Myc (N-262), anti-Max (C-19), anti-c-Myb (H141), anti-USF-1 (C-20), anti-USF-2 (C-20) (all from Santa Cruz), and polyclonal sera specific for USF1 or USF2 obtained from M Sawadogo.

The following PCR primers were used:

hTFF1-f: 5'-GGCCTCTCAGATATGAGTAG,
hTFF1-r: 5'-TCCTCTGAGACAATAATCTCC,
hTFF2-f: 5'-TGTGGTCCCTGCCCACTC,
hTFF2-r: 5'-TCTCCCTGCTCGGTGATAC,
hTFF3-f: 5'-GGCTCTCTTGTCATGGGAC,
hTFF3-r: 5'-AAGCGTAAGGGCGGATTC,
hADA-1: 5'-CCCTCCTCCTTTTGTCTTCCTG,
hADA-2: 5'-GAAACTCAGTCTCCTTTGTCCCC,
ODC-h1: 5'-GAGCAGAGCGCACCGGATCA,
ODC-h2: 5'-CAGTACCTCGTGCCCGAGAGC,
eIF2α-h1: 5'-TTCTCGGAGGACCCAGACTCTATG,
eIF2α-h2: 5'-TCACAGAGACCAGACTTGCTTCCC.

Electrophoretic mobility shift assay (EMSA)

EMSA were performed as described²³ using the following double stranded oligonucleotides:

CMD: 5'-TCAGACCACGTGGTCGGG,
TFF1: 5'-GATGACCTCACCACATGTCGTCTC,
TFF2: 5'-CAGACCTGCACGTGGCCGGTTTTC,
TFF3: 5'-CTGCCACCCACATGGCTCCTGCAC.

RESULTS

Activation of *TFF* reporter genes by USF

In gastric MKN45 cells, in colonic LS174T cells, and in several other gastrointestinal cell lines a strong activation of a *TFF2* reporter gene construct by USF was observed whereas c-Myc showed little activity (fig 2A and B and data not shown). In both cell lines USF1 activated less efficiently than USF2 while coexpression of both USF proteins resulted in higher activation than either factor alone. This activation was specific as mutation of the E box strongly decreased activation (fig 2C). Furthermore, a mutant of USF1 that cannot bind DNA did not activate the *TFF2* reporter (fig 2C).

To determine whether the difference between the activation of *TFF2* by USF and by c-Myc was promoter specific or cell line dependent, we measured the activity of these factors on M4-mintk-luc, a reporter gene with 4 high affinity Myc/Max binding sites (equivalent to the CMD oligonucleotide, see below). In MKN45 cells activation by USF and c-Myc was comparable while in LS174T cells USF was more active than c-Myc (fig 2D and E). This suggested that c-Myc is capable of activating transcription to an extent that is comparable to other cell systems (see, for example, Sommer *et al*²⁴) and that strong activation by USF appears specific for the *TFF2* promoter.

In addition to *TFF2* we tested whether the two other *TFF* promoters were responsive to USF and Myc/Max/Mad proteins. Moderate activation of the *TFF3* reporter gene was

Figure 4 Binding of USF, c-Myc/Max, and Max/Max complexes to *TFF* derived E box sequences. (A) Binding of COS-7 derived USF1/USF1 homodimers to radiolabelled CMD was competed with a 3-fold, 10-fold, 30-fold, or 100-fold excess of unlabelled CMD, or oligonucleotides spanning the E boxes of either *TFF1*, *TFF2*, or *TFF3* as indicated. Supershift experiments were performed with an antibody specific for USF1 (α-USF1). Control: extract of mock transfected cells was used. * Non-specific complex. (B) Binding of COS-7 derived c-Myc/Max and Max/Max complexes were analysed as in (A). Supershift experiments were done with specific antibodies to c-Myc or Max (α-Myc or α-Max, respectively). The control is as in (A). * Non-specific complex.

observed in three different cell lines (MKN45, LS174T, and HT-29) with USF1 while the *TFF1* reporter gene was not activated (fig 3). c-Myc activated neither the *TFF1* nor the *TFF3* reporter gene (data not shown). Furthermore, other components of the Myc/Max/Mad network, including Max and Mad1, did not affect expression of any of the *TFF* reporter genes (data not shown). Together these findings identify USF proteins as potential activators of the *TFF2* gene.

Distinct reporter gene activation is not attributable to differences in DNA binding

The difference in regulation of the three *TFF* reporters by USF and Myc/Max/Mad network members might be the result of distinct binding to the respective E boxes. Therefore binding of USF1/USF1, c-Myc/Max, and Max/Max complexes derived from COS-7 cells to CMD was compared in EMSA.²³ Relative binding was determined in competition experiments that revealed comparable binding affinities of USF to the three *TFF* E box sequences whereas binding to CMD was slightly stronger (fig 4A). Thus DNA binding is unlikely to be the reason for the differences in *TFF* reporter gene and M4-mintk-luc activation. c-Myc/Max and Max/Max complexes bound less efficiently to any of the three *TFF* derived E box elements than CMD (fig 4B). Therefore weak binding of these complexes might explain in part the inability of c-Myc to activate the *TFF* reporter genes.

Figure 5 Binding of USF to the *TFF2* promoter in cells. (A) ChIP analyses were performed on lysates of formaldehyde crosslinked HT-29 and MKN45 cells. One of 20 of the purified DNA from individual immunoprecipitations was subjected to PCR analysis (34 cycles). Fourfold dilution series of purified input DNA served as normalisation controls for the PCR reactions. (B) Quantification of ChIP reactions from HT-29 cells. Product intensities of samples and adequate twofold dilution series of purified input DNA were compared. Relative DNA binding defines the amount of PCR product obtained from immunoprecipitates compared with total input DNA with the amount of PCR product equivalent to 80 pg arbitrarily set as 1. The graph shows the mean value of two independent determinations. Insert: Lower amounts of input DNA were used to determine c-Myc and c-Myb binding equivalents (39 cycles). (C) ChIP analyses were performed as described under (A).

The *TFF2* promoter is occupied and regulated by USF but not c-Myc

To determine whether endogenous USF is associated with the chromosomal *TFF2* promoter, we performed chromatin immunoprecipitation (ChIP) experiments.²⁸ We observed strong signals for USF1 and USF2 on the *TFF2* promoter while binding to *TFF1* and *TFF3* was considerably weaker (fig 5A). Rough quantification was obtained by comparing the signals from the ChIP with a dilution series of sonicated total input DNA. USF1 binding to *TFF2* was about eightfold higher than binding to *TFF1* and *TFF3* whereas USF2 binding was about sixfold to 10-fold stronger (fig 5A and B). No binding of USF factors was detectable to the locus control region of the ADA

gene,²⁹ which served as control (fig 5A). The binding of c-Myc to either of the three *TFF* promoters was not significantly above the non-specific c-Myb control (fig 5B) despite considerable expression of c-Myc in both cell lines (data not shown). In contrast, comparable binding of c-Myc and USF was observed to the E box of the ODC promoter and c-Myc binding was stronger than USF binding to the E box of the *eIF2 α* promoter¹⁶ (fig 5C). Interestingly Max was found on the *TFF2* and *TFF3* E boxes suggesting that other Myc/Max/Mad network complexes can bind to these promoters (fig 5C). This defines specific interaction of USF factors preferentially with the *TFF2* promoter.

Finally, we determined whether USF factors could activate the endogenous *TFF2* gene. USF1 and USF2 were expressed transiently and the expression of endogenous *TFF* genes monitored by multiplex RT-PCR. USF1/USF2 but not c-Myc were sufficient to activate the endogenous, chromosomal *TFF2* gene in LS174T cells as well as other cell lines while little or no effect on *TFF1* and *TFF3* was observed (fig 6 and data not shown). Together our findings identify USF as a critical and specific modulator of *TFF2* transcription.

Figure 6 Exogenous USF stimulates the expression of the chromosomal copy of *TFF2*. LS174T cells were transiently transfected with empty vector or with plasmids encoding USF1 and USF2 or c-Myc as indicated. Endogenous *TFF* mRNA expression was analysed after 24 cycles of multiplex RT-PCR. Lanes 1: TFF1-PCR, GAPDH-PCR, TFF3-PCR reactions; lanes 2: TFF1-PCR, GAPDH-PCR, TFF2-PCR reactions.

DISCUSSION

We have identified the *TFF2* gene as a target for USF transcription factors. An E box DNA sequence element in the promoter of the *TFF2* gene mediates this activation. Despite E box elements in *TFF1* and *TFF3* no significant stimulation by USF was observed. In addition, the Myc/Max/Mad network members c-Myc and Mad1 were unable to regulate any of the *TFF* genes. Thus it seems that the *TFF2* promoter is unique among the *TFF* genes in that it supports strong activation by USF.

Our studies using EMSA suggest that this is most probably not attributable to differences in DNA binding affinity (fig 4). In contrast with these in vitro studies, DNA binding analyses

in cells by ChIP showed striking differences. USF binding was predominantly found on *TFF2* while binding to *TFF1* and *TFF3* was low (fig 5). Binding of c-Myc to any of these three genes was not significantly above background (fig 5). Thus DNA binding selectivity is considerably more pronounced in cells than in vitro offering a mechanistic basis for the observed specificity. This might reflect distinct accessibility to chromatin embedded binding sites or cooperative DNA binding effects resulting from interaction with other as yet unidentified transcriptional regulators.

Previous studies have shown that USF can activate a number of different promoters in reporter gene assays in many different cell types. In general this effect is rather modest (threefold to fivefold)^{24 30 32} while the activation of the *TFF2* reporter is significantly higher (fig 2). Most probably this is attributable to cooperative effects with other transcriptional regulators. Indeed, cooperation of USF with other factors, including Egr-1, STAT1, and cAMP response element binding proteins, has been described.^{24 33 34} We postulate that these function at least in part in a tissue specific manner. This is indicated by the high tissue specific expression of *TFF2*, predominantly in the antral glands and the Brunner's glands of the duodenum,⁴ whereas USF is expressed ubiquitously.

The endogenous levels of USF in LS174T as well as several other cell lines tested seem not to be sufficient to support high *TFF2* expression²² despite the ability of USF to bind to the promoter of the *TFF2* gene (fig 5 and data not shown). It is possible that the cooperative activation is inefficient in tumour cell lines because of insufficient factors available, which can be compensated by overexpressing USF (fig 6), or because of the lack of or the reduced sensitivity to activating signals. Indeed, the *TFF2* gene is regulated in response to a number of different insults, to drugs such as 5-amino salicylic acid used for treatment of inflammatory bowel disease (EA and PG, unpublished observation) or aspirin and to circadian rhythm and food intake.^{35 36} *TFF2* expression is predominantly controlled by transcriptional initiation,^{13 22 37} but it remains largely unclear how these stimuli affect the activity of the *TFF2* promoter. USF might be involved in recognising stress signals³⁸ or cooperate with factors targeted during signalling events, or both.²⁴ As USF constitutes a major E box binding activity in most cell lysates, its interaction with the *TFF2* promoter might be a prerequisite for signal stimulated activation. In addition regulatory elements that are not located within the reporter constructs could be important. This may be relevant for *TFF1*. In contrast with the reporter gene, the endogenous *TFF1* gene was activated to a small extent (figs 3 and 6) suggesting the presence of activities that may cooperate with USF in regulating this gene.

USF is known to evoke anti-proliferative effects.¹⁸ In this respect it is noteworthy that several USF target genes, including BRCA2, p53, and transforming growth factor β 2, are involved in inhibition of cell proliferation.¹⁸ The identification of USF as a potent regulator of *TFF2* is in line with a more general role of this transcription factor in negative growth control. Together the data summarised above suggest that defining the control of *TFF2* expression in more detail will potentially generate opportunities for therapeutic approaches of gastrointestinal diseases.

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