Cytokine Regulation of the Trefoil Factor Family Binding Protein *GKN2* (GDDR/TFIZ1/blottin) in Human Gastrointestinal Epithelial Cells

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Key Words

TFFs • GDDR • TFIZ1 • Blottin • Gastrokine-1 • Transcription factors • Gene regulation • Duodenal gastric metaplasia

Abstract

Trefoil factor family (TFF) peptides are major secretory products of mucous epithelia and play a multifunctional role in cytoprotection, apoptosis, and immune response. Recently, a TFF2-binding protein was discovered in mice and named blottin. It is down-regulated in gastric cancer (GDDR), abundant in human gastric surface (TFIZ1) and its similarity to gastrokine-1 led to the gene's name *GKN2*. To investigate the mode of *GKN2* regulation activity of a luciferase reporter gene, controlled by the *GKN2* promoter, was monitored upon treatment with various pro-inflammatory (TNF- α , IL-1 β , IL-6, IFN- γ) and anti-inflammatory (TGF- β 1) cytokines using gastric (AGS, KATO III) and colonic (HT-29) cell lines. To assess the direct role of transcription factors (NF κ B, HNF-3 β , hGATA6) in regulating *GKN2* we performed transient co-transfection of their expression plasmids and the reporter gene construct. *GKN2* gene was down-regulated by pro-inflammatory cytokines in all tested

cell lines while up-regulated by TGF- β 1 only in the colonic cell line. *GKN2* expression was significantly reduced in both gastric adenocarcinoma cell lines by the active form of NF κ B transcription factor, whereas in the colonic cell line an up-regulation was noticed. Down-regulation by IL-6 was mediated by C/EBP β transcription factor in case of HT-29 but not of KATO III cells. We conclude that the regulation of *GKN2* parallels that of *TFF* genes, indicating that together they may play an important role in maintaining the homeostasis of the gastrointestinal tract.

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Introduction

The physiological function of the gastrointestinal (GI) mucosa is dependent on the persistent integrity of the GI epithelium. The upper gastrointestinal tract must resist a variety of injuries, including those caused by ingested noxious substances and infections. As a result, inflammation, ulceration, or metaplasia/dysplasia can occur [1]. Gastric mucosa separates the underlying tissue from the vast array of antigens that traffic through the stomach lumen. While the extreme pH of this environment is es-

sential in aiding enzymes activation and food digestion, it also renders the gastric epithelium relatively free from bacterial colonization [2]. Many common cancers develop as a consequence of chronic inflammation that may result from persistent mucosal or epithelial cell colonization by microorganisms, including viruses (hepatitis B and C) and bacterium (*Helicobacter pylori*). *H. pylori* has been recognized as the casual agent for chronic gastritis and gastric and duodenal ulcers [3]. Chronic mucosal ulceration with inflammation of sub-epithelial tissues is typical for inflammatory bowel diseases (IBD) [4]. The GI tract has developed a complex network of immunological and non-immunological mechanisms to protect the host from potentially harmful agents that can destroy the integrated structure of the mucosal epithelia. The protective and reparative mechanisms rapidly restore mucosal integrity by stimulating migration of epithelial cells over denuded areas ('restitution'), increasing mucus production and re-establishing epithelial proliferation and differentiation programmes. These processes are regulated by various factors including small proteins (7-14 kDa) belonging to the trefoil factor family (TFF). In mammals this family has three major secretory products of mucous epithelia: TFF1, TFF2, and TFF3 [5]. These cytoprotective TFF peptides [6] promote healing in response to GI injury in humans and animal models by inhibiting epithelial apoptosis [7] and enhancing the barrier function of mucus [8]. They share similar cis-regulatory motifs which suggest a coordinated control of expression. However they also display specific signaling sequences which allow a unique regulation [9]. Each TFF peptide shows a particular expression pattern, they participate in the immune response [10, 11] and modulate inflammatory processes [12]. Their expression is induced in reaction to mucosal damage as well as during chronic inflammatory diseases, various types of metaplasia and many tumours [13]. It is known that the trefoil peptides are rapidly up-regulated after experimental gastric damage [14].

These different tasks within and outside the cell probably require additional interacting partners for these peptides. Recently a new TFF2 binding protein was discovered in mice and named blottin [15]. Blottin was found in gastric surface and foveolar epithelial cells of mouse stomach. The protein is well conserved in mammals, has a human homologue TFIZ1/GDDR and similarities with another gastric foveolar protein termed gastrokine-1 [16]. The expression pattern of gastrokine-1 and blottin is the same and this similarity led to the gene's name *GKN2*. Electron microscopy showed that mouse blottin (Gkn2) is being secreted with mucin granules and co-localized

with TFF1. Interestingly TFF2 is found deeper in the gastric glands [17]. Blottin's human homologue TFIZ1/GDDR, is down-regulated in gastric cancer, as suggested by original reports [18, 19]. Gkn2 protein contains one BRICHOS domain [20] that is involved in the interaction with trefoils. It is known that human TFIZ1 binds to hTFF1 monomer by a covalent disulphide bond which stabilises the heterodimer of the two proteins [19]. Gkn2 binds to mouse or human TFF2 directly [15].

The mucosal immune system plays an important role in the GI tract by preventing the uptake of harmful agents while at the same time allowing the passage of nutrients from the intestinal lumen into the systemic system [21]. Trefoil peptides co-operate in the protection of the mucosal barrier and are regulated by both pro-inflammatory [22] and anti-inflammatory [23-25] cytokine expression. A number of regulatory peptides including transforming growth factor (TGF)- β , interleukin (IL)-1 β , and interferon (IFN)- γ stimulate restitution of GI epithelia. These peptides differentially regulate the regeneration of mucous epithelia through specific modulation of restitution. A central role has been reported for TGF- β at least *in vitro* [see review 26]. TGF- β is a member of the superfamily of cytokines that affect a variety of cell types and elicit a wide array of cell-type-specific biological effects such as differentiation, migration, cell-cycle arrest, adhesion, extracellular matrix production and apoptosis [27]. Production of the anti-inflammatory cytokine TGF- β is often significant during immune responses at mucosal surfaces, and this may be an important adaptive mechanism to prevent the development of chronic inflammation and defects of the immune system [28]. Conversely TGF- β deficiency results in severe pathology associated with dysfunction of the immune and inflammatory system [29]. Deregulated expression of, or response to TGF- β has been implicated in a variety of disease processes, including autoimmune disease, fibrosis and chronic inflammation, parasitic infection, neurodegenerative disease, and carcinogenesis [30]. Cytokine-mediated changes in epithelial cell integrity may initiate pathological events in the GI tract. Pro-inflammatory cytokines such as IL-1 β , IL-6 and tumour necrosis factor (TNF)- α have been reported to be increased in serum from patients with inflammatory bowel disease (IBD) [4] and with chronic inflammation of the gastric mucosa. Chronic gastritis caused by *H. pylori* increases a T-helper (Th)1 immune response characterised by the recruitment of T lymphocytes expressing IFN- γ , TNF- α and IL-1 β [31]. The IL-6 family of cytokines plays an important role in maintaining the normal physiological func-

tion of the GI tract. IL-6 enhances the high affinity immune responses of the gut mucosa [32] and is also involved in early wound-healing regulation [22]. IL-6 can repress *TFF1* promoter activity and gene expression synergistically via CCAAT enhancer binding protein C/EBP β transcription factor [24]. C/EBPs act as pivotal regulators of response to inflammatory insult [33]. Other important cytokines that regulate TFFs are IL-1 β and TNF- α (acting via NF κ B transcription factor). IL-1 is a key pro-inflammatory driver of several autoimmune diseases [34], gastritis [35] and Crohn's disease [36]. Like IL-1 β and IL-6, which can also inhibit *TFF2* and *TFF3* transcription [24], the effects of TNF- α lead to acutely reduced trefoil expression in inflammation, resulting in delayed cytoprotection, restitution and mucosal renewal [37].

The discovery of TFF-interacting proteins has revealed an additional mechanism by which TFF function in the GI tract may be regulated. Here, we investigated the effects of several pro-inflammatory (TNF- α , IL-1 β , IL-6, IFN- γ) and anti-inflammatory (TGF- β 1) cytokines on expression of the *GKN2* in gastric (AGS, KATO III) and colonic (HT-29) cell lines. In order to elucidate the regulatory interplay we performed experiments using transcription factors with binding motifs within the *GKN2* promoter: NF κ B, C/EBP β , HNF-3 β , hGATA6.

Materials and Methods

Cloning

The promoter region of the *GKN2* (position -1906 to -20) (accession number AC114802) was amplified from human genomic DNA using primers GKN2_For: 5'CAA GGC AAG GAG AGG CTG TAT CAT 3' and GKN2_Rev: 5'CTG GAG TAA GTA AAG CTG CCC TGT 3'. The resulting fragment was re-amplified using a primer pair introducing Acc65I and BglII restriction sites at the 5' and 3' ends respectively (CL For4: 5'GGG GTA CCC CCA AGC AAG GAG AGG CTG TAT CAT 3' and CL Rev1: 5'GAA GAT CTT CCT GGA GTA AGT AAA GCT GCC CTG T 3'). This adapted product was first cloned by using TA-cloning procedure [38] into the pGEM-T vector (Promega) and then into a luciferase reporter vector (pGL3 basic, Promega). Transformation into JM109 competent cells (Promega) was performed according to the manufacturer's protocol. The identity of clones was verified by cycle-sequencing using primers LucR: 5'GTT TTT GGC GTC TTC CAT GGT GG 3', Rvprimer3: 5'CTA GCA AAA TAG GCT GTC CC 3', LucF-2: 5'CAG GTG CCA GAA CAT TTC TC 3', seqfor1: 5'CCC TTA ACT TGC CCA GAT GTC 3' and seq3rev: 5'AGG AAC AAG ACT CAG AAA GCA TTC 3', and performed on a Beckman CEQ2000 DNA Sequencer.

Cell culture

The gastric adenocarcinoma cell line AGS was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). The gastric adenocarcinoma cell line KATO III was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The colon adenocarcinoma cell line HT-29 was cultured in Waymouth medium with Glutamax and 12% FCS. All cells were incubated at 37°C in a humidified 5% CO₂ water-jacketed incubator.

Transfection, co-transfection and reporter gene assay

Reporter gene assay was performed by using the Dual-Luciferase Reporter Assay system (Promega, Mannheim, Germany). The *GKN2* gene promoter was cloned in front of the luciferase gene in the pGL3 basic vector (Promega, Mannheim, Germany). Transfection and co-transfection experiments were performed using the cationic polymer transfection reagent ExGen 500 (MBI Fermentas, St. Leon-Rot, Germany). Cells were seeded at a density of 4×10^4 to 6×10^4 cells per well in a 96-well culture plate at subconfluent density and transfected after 24h with a mixture of the pGL3 reporter plasmid (500 ng/ μ l) and control Renilla luciferase pRL-CMV plasmid (5 ng/ μ l) per well. Six hours after transfection, medium was removed and replaced with serum-free medium (control) or serum-free medium containing different cytokines (treatment). The following cytokines were used: TNF- α , IL-1 β , IL6, INF- γ (Biomol, Hamburg, Germany) and TGF- β 1 (obtained from S. Dooley, Mannheim, Germany). A concentration range of each cytokine was tested (0.1–100 ng/ml) to determine the minimum concentration affecting *GKN2* gene expression, which was used in further experiments. To test the specific effect of transcription factors on *GKN2* gene regulation, cells were co-transfected with the reporter gene vector and plasmids encoding specific transcription factors (NF κ -B subunit p65, C/EBP β , hGATA-6, HNF-3 β) or its inhibitor (IkB). 320 ng/ μ l of the reporter plasmid containing the *GKN2* promoter and 100 ng/ μ l of the following expression plasmids were used: NF κ -B subunit p65 (pMT2T-p65) [39], IkB (degradation resistant mutant form CMV-MAD3, obtained from D. Kube, Tübingen), C/EBP β (MSV/EBP β obtained from B. Lüscher, Aachen), hGATA-6 (pCDNA1) [T. Evans, E. Al-Azzeh, Ulm], and HNF-3 β (pGEM-2) [R.H. Costa, Illinois]. Expression of luciferase protein regulated by the *GKN2* promoter was measured after 48h according to the manufacturer's protocol (Berthold Technologies, Bad Wildbad, Germany). All of the transfection studies were performed in 3–5 separate experiments, where triplicate dishes were transfected. The results are presented as means $\pm$ S.D. Statistical analysis was performed using one-way ANOVA and a post-hoc Tukey HSD test. P value of <0.05 was used to determine statistical significance.

RNA isolation and quantitative real time PCR (qPCR)

After micro-homogenisation (B. Braun Biotech International) of various mouse tissues, total RNA was isolated from: stomach, small intestine, spleen, liver, lung, brain and heart with the RNeasy Mini Kit (Qiagen), and transcribed into cDNA (iScript cDNA Synthesis Kit; Bio-Rad). Quantitative Real Time PCR (qPCR) was performed using an iCycler (Bio-Rad).

Fig. 1. Schematic representation of the human promoters of the *GKN2* and of the three *TFF* genes. The locations and sequences (deviation from the consensus are underlined) of 7 E box elements in relation to the core promoters are shown (elipses). Binding sites for C/EBP β factors (C), HNF-3 β (H), GATA-6 (G) and NF κ B (N) are indicated. Note that the length of upstream DNA analysed was greater for the *GKN2* gene than those of the previously reported *TFF* genes. To find transcription sites for those transcription factors a TRANSFAC database was used. The TATA box in *TFF3* is CAT AAA [Adapted from E. Al-azzeah et.al 2002].

Specificity and efficiency of PCR reactions were verified by using the melting curve method and gel electrophoresis respectively. PCR was performed using the specific primer pair *Gkn2_for1*: 5' CCT CTA CGA GAA GCA GAC AAT G 3', and *Gkn2_rev2*: 5' CCT TCG TGA TCA GAG ACT TCA G 3'. The PCR conditions were: denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 1 min, 58°C for 30 sec and 72°C for 10 sec. To check the effect of knocking out the *Tff2* gene we compared *Gkn2* expression in the stomach of *Tff2* wild type and knock out mice. As an internal control we used *GAPDH* since it did not show significant variation in the stomach of wild type and knock out mice. No significant difference was noticed in *Gkn2* gene expression due to *Tff2* protein deficiency (data not shown). For *GAPDH* amplification the following primer pair was used: *mGAPDH_For4*: 5' AAC GAC CCC TTC ATT GAC 3', *mGAPDH_Rev4*: 5' TCC ACG ACA TACT CAG CAC 3'. The PCR product was first denatured at 95°C for 1 min, followed by 30 cycles at 95°C for 20 sec, 59°C for 40 sec and 72°C for 10 sec.

In situ hybridisation

Paraffin blocks were cut at 5 μ m for ³⁵S riboprobe *in situ* hybridisation using our previously published method [40, 17] and brightfield and darkfield image pairs, of human duodenal gastric metaplasia and normal tissue, were captured in Photoshop using a Micropublisher 5 digital camera (QImaging, Burnaby, BC, Canada) mounted on a Nikon Eclipse ME600 microscope (Nikon UK, Kingston on Thames, UK). Manual image manipulations were limited to brightness and contrast. Normal tissue was used as a control as published [17]. All

human tissues were anonymous archival paraffin blocks used under the Human Tissue Act UK License Number 12271 for the London Research Institute, Cancer Research UK.

Results

Searching for putative transcription factor binding sites

The promoter region of the *GKN2* gene was analyzed by using the TRANSFAC data base (www.cbil.upenn.edu), and we found many consensus sites for various transcription factors, among them GATA-6 (17 sites), HNF-3 β (2), C/EBP β (4) and NF κ B (1) and 7 E-box elements were also noted (elipses, Figure 1). It was interesting that each E-box element in *GKN2* differed from the single E-boxes found in the *TFF* genes. We found a total of 31 upstream controlling elements in the analysed segment of the *GKN2* gene, but these were found in a larger upstream fragment (1800bp) than those previously described for the *TFF* genes (800bp). Of those comparable to the *TFF* genes (to -800bp), the *GKN2* gene has 3 E-boxes (*TFF* genes have 1), but no NF κ B or HNF-3 β controlling elements; these were found further upstream (Figure 1).

Fig. 2. In situ hybridisation for *GKN2* mRNA in a case of human duodenal gastric metaplasia (A, B) and normal tissue (C, D). The upper panel shows the Giemsa stained autoradiograph brightfield image, with *GKN2* mRNA as black silver grains over the surface and upper gland epithelial cells. The lower panel shows a corresponding reflected light image, where silver grains appear white. No expression was found in normal human duodenal tissue (C, D). Original magnification 200X.

Fig. 3. Regulation of the human *GKN2* gene by pro-inflammatory (TNF- α , IL-1 β , IL-6, IFN- γ , all at a concentration of 40ng/ml) and anti-inflammatory (TGF- β 1, 1ng/ml) cytokines in the (A) gastric (KATO III) and (B) intestinal (HT-29) cell lines. (C) Down-regulation of human *TFF* reporter gene constructs by IFN- γ (20 ng/ml) in the same cell lines. The *GKN2* reporter gene activity 48 hours after stimulation with different cytokines is expressed as % of the non-stimulated control. *Statistically significant ($p < 0.05$), **($p < 0.01$).

Gastric *GKN2* expression pattern

GKN2 has been shown to be abundantly expressed in gastric surface and foveolar epithelia at mRNA and protein levels. It is down-regulated in gastric cancer, but up-regulated in the reparative monolayer in Crohn's disease [17]. We have further examined the expression of *GKN2* mRNA in gastric metaplasia, as this is a condition associated with *H. pylori* infection that may increase the risk of inflammation and pre-neoplastic changes. As seen in Figure 2, *GKN2* mRNA was clearly present in the cells of gastric surface phenotype but not in the cells of normal crypts.

Regulation of *GKN2* gene expression by various cytokines

To investigate whether *GKN2* gene expression in the GI tract is modulated by pro-inflammatory (IL-1 β , IL-6, TNF- α and IFN- γ) and anti-inflammatory (TGF- β 1) cytokines we applied a reporter gene assay in epithelial cell lines of gastric and colonic origin. While the

Fig. 4. Effect of transcription factors NF κ B and C/EBP β on regulation of the *GKN2* gene. Results are expressed as % of the non-transfected control. ** Statistically significant ($p < 0.01$).

pro-inflammatory cytokines caused down-regulation of *GKN2* in the range of 50%-70% compared to control, TGF- β 1 displayed a most pronounced up-regulatory effect in the colonic cell line (HT-29), but less effect in the gastric (KATO III) cell line (Figure 3 A, B).

Since *GKN2* co-localizes with *TFF1* expression in gastric epithelia, and interacts with *TFF2* [17], we wished to compare the influence of cytokines on *GKN2* expression to their effect on the *TFF* genes regulation by apply-

Fig. 5. Regulation of the *GKN2* gene by C/EBP β and NF κ B transcription factors and additional stimulation with IL-1 β (40ng/ml) and IL-6 (40ng/ml) in HT-29 (A) and KATO III (B) cell lines. Statistically significant * $p < 0.05$, ** $p < 0.01$ versus non-stimulated controls; † $p < 0.05$, ‡ $p < 0.01$, ††† $p < 0.001$ versus C/EBP β -treatment alone. Results are expressed as % of the non-stimulated control.

ing previously constructed *TFF1*, *TFF2*, *TFF3* promoter/reporter gene plasmids [41]. While the anti-inflammatory TGF- β 1 exerted no regulatory effect on the *TFF* genes, the pro-inflammatory cytokines showed a different picture: in the gastric cell line KATO III and colonic cell line HT-29, *TFF2* (which is also expressed in Brunner's glands and duodenum) was down-regulated by IFN- γ to 57% and 78% respectively (Figure 3C). The reductions in *TFF1* and *TFF3* expression did not reach statistical significance.

Fig. 6. Up-regulation of the human *GKN2* gene by HNF-3β and hGATA-6 transcription factors in the gastric (AGS and KATO III) and intestinal (HT-29) cell lines. Results are expressed as % of the non-transfected controls. ** Statistically significant ($p < 0.01$).

Regulation of *GKN2* gene by transcription factors NFκB and C/EBPβ

Among the pro-inflammatory cytokines tested above, IL-1 and IL-6 are the primary signals in various cascades that usually cause activation of transcription factors NFκB (IL-1 β) and C/EBPβ (IL-6), and members of the STAT family [24]. To check whether the effect of TNF-α, IL-1β and IL-6 on *GKN2* expression is mediated by NFκB or C/EBPβ, we performed a co-transfection experiment with expression plasmids for these transcription factors. The *GKN2* gene expression was significantly reduced in both gastric adenocarcinoma cell lines by the p50 and p65 subunits of NF κB (Figure 4 A, B). Reduction was more prominent in the AGS cell line where p50/p65 caused down-regulation to 20% relative to the control. It is interesting to see that IL-1β (normally acting through NFκB pathway) caused down-regulation only to 70% relative to the control, pointing to its interplay with another regulation pathway. Co-transfection with the specific NFκB inhibitor (IκB) expression plasmid showed strong up-regulation of the *GKN2* gene, suggesting that the down-regulation is due to NFκB activation. It was interesting to notice that in the colonic cell line we observed significant up-regulation of the *GKN2* gene by NFκB subunits as opposed to the effect seen in gastric cell lines. Co-transfection with its specific inhibitor (IκB) resulted in further up-regulation of the *GKN2* gene. HT-29 cells co-transfected with NFκB, and additionally stimulated with TNF-α or IL-1β, did not display down-regulation as observed in AGS and KATO III cell lines, but

Tissue	% of expression
Stomach	100
Small intestine	0.08
Heart	0.6
Brain	1
Kidney	0.1
Liver	0.04
Lung	9
Spleen	0.6

Table 1. Expression of *Gkn2* mRNA in mouse tissues studied by quantitative real time PCR (qPCR), adjusted to the expression level in the stomach which was set as 100.

rather showed up-regulation of the *GKN2* gene activity (Figure 4 C). In summary, these results indicate that NFκB was responsible for down-regulation of the *GKN2* gene expression in both stomach cancer cell lines but it caused a different response in the colonic cell line.

IL-6 stimulation caused a statistically significant down-regulation of the *GKN2* gene in both tested cell lines (HT-29 and KATO III) (Figure 3 A, B). We therefore monitored whether the IL-6 effect is caused by activation of the C/EBPβ transcription factor (Figure 5). We noticed that C/EBPβ caused down-regulation only in the HT-29 cell line that was little affected by further IL-1β or IL-6 treatment. Co-transfection with NFκB subunits p50 and p65 and additional stimulation with IL-6 and IL-1β resulted in a return to unstimulated control values in HT-29 cells (Fig 5A). In KATO III cells we observed a significant down-regulation of the *GKN2* gene after IL-1β, while the reduction caused by TNF-α and IL-6 did not reach statistical significance in this experiment. In contrast to HT-29, transfection of KATO-III cells with C/EBPβ resulted in a large rise in *GKN2* expression that was almost unaffected by IL-1β and IL-6. Co-transfection with p50 and p65 highly significantly reduced this level of expression relative to both untreated controls and C/EBPβ-treated KATO-III cells, and IL-6 further reduced this expression of the *GKN2* gene. In conclusion, down-regulation by IL-6 is mediated by the C/EBPβ transcription factor in the case of HT-29 but not of KATO III cell line.

Regulation of GKN2 gene by hGATA-6 and HNF-3 β

The transcription factor GATA-6, a factor controlling gut development, up-regulates *TFF1* and *TFF2*, but exerts no activation of *TFF3* [42], while HNF-3 α and β (other factors stimulating GI differentiation) induce *TFF1* gene transcription in pancreatic and gastric cancer cell lines [43]. To check whether the regulatory pattern of the *GKN2* gene is similar to that of the TFFs, we performed co-transfection experiments with expression plasmids for HNF-3 β and hGATA-6 (Figure 6). Both transcription factors caused a marked 10-15 fold up-regulation of the *GKN2* gene in all three tested cell lines.

Expression of Gkn2 in mouse tissues

Comparison of *Gkn2* expression by using quantitative real-time PCR (qPCR) revealed a strong presence of *Gkn2* mRNA in stomach and minute amounts in lung (Ct value respectively 18 and 29) of normal mouse tissues. No expression was found in small intestine, heart, brain, kidney, liver and spleen (Table 1).

Discussion

In terms of providing protection, the gastric epithelium has typically been regarded as a physical barrier; however, multiple studies have provided evidence to suggest that the gastric epithelium plays a key role in the inflammatory and immune responses. The gastric epithelium is an active player in the response while performing functions associated with antigen presenting cells [2]. Ye et al. reported that gastric epithelial cells constitutively expressed the class II major histocompatibility complex and that the constitutive expression of the class II major histocompatibility complex on gastric epithelial cells increased markedly during infection with *H. pylori*. They also suggested a role of IFN- γ in enhancing the antigen-presenting cell function [44]. Additionally, the epithelium has the ability to produce cytokines (IL-1 β , IL-6, IL-8, TNF- α and also macrophage migration inhibitory factor (MIF)), important cytokine that bridges the innate and adaptive immune responses [2]) that trigger the recruitment of inflammatory cells into the gastric lamina propria [35]. The GI tract represents a large interface between the immune system and the outside world [29]. Specific anatomical features and the presence of specialised immune cells ensure rapid and local responses to contain and eradicate pathogens without the development of sustained inflammatory responses to harmless GI antigens

[45]. The mucosal epithelial cells are major actors in coordinating defences [46]. Perturbed homeostasis between GI antigens and host immunity represents a critical determinant in the development of inflammation [47]. Chronic inflammation, which is driven by immune responses to the enteric flora, establishes a vicious cycle because tissue injury further compromises the ability to exclude immunostimulatory molecules [48]. This type of inflammatory cycle is centrally involved in the pathogenesis of digestive diseases and may also predispose to subsequent development of cancer [29, 49]. Although separate cellular or molecular events may be involved in inflammatory processes, it has been accepted that locally produced cytokines may play a critical role, comparable to their effects in a number of other chronic inflammatory disorders [50].

The results of our study present for the first time information on the regulatory control of the *GKN2* gene. Mouse blottin (*Gkn2*) is homologous with the human TFF1-binding protein (TFIZ1), which was first described as *GDDR* [18, 19]. *Gkn2* binds to mouse TFF2 and is found in gastric surface and foveolar epithelial cells. The protein is also seen in the reparative surface epithelium of the colon in the ulcer-associated cell lineage (UACL) of Crohn's disease, in a location similar to that which was previously reported for TFF1 [17]. The UACL is a unique glandular structure occurring at sites of chronic gastrointestinal ulceration containing neutral mucin secreting cells and synthesises and secretes EGF, TGF- α and TFF peptides [51]. Trefoil factors play an important role in the homeostasis of the GI tract. These secreted peptides maintain epithelial integrity, are important for protection and wound healing. They are expressed rapidly in response to injury and are up-regulated in inflammation and ulceration [52]. TFFs are GI factors that allow the healthy mucosa to restrain inflammation and live harmoniously with its abundant flora. Their expression is modulated by pro- and anti-inflammatory cytokines [22-25]. Cytokines such as IL-1 β and IL-6 down-regulate *TFF* genes expression by transcriptional repression in GI cells. Since these cytokines are over-expressed in IBD and some gastric carcinomas, expression of the gastro-protective TFF may be reduced *in vivo* [24, 37, 42, 43]. It was interesting to explore whether *GKN2* shows similar or even the same regulation pattern as members of the TFF.

GKN2 is expressed in gastric surface epithelial cells but down-regulated in gastric cancer [17, 18]. We have examined its expression in duodenal gastric metaplasia. Remarkably, while normal duodenal crypts were devoid

of *GKN2* mRNA, it was clearly visible in cells of the gastric surface phenotype.

Duodenal gastric metaplasia (DGM) is often considered a precursor lesion to *H. pylori*-induced ulceration, insofar as a gastric-like niche is established into which the bacteria invade. This is probably correlated with a down-regulation of *Cdx2* and thus of sucrase-isomaltase, a target gene, and an up-regulation of the gastric mucins *Muc5AC* and *Muc6* [53]. There is a close match between the expression of gastric mucins *Muc5AC* and *Muc6*, as well as *TFF1* and *TFF2* in DGM and in normal gastric mucosa [54, 55]. The relationship between *H. pylori* infection and DGM is still not clear, since some patients with this condition may be *H. pylori*-negative [56], while other studies show a correlation between DGM and *H. pylori* presence [57]. Whatever the causality, there was clear expression of the *GKN2* gene in the surface gastric metaplastic epithelium of DMG. In this regard, this observation augments our earlier report [17] that this gene is frequently found at sites of *TFF1* expression.

Since *GKN2* is also expressed in the GI cell lines [15] we tested its regulation in gastric (AGS and KATO III) and colonic (HT-29) cell lines. We used various cytokines which have been shown to regulate TFF genes expression [24, 58] and we tested different transcription factors that have been shown to mediate the action of these cytokines in TFF genes. Looking at results obtained from the TRANSFAC database (Figure 1), we notice that in promoters of *GKN2* and *TFF* genes there are several predicted consensus sites for different but, in part, shared transcription factors: GATA-6, NF κ B, HNF-3 β , C/EBP β and E-box elements. E-box elements are enhancer elements with the consensus sequence CANNTG that play a regulatory role in the control of transcription, and bind with basic helix-loop-helix type transcription factors [59]. We note that of the 31 total control elements for the *GKN2* gene segment that we have analysed, only 12 were proximal to the TATA box, whereas *TFF1/2/3* genes have 14, 9 and 12 elements respectively in a similar position. There was no clear pattern to the distribution of the elements. These data suggest a more complex regulatory pattern for *GKN2* and the *TFF* genes than has hitherto been recognised.

We observed down-regulation of the *GKN2* gene by pro-inflammatory cytokines. These results are similar to those which were reported for TFFs, especially for *TFF1* and *TFF2* [22]. The inhibitory effect of pro-inflammatory cytokines on TFFs has been further investigated since they are up-regulated in IBD [60]. The inhi-

bition of TFFs is mediated by NF κ B and C/EBP β transcription factors [24, 25]. NF κ B is a mediator of inflammatory responses, plays a significant role in carcinogenesis and is emerging as a link between inflammation and cancer [61]. Aberrant NF κ B activation leads to tumourgenesis via antiapoptotic gene expression [62]. It is known that *H. pylori* persistently infects the human stomach and activates NF κ B [63]. This factor is normally held in an inactive state in the cell cytosol as a complex with its inhibitor, I κ B α . Degradation of the inhibitor in response to a variety of extracellular signals frees NF κ B to translocate to the nucleus, where it binds to the promoter region of target genes. Activation of NF κ B results not only in up-regulation of genes involved in inflammation and cell survival but also in synthesising/resynthesising NF κ B-dependent negative regulators of NF κ B signalling [64]. We found that the inhibitory effect of TNF- α and IL-1 β on *GKN2* gene expression was mediated by NF κ B in two gastric cell lines, whereas in the colonic cell line (HT-29) we did not observe such an effect. In contrast, inhibition of NF κ B pathway by its specific inhibitor I κ B caused further up-regulation of the *GKN2* gene in this cell line. Previously it was shown that down-regulation of *TFF3* by IL-1 β and IL-6 was mediated in the colonic cell line HT-29 by NF κ B and C/EBP β transcription factors respectively [24, 25]. It is known that NF κ B can interact with other transcription factors such as AP-1 and C/EBP β [65]. We noted down-regulation of the *GKN2* gene by IL-6 in HT-29 and KATO III cell lines. Suspecting C/EBP β as a mediating factor we performed co-transfection experiments with this transcription factor and with NF κ B, and stimulated cells with pro-inflammatory cytokines (IL-1 β and IL-6). C/EBP β is a direct mediator of the acute phase response in intestinal epithelial cells [66], and it can function as both a transcriptional activator and repressor [67]. In gastric cell lines, C/EBP β up-regulated the *GKN2* gene while in the colonic cell line it induced its down-regulation. Co-transfection with the active form of NF κ B (p50/65) and additional stimulation with cytokines caused further down-regulation in KATO III cells but up-regulation in the HT-29 cell line (statistically significant versus C/EBP β as a control). From our studies we conclude that C/EBP β is a mediating factor for IL-6 and IL-1 β , whereas co-transfection with an active form of NF κ B and further stimulation with cytokines neutralised this effect in HT-29 cells. These data together suggest the existence of a complex regulatory role of NF κ B that may differ with target tissue.

It has been shown that in the 5'-flanking region of

the *TFF* genes there are several predicted consensus sites for members of the GATA transcription factor family, known to control gut-specific gene expression. HNF-3 alpha and beta bind predominantly to the sites in *TFF1*, and to a lesser extent to the sites in *TFF2* or *TFF3*; HNF-3β and GATA6 induced up-regulation of *TFF* genes [42, 43]. We observed up-regulation of *GKN2* expression by both transcription factors in all tested cell lines (AGS, HT-29, and KATO III). In the promoter region of the *GKN2* gene we found consensus sites for members of the GATA family and HNF-3β transcription factors, which indicate that they play a role in its regulation.

We also observed a significant up-regulation of *GKN2* expression by the anti-inflammatory cytokine TGF-β1 in the HT-29 cell line. Many observations indicate a role of TGF-β in both inflammation and tissue repair. TGF-β deficiency results in severe pathology (massive multifocal inflammation affecting multiple organs) associated with dysfunction of the immune and inflammatory system (autoimmune mechanisms), indicating its essential role as a potent regulator [68]. Increased expression of TGF-β has been demonstrated following acute epithelial injury *in vivo*, and in patients with active inflammatory bowel diseases. It is generally accepted that almost all expression of TGF-β is observed in epithelial cells of the intestine and only minor amounts within constituents of the mucosal immune system. Three isoforms of TGF-β (TGF-β_{1,2,3}) were detected in all GI tract tissues. Data suggest that TGF-β1 may play a role by regulating the activation and inactivation of immune cells and other cell populations present within the intestinal mucosa [see review 69].

In conclusion, we describe the regulation of the *GKN2* gene by various cytokines. Previously it was shown that the location of *GKN2* expression is distinct from its

binding partner TFF2, which is found deeper in the gastric glands. It was implied that GKN2 may modulate the process of maintaining the integrity of the mucus barrier and its mode of action may be enhanced in a wound situation, perhaps by influencing mucus viscosity [17]. The transcription factor regulatory pattern of *GKN2* expression shows similarities to that of the *TFF* genes. Our data, together with previous report [17], suggest that GKN2 and the TFFs may play an important role in maintaining the homeostasis of the GI tract.

Abbreviations

C/EBPβ (CCAAT enhancer binding protein); DGM (duodenal gastric metaplasia); FCS (foetal calf serum); GI (gastrointestinal); *H. pylori* (*Helicobacter pylori*); IBD (inflammatory bowel disease); IκB (inhibitor κB); IL-1β (interleukin 1β); IL-6 (interleukin 6); INF-γ (interferon γ); MIF (macrophage migration inhibitory factor); NFκB (nuclear factor κB); qPCR (quantitative real time PCR); TGF-β (transforming growth factor β); TFF (trefoil factor family); TNF-α (tumour necrosis factor α); UACL (ulcer associated cell lineage).

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