

The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis

Carine Bossenmeyer-Pourié,¹ Rama Kannan,¹ Stéphane Ribieras,¹ Corinne Wendling,¹ Isabelle Stoll,¹ Lars Thim,² Catherine Tomasetto,¹ and Marie-Christine Rio¹

¹Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur, 67404 Illkirch Cedex, C.U. de Strasbourg, France

²Novo Nordisk, Novo Allé, DK-2880, Bagsvaerd, Denmark

Trefoil factor (TFF)1 is synthesized and secreted by the normal stomach mucosa and by the gastrointestinal cells of injured tissues. The link between mouse TFF1 inactivation and the fully penetrant antropyloric tumor phenotype prompted the classification of TFF1 as a gastric tumor suppressor gene. Accordingly, altered expression, deletion, and/or mutations of the TFF1 gene are frequently observed in human gastric carcinomas. The present study was undertaken to address the nature of the cellular and molecular mechanisms targeted by TFF1 signalling. TFF1 effects were investigated in IEC18, HCT116, and AGS gastrointestinal cells treated with recombinant human TFF1, and in stably transfected HCT116 cells synthesizing constitutive or doxycycline-induced human TFF1. We observed

that TFF1 triggers two types of cellular responses. On one hand, TFF1 lowers cell proliferation by delaying G1-S cell phase transition. This results from a TFF1-mediated increase in the levels of cyclin-dependent kinase inhibitors of both the INK4 and CIP subfamilies, leading to lower E2F transcriptional activity. On the other hand, TFF1 protects cells from chemical-, anchorage-free-, or Bad-induced apoptosis. In this process, TFF1 signalling targets the active form of caspase-9. Together, these results provide the first evidence of a dual antiproliferative and antiapoptotic role for TFF1. Similar paradoxical functions have been reported for tumor suppressor genes involved in cell differentiation, a function consistent with TFF1.

Introduction

Trefoil factor (TFF)*1 (pS2) is a small secreted protein. It belongs to the TFF protein family that is characterized by a clover leaf-like disulphide structure named the TFF domain (Thim, 1997; Ribieras et al., 1998; Hoffmann et al., 2001). This domain, highly conserved from amphibians to mammals, is likely to have an important function. In mammals, two other TFFs have been identified, TFF2 (spasmolytic polypeptide [SP]) and TFF3 (intestinal trefoil factor [ITF]). TFFs are normally synthesized in the gastrointestinal mucosa. In addition to transcriptional regulation via the Gata 4 and HNF3 factors (Beck et al., 1999), the tissue-specific expression of TFFs along the gastrointestinal tract is dependent on

the methylation status of their proximal promoter regions (Ribieras et al., 2001).

In mouse embryos, TFF1 starts to be expressed at day 17 in the epithelial cells lining the surface and the nascent stomach pits and glands. In addition, 18-d embryos show expression of TFF1 in the glands of the gastroduodenal junction corresponding to the developing Brunner's glands. This latter expression of TFF1 is no longer observed beyond postnatal day 14 (Lefebvre et al., 1993; Otto and Patel, 1999). In human and mouse adult tissues, TFF1 expression is normally restricted to the stomach. TFF1 is observed in the fundus, antrum, and antrumpyloric mucosa. It is located in the epithelial cells of the upper part of the pits, an area where cells undergo commitment and differentiate to give rise to a functional secreting mucosa and to limit gland proliferation. TFF1 is observed in the cytoplasm of producing cells with a preferential perinuclear accumulation, a subcellular localization appropriate for a secreted protein. Accordingly, TFF1 is detected in the gastric juice and urine (Rio et al., 1988).

Address correspondence to Marie-Christine Rio, IGBMC, BP 163, Illkirch Cedex, 67404, France. Tel.: 33-3-88-65-34-34. Fax: 33-3-88-65-32-01. E-mail: rio@igbmc.u-strasbg.fr

*Abbreviations used in this paper: ITF, intestinal trefoil factor; SP, spasmolytic polypeptide; TFF, trefoil factor.

Key words: trefoil factor 1; pS2; cell cycle; gastrointestinal differentiation; apoptosis

In ulcerative and acute inflammatory bowel diseases such as Crohn's disease, duodenal peptic ulcer, or hemorrhagic rectocolitis, TFF1 is ectopically expressed along the entire human gastrointestinal tract. TFF1 is observed in cells of regenerating tissues surrounding areas of damage (Rio et al., 1991; Wright et al., 1993). These observations raised the possibility that TFF1 might be involved in the repair process and the maintenance of mucosal integrity. In this context, transgenic mice overexpressing human TFF1 in the jejunum have a lower rate of induced ulceration in this part of the gastrointestinal tract, showing that TFF1 exerts a protective effect (Playford et al., 1996). It has been shown that stable expression of TFF1 promotes invasiveness of human colonic HCT8/S11 cells and cell scattering (Emami et al., 2001). Because the TFF domain of TFF1 is able to directly interact with the von Willebrand factor C cysteine-rich domains of mucins (Tomasetto et al., 2000), TFF1 might also protect the gastrointestinal tract through its participation in the formation of the mucus layer covering all of the gastrointestinal mucosa. Together, these *in vitro* and *in vivo* data suggest that TFF1 could serve as a valuable pharmacological tool for the prevention and healing of gastrointestinal tissue ulcerations.

Using transgenic mice overexpressing human TFF1 in the mammary gland, it has been shown that TFF1 does not favor cell proliferation and is not an oncogene (Tomasetto et al., 1989). In contrast, 100% of TFF1-null mice exhibit antropyloric hyperplasia and dysplasia, leading to adenomatous tumors with occasional foci of intraepithelial or intramucosal carcinoma. Thus, at least in the stomach, TFF1 acts as a tumor suppressor gene (Lefebvre et al., 1996). Accordingly, 50% of human gastric tumors are devoid of TFF1 (Luqmani et al., 1989). TFF1 gene deletion and alterations (Park et al., 2000a, 2000b) as well as abnormal gene methylation (Fujimoto et al., 2000) have been reported to be associated to gastric tumors. Finally, a recent report showed that TFF1 induces growth inhibition in the human AGS gastric adenocarcinoma cell line (Calnan et al., 1999).

How TFF1 acts at the molecular and cellular levels remains elusive. Tumor suppressors have been shown to exert various functions to either lower cell proliferation, increase cell death, or favor cell differentiation (Kinzler and Vogelstein, 1998). Therefore, TFF1 might be involved in such processes. In the present study, we address these hypotheses in the IEC18, HCT116, and AGS gastrointestinal cell lines treated with recombinant human TFF1, and in HCT116 cells stably transfected with vectors allowing either constitutive or induced expression of human TFF1.

Results

TFF1 treatment or expression lowers gastrointestinal cell number

We first tested the effect of TFF1 on the viability of gastrointestinal rat IEC18 (diploid intestinal cells), and human HCT116 (colon cancer), and AGS (gastric cancer) cells, that do not express endogenous TFF1. The addition of increasing quantities (0.1–50 μ M) of recombinant human TFF1 (Kannan et al., 2001) in the culture medium lowered, in a dose-dependent manner, the cell number of these cell lines

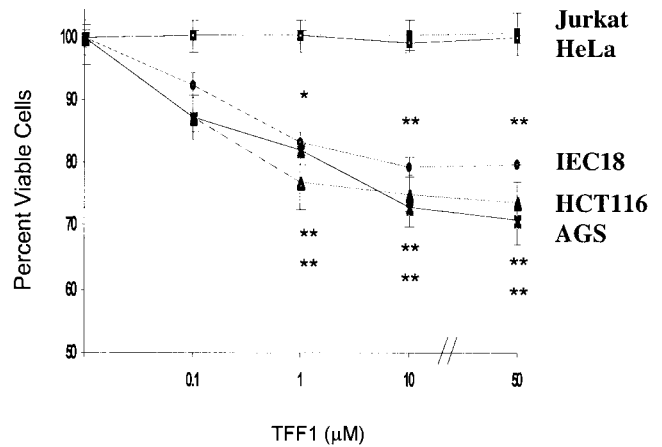


Figure 1. Human recombinant TFF1 decreases IEC18, HCT116 and AGS gastrointestinal cell numbers. The number of live cells was estimated by the MTT method. Human recombinant TFF1 addition to the culture medium (0.1–50 μ M) induces a dose-dependent reduction in the number of HCT116 (\blacktriangle), IEC18 (\bullet) and AGS (cross) cells but has no effect on that of hematopoietic Jurkat (\blacksquare) and cervix HeLa (\square) cells. Standard deviations are indicated. * $P < 0.05$; ** $P < 0.01$.

(MTT test) (Fig. 1). This effect was already observed at 0.1 μ M TFF1. The maximum decrease in cell number was 20% for IEC18, 25% for HCT116, and 30% for AGS cells. Moreover, this effect was TFF1 specific, as the addition of albumin or of an unrelated peptide at the same concentration did not alter cell number (unpublished data). Similar experiments were performed using the human HeLa (cervix) and Jurkat (T cell) cancer cell lines. In the latter case, no alteration in cell number was observed after TFF1 treatment (Fig. 1), indicating that the decrease observed in the IEC18, HCT116, and AGS cells was cell specific and did not result from a nonspecific cytotoxic effect of the recombinant human TFF1. Moreover, because IEC18 cells are nontransformed cells, TFF1 function is not due to cell malignancy. Finally, human TFF1 can exert effects on rat IEC18 cells, showing that the TFF1 signalling pathway has been well conserved during species evolution.

In vivo, TFF1 is expressed in response to injury in intestine and colon (Rio et al., 1991). Thus, to further study TFF1 function, we established, in the human colon cancer cell line HCT116, clones constitutively expressing human TFF1 (HCT116/TFF1) (Fig. 2, lanes 3 and 4), and clones expressing the human TFF1 under doxycycline induction (HCT116/iTFF1) (Fig. 2, lanes 11 and 12). HCT116/CMV (Fig. 2, lanes 1 and 2) and HCT116/UHD clones (Fig. 2, lanes 5, 6, 9, and 10), transfected with vector alone, were used as a negative control. Moreover, in order to avoid doxycycline-specific effects (Fife et al., 1997), HCT116/UHD clones grown in the presence of doxycycline were also used as negative controls in all of the following experiments. Constitutive or induced TFF1 expression significantly reduced the number of viable cells to ~ 20 ($83 \pm 6.2\%$ vs. 100%, $P < 0.01$) and 30% ($68 \pm 7.1\%$ vs. 100%, $P < 0.01$) of control levels, respectively.

Together, these results indicate that TFF1 lowers gastrointestinal cell number in both a paracrine and autocrine manner, and suggest a possible role for TFF1 in the regulation of either cell proliferation or cell death.

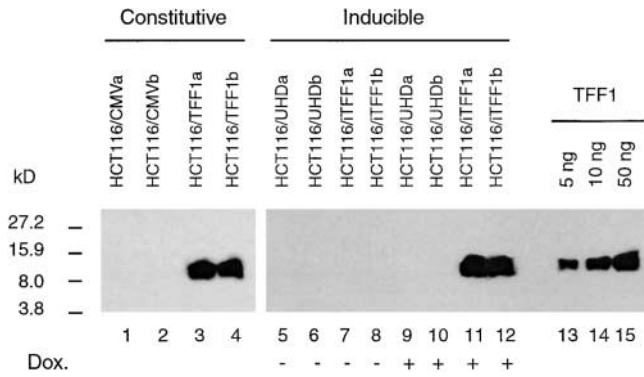


Figure 2. Western blot analysis of constitutive and doxycycline-induced TFF1 synthesis in stably transfected HCT116 cell lines. 10 μ l of conditioned culture medium from two clones of each HCT116 cell line were loaded. TFF1 detection was done using the p2802 specific antibody. (Lanes 1 and 2) pCMV-transfected control clones. (Lanes 3 and 4) Clones transfected with the pCMV-hTFF1 constitutive expression vector. (Lanes 5, 6, 9, and 10) pUHD-transfected control clones. (Lanes 7, 8, 11, and 12) Clones transfected with the pUHD-hTFF1 inducible vector. (Lanes 5–8) Absence (Dox. –) of doxycycline treatment. (Lanes 9–12) Presence (Dox. +) of doxycycline treatment. (Lanes 13–15) 5, 10, and 50 ng of human recombinant TFF1. Molecular weight scale is indicated on the left in kD.

TFF1 delays G1-S phase transition

Therefore, we investigated the effect of TFF1 on the cell cycle. Addition of recombinant human TFF1 to parental HCT116 cells led to a slight increase in the number of cells in G1 phase, suggesting that TFF1 might delay the passage of cells to S phase (unpublished data). This hypothesis was further studied using G1-enriched parental HCT116 cells (85% of cells in G1 phase, 4% in S, and 11% in G2/M). In the absence of TFF1 treatment, after a 1-h release into cell cycle, 42% of the cells (34% S and 8% G2/M) had passed through the G1-S checkpoint. In contrast, TFF1 treatment (1 μ M) resulted in only 19% of the cells (16% S and 3% G2/M) proceeding through the G1/S transition. Thus, under these conditions, TFF1 cell treatment diminishes by 50% the S phase cell entry (Fig. 3). Similar experiments were performed using G1-enriched TFF1-transfected HCT116 cell lines expressing either constitutive or inducible (Table I) TFF1. Whereas control clones (HCT116/CMV and HCT116/UHD) showed ~50% of the cells in S and G2/M phases, constitutive (HCT116/TFF1 clones) or

TFF1 treatment

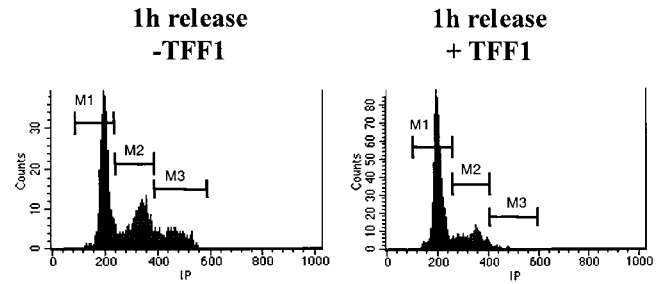


Figure 3. TFF1 induces the accumulation of cells in the G1 phase of the cell cycle. Analysis of the cell cycle patterns was done using propidium iodide. Cell cycle profiles of G1-enriched HCT116 parental cells after a 1 hour release into the cell cycle in the absence (control) or presence (TFF1, 1 μ M) of human recombinant TFF1 (1 μ M). Cells in the G1 (M1), S (M2), and G2/M (M3) phases are indicated. TFF1 delays G1/S transition.

doxycycline-induced (HCT116/iTFF1 clones) TFF1 expression led to <30 and 40% of cells in S and G2/M phases, respectively. Thus, constitutive and doxycycline-induced TFF1 expression reduce S phase entry by 40 and 20%, respectively.

Consistent with the higher number of cells in G1 phase, FACS screening analyses (Table I) showed that the number of cells positive for cyclin D1, a cell cycle protein specific of the G1 phase, was increased in the presence of TFF1 (1.4- to 2.5-fold). In contrast, TFF1 led to a decrease of cell cycle proteins expressed later in the cell cycle such as PCNA (S phase) (1.5- to 30-fold) and cyclin B1 (G2 phase) (1.2- to 9-fold).

Thus, in both a paracrine and autocrine manner, TFF1 retains cells within the G1 phase of the cell cycle, thereby delaying their S phase entry and reducing their proliferation.

TFF1 leads to increased expression of cyclin-dependent kinase inhibitors

By controlling the level of cyclin-dependent kinase (cdk) activity, cdk regulators control cell cycle commitment (Malumbres and Barbacid, 2001). Thus, overexpression of the cdk activator cyclin D1 favors the passage of cells through G1 and promotes proliferation. However, in our experiments, S phase transition is repressed in the presence of TFF1 despite

Table I. Percentage of cells in G1, S, and G2/M phases, and positive for cyclin D1, PCNA, and cyclin B1 proteins after TFF1 treatment and constitutive or induced TFF1 expression

Cell cycle	TFF1 treatment			Constitutive			Inducible		
	- ^a	+ ^b	P	-	+	P	-	+	P
Phase									
G1	58 ± 1.9	81 ± 2.1	<0.01	49 ± 3.4	72 ± 4.0	<0.01	50 ± 2.6	61 ± 2.5	<0.01
S	34 ± 2.2	16 ± 2.5	<0.01	38 ± 3.7	23 ± 3.3	<0.01	40 ± 1.3	28 ± 1.5	<0.01
G2/M	8 ± 1.6	3 ± 0.7	<0.01	13 ± 1.7	5 ± 0.8	<0.01	10 ± 1.1	11 ± 1.2	NS
Protein									
D1	8 ± 1.2	20 ± 5.7	<0.01	12 ± 3.6	17 ± 0.7	NS	8 ± 1.9	16 ± 1.6	<0.01
PCNA	62 ± 2.5	2 ± 0.6	<0.01	56 ± 10.2	30 ± 6.2	<0.05	50 ± 2.3	34 ± 5.6	<0.01
B1	5 ± 1.6	1 ± 0.8	<0.01	6 ± 2.5	5 ± 2.1	NS	9 ± 1.2	1 ± 0.6	<0.01

^a–, Absence of TFF1 treatment or synthesis.
^b+, Presence of TFF1 treatment or synthesis.

Figure 4. TFF1 modifies pRb and E2F transcriptional activity. Parental HCT116- and HCT116-expressing TFF1 constitutively or under induction were transfected with vectors containing a specific response element for pRb (pRb-Ta-Luc) or E2F (pE2F-Ta-Luc) upstream from a TATA-like promoter driving a luciferase reporter gene. The pTA-Luc vector was used as a negative control. Luciferase activities were corrected for β -galactosidase activities. pTA-Luc luciferase activity was set at 1. TFF1 treatment (10 μ M), constitutive or inducible TFF1 synthesis increased pRb transcriptional activity and decreased E2F transcriptional activity. (Gray bars) Absence of TFF1. (White bars) Presence of TFF1. Standard deviations are indicated. $**P < 0.01$.

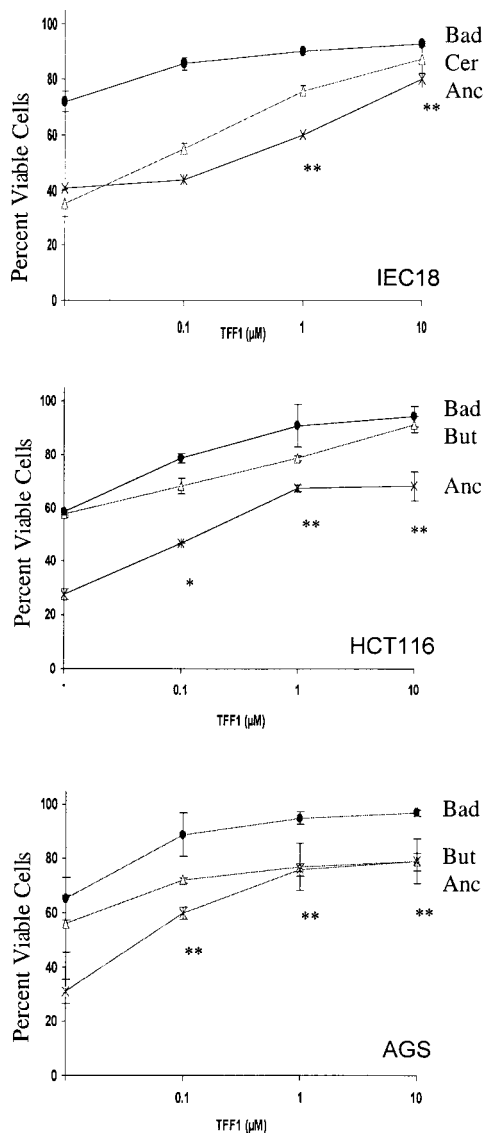
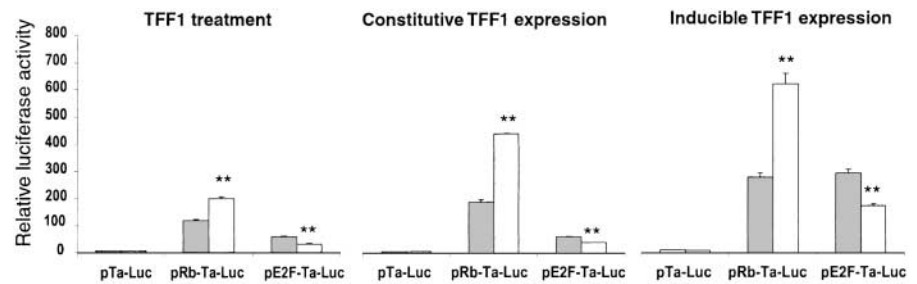


Figure 5. TFF1 prevents induced apoptosis of IEC18, HCT116, and AGS gastrointestinal cells. Apoptosis was induced by treatment with 5 mM sodium butyrate (But), 50 μ M ceramide (Cer), anchorage-free condition (Anc), or Bad expression (Bad), as indicated. The number of live cells was estimated by the MTT method. Independent of the nature of the apoptosis inducer, addition of recombinant TFF1 (0.1 to 10 μ M) protected gastrointestinal IEC18 (top), HCT116 (middle) and AGS (bottom) cells from apoptosis in a dose-dependent manner. Standard deviations are indicated. $*P < 0.05$; $**P < 0.01$.

the observation that cyclin D1 levels were increased. This observation suggests that higher levels of cyclin D1 reflect the higher number of cells in G1, and that cdk inhibitors that negatively regulate the cell cycle (Ekholm and Reed, 2000) might be involved in TFF1 signalling. Therefore, the expression of two cdk of the INK4 family, p15 and p16, and two cdk inhibitors of the CIP family, p21 and p27, were studied by flow cytometry. All data obtained from the three conditions, TFF1 treatment, constitutive, and induced TFF1 expression, are compiled in Table II. Upon TFF1 treatment or expression, most of the cell lines showed significantly increased levels of p15 (1.5- to 2.5-fold), p16 (1.4-fold), p21 (1.4- to 2.3-fold), and p27 (1.4- to 2.5- fold).

Thus, the presence of TFF1 resulted in the upregulation of all cdk inhibitors studied, indicating that TFF1 delays the G1-S phase transition via cdk inhibitors of both INK4 and CIP subfamilies.

TFF1 increases pRb activity and decreases E2F activity

The retinoblastoma protein pRb, a target of cdk, is a major player in the G1-S cell transition (Weinberg, 1995; Herwig and Strauss, 1997; Ross et al., 1999). pRb activity during the cell cycle is regulated in a phosphorylation-dependent manner. In order to test whether TFF1 affects this protein, we studied the level of pRb expression and activity in response to TFF1. FACS analysis showed a significant increase in pRb expression in all cases: TFF1 treatment (15 ± 1.2 to 31 ± 4.6 , $P < 0.01$), constitutive TFF1 (18 ± 1.5 to 53 ± 4.5 , $P < 0.01$), and inducible TFF1 expression (4 ± 0.8 to 14 ± 4.5 , $P < 0.01$). Transcriptional activity of pRb was then investigated by cotransfecting cells with a luciferase reporter gene under the control of regulatory promoter regions shared by genes responding to pRb (pRb-Ta-Luc). TFF1 treatment of the parental HCT116 cells led to a twofold increase in pRb activity (Fig. 4). Similarly, in stably transfected HCT116 cell lines expressing TFF1, pRb activity was increased about 2.5-fold (Fig. 4). These results indicate that the pRb synthesized in response to TFF1 is transcriptionally active.

The transcription factor E2F is a major regulatory factor determining entry into S phase. Its interaction with the unphosphorylated form of pRb gives rise to pRb/E2F heterodimers, leading to decreased E2F transcriptional activity, and prevents cells from entering S phase (Herwig and Strauss, 1997; Ross et al., 1999). Therefore, the effect of TFF1 on E2F transcriptional activity was investigated in HCT116 cell lines transfected with a luciferase reporter gene under the control of a

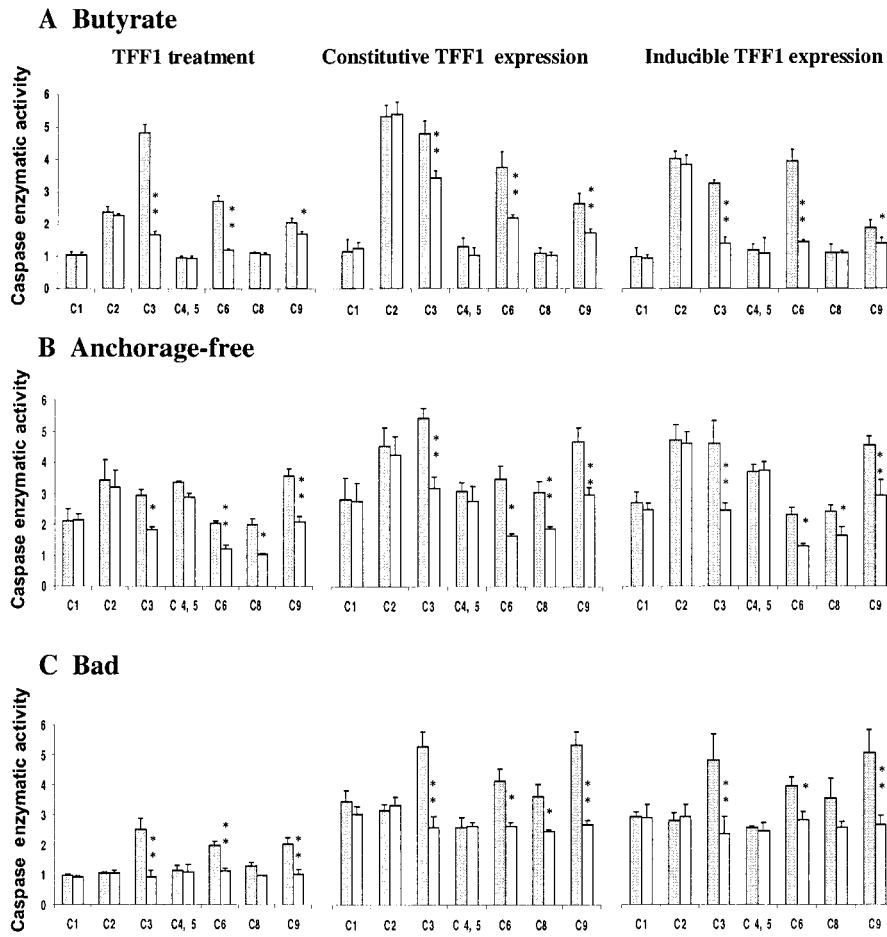


Figure 6. TFF1 reduces caspase activities. Apoptosis of HCT116 cells was induced using either 5 mM sodium butyrate treatment (A), anchorage-free condition (B), or Bad expression (C). Enzymatic activities of caspase-1, -2, -3, -4/5, -6, -8, and -9 (C1 to C9) were investigated by quantification of the cleavage of specific fluorometric substrates. Results were given as fold increase compared with controls. TFF1 treatment (10 μ M), constitutive or induced TFF1 expression reduces activity of several caspases. (Gray bars) Absence of TFF1. (White bars) Presence of TFF1. Standard deviations are indicated. * $P < 0.05$; ** $P < 0.01$.

regulatory promoter region shared by genes responding to E2F (pE2F-Ta-Luc). The level of luciferase expression was approximately twofold lower in the presence of TFF1, indicating that TFF1 leads to a twofold decrease in E2F activity (Fig. 4).

Thus, TFF1 delays HCT116 cells from entering S phase of the cell cycle, at least partially, via a reduction in E2F transcriptional activity.

TFF1 reduces gastrointestinal cell-induced apoptosis

In addition to studying the effect of TFF1 on proliferation, we also investigated the possible role of TFF1 in apoptosis. Although reduced in number (Fig. 1), TFF1-treated or -expressing cells appeared healthy, with no morphological evidence of cell death (unpublished data). However, as another member of the TFF family was reported to have antiapoptotic properties (Kinoshita et al.,

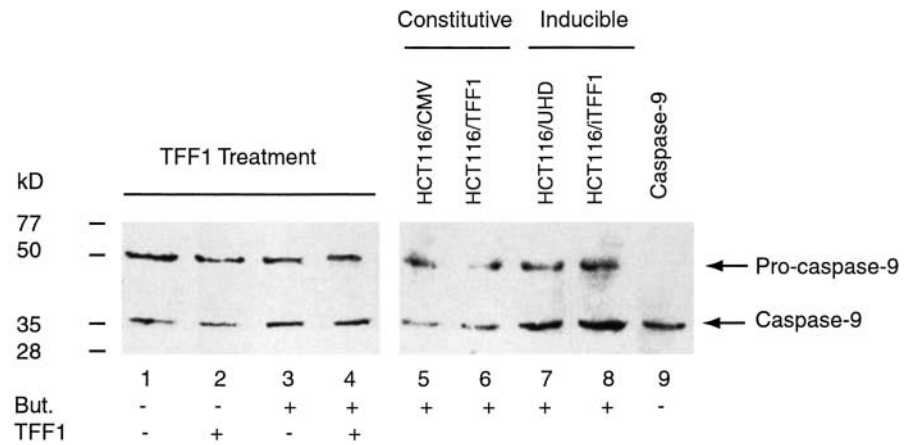
2000), the effect of TFF1 was also tested on apoptotic cells. Apoptosis was induced using either C2-ceramide (IEC18, HeLa), butyrate (HCT116 and AGS), staurosporine (Jurkat), Bad expression (IEC18, HCT116, and AGS), or anchorage-free conditions (IEC18, HCT116, and AGS). The number of viable IEC18, HCT116 and AGS cells decreased dramatically (30–75%) regardless of the nature of the apoptotic stimulus (Fig. 5). The most effective inducer of apoptosis was the anchorage-free condition (60–75% cell death).

The simultaneous addition of increasing concentrations of recombinant human TFF1 (0.1–10 μ M) to apoptotic inducers reduced the magnitude of the apoptosis. This antiapoptotic effect first detected at 0.1 μ M TFF1 was optimal at 10 μ M TFF1, and was more pronounced in HCT116 and AGS than in IEC18 cells (Fig. 5). Similarly,

Table II. TFF1 treatment and constitutive and induced TFF1 expression induce increased levels of cdk inhibitors

Ckis	TFF1 treatment			Constitutive			Inducible		
	-	+	P	-	+	P	-	+	P
INK4									
P15	2 \pm 0.8	5 \pm 0.2	<0.01	15 \pm 4.1	37 \pm 3.5	<0.01	17 \pm 0.4	18 \pm 0.5	NS
P16	44 \pm 6.1	61 \pm 2.3	<0.05	50 \pm 2.5	64 \pm 1.8	<0.05	37 \pm 2	46 \pm 1.5	<0.05
CIP									
P21	3 \pm 1	7 \pm 1.1	<0.05	14 \pm 3.5	20 \pm 4.5	NS	11 \pm 0.5	19 \pm 1.5	<0.05
P27	13 \pm 1.3	18 \pm 0.8	<0.05	3 \pm 0.2	5 \pm 1.5	<0.05	3 \pm 1	8 \pm 1.5	<0.05

Figure 7. TFF1 does not alter procaspase-9 cleavage. Western blot analysis of cell extracts (15 μ g protein) from butyrate-treated (But. +) parental (lanes 3 and 4) and stably transfected HCT116 cell lines expressing constitutive (lane 6) or doxycycline-induced (lane 8) TFF1. TFF1 treatment (10 μ M, TFF1 +) was as indicated. (Lane 9) Cleaved caspase-9 as positive control. Arrows indicate the inactive procaspase-9 (47 kD) and the active caspase 9 (37 kD). TFF1 does not modify procaspase-9 cleavage. Molecular weight scale is indicated on the left in kD.



transfected HCT116 cells expressing constitutive ($65 \pm 6.2\%$ vs. $50 \pm 6.2\%$ viable cells, $P < 0.01$), or induced ($67 \pm 6.7\%$ vs. $54 \pm 4.6\%$ viable cells, $P < 0.05$) TFF1 were less sensitive to butyrate-induced apoptosis than control cells. We noted that, for the doxycycline-induced TFF1, this represents a complete abolishment of the butyrate-induced apoptosis, as we previously observed that TFF1 expression alone reduces the cell number by $\sim 30\%$ (see first paragraph). TFF1 inhibition of induced apoptosis was not observed for the nongastrointestinal HeLa and Jurkat cells (unpublished data).

Thus, TFF1 treatment or synthesis prevents chemical-, Bad-, or anchorage-free-induced gastrointestinal cell apoptosis.

TFF1 decreases caspase-3, -6, -8, and -9 activities

Because apoptotic signals converge on the caspase cascade, the activities of caspase-1, -2, -3, -4/5, -6, -8, and -9 were investigated in HCT116 cell lines (Fig. 6). Butyrate-induced apoptosis of parental HCT116 cells led to an increase in the activities of caspase-2 (twofold), -3 (fivefold), -6 (threefold), and -9 (twofold), whereas there was no modification of caspase-1, -4/5, and -8 activities. Addition of TFF1 (10 μ M) almost completely inhibited butyrate-induced caspase-3 and -6 activities, and partially reduced caspase-9 activity, but had no effect on caspase-2. Anchorage-free conditions led to increased activity of all the caspases studied: caspase-1 (twofold), -2 (threefold), -3 (threefold), -4/5 (threefold), -6 (twofold), -8 (twofold), and -9 (3.5-fold). TFF1 treatment completely abolished the induced activity of caspase-6 and -8, and partially reduced that of caspase-3 and -9. However, TFF1 treatment had no obvious effect on caspase-1, -2, and -4/5 activities. Finally, Bad expression led to an increase in caspase-3 (2.5-fold), -6 (twofold), and -9 (twofold) activities that were totally abolished by TFF1 treatment. Similar results were observed in the apoptotic TFF1-transfected HCT116 cell lines. Both constitutive and doxycycline-induced TFF1 expression significantly reduced caspase-3, -6, -8, and -9 activities (Fig. 6). In the absence of apoptosis induction, TFF1 treatment of parental cells (10 μ M) did not modify the basal level of any of the caspase activities tested (unpublished data).

Thus, the antiapoptotic function of TFF1 occurs via the decrease of caspase-3, -6, -8, and -9 activities.

TFF1 does not prevent procaspase-9 activation

Caspases are produced from inactive procaspase forms that are activated by proteolytic cleavage by other caspases, leading to the caspase cascade. Caspase-9, an initiator of this cascade, is processed at the level of the apoptosome (Slee et al., 1999). We checked if TFF1-reduced activity of caspase-9 was due to a TFF1-induced inhibition of its proteolytic activation. The effect of TFF1 on the cleavage of pro-caspase-9 was studied by Western blot analysis of butyrate-treated cell extracts using an antibody that recognizes both the inactive procaspase-9 (47 kD) and the active caspase-9 (37 kD). Neither TFF1 treatment (10 μ M) of parental HCT116 cells (Fig. 7, lane 4), nor constitutive or induced TFF1 in transfected HCT116 cells (Fig. 7, lanes 6 and 8) reduced the quantity of mature caspase-9.

Together, these results indicate that TFF1 does not inhibit the processing of the inactive pro-caspase-9 to the active form.

Discussion

From in vivo and in vitro data, a tumor suppressor function has been proposed for TFF1 (Lefebvre et al., 1996; Calnan et al., 1999). In the present study, we investigated in IEC18, HCT116, and AGS gastrointestinal cells the possible involvement of TFF1 in regulating cell proliferation and cell death.

TFF1 exerts an antiproliferative effect via the pRb/E2F pathway

We have demonstrated that TFF1 represses cell progression towards the S phase of the cell cycle, and therefore proliferation. The transition between the G1 and S phase is tightly regulated (Herwig and Strauss, 1997; Ross et al., 1999; Malumbres and Barbacid, 2001; Zheng and Lee, 2001). At this checkpoint, a decision is made by the cell whether to proceed or not. pRb and E2F are major players in a regulatory circuit in the late G1 phase. E2F triggers S phase entry by activating promoters of S phase-regulated genes. A prerequisite for the pRb growth-suppressor function is binding of its unphosphorylated form to the E2F transcription factor, thus inhibiting E2F transcriptional activity, and arresting cells in G1. Consistently, the presence of TFF1 leads to a twofold increase in pRb activity that is associated with a twofold decrease in E2F transcriptional activity. These observations provide the first

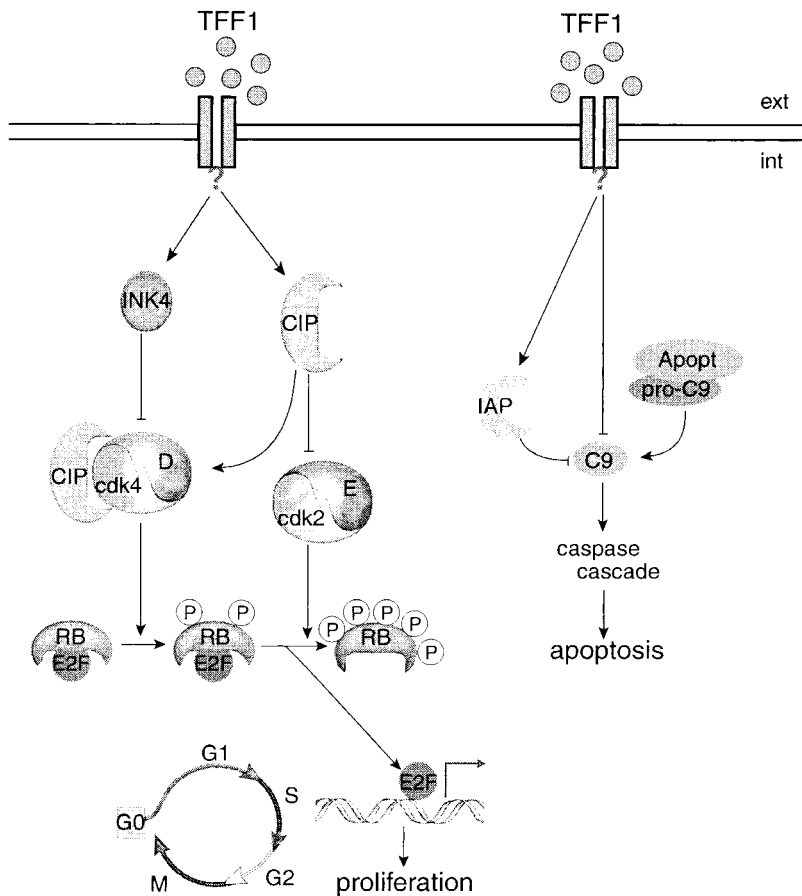


Figure 8. Schematic model of signaling pathways mediating TFF1 antiproliferative and anti-apoptotic function. Cells integrate external mitogenic or antimitogenic stimuli to decide whether or not to proliferate. Although the number and the nature of the molecular mechanisms participating in transduction of the external TFF1 stimulus remain unknown (?), we demonstrate that TFF1 triggers two types of responses. One function of TFF1 is to delay cell cycle commitment. TFF1 signaling results in increased levels of INK4 and CIP cdk inhibitors. Increased amounts of INK4 alter the distribution of cdk4 in favor of INK4/cdk4 and thereby reverse the sequestration of CIP. The release of CIP proteins leads to cyclinE/cdk2 inhibition, and a lower rate of pRb phosphorylation. This event results in the formation of pRb/E2F complexes, and subsequently, to the reduction of E2F transcriptional activity and reduced expression of E2F-responsive genes needed to drive cells through the G1/S transition. On the other hand, TFF1 signaling targets the active caspase-9, possibly via inhibitors of the IAP family, thereby down-regulating apoptosis. Apopt: apoptosome; C9, caspase-9; D and E, cyclins D and E; ext and int: extra- and intracellular.

evidence of a relationship between a member of the TFF family and cell cycle regulation. Moreover, it indicates that the effector of the antimitotic function of TFF1 is the pRb/E2F pathway (Fig. 8).

TFF1 signalling pathway targets Cdk inhibitors

Cdks associated with D-type cyclins, are the early executors that trigger the cell transition to S phase, via phosphorylation of target proteins including pRb. Our data suggest that TFF1 reduces the function of these cdks. Accordingly, TFF1 leads to an increased level of cdk inhibitors of both INK4 and CIP subfamilies. Members of the INK4 family (p16, p15, p18, and p19) specifically bind to cdk4 and inhibit pRb phosphorylation, thereby causing G1 phase arrest (Sherr and Roberts, 1999; Ashizawa et al., 2001). Interestingly, loss of p16 has been associated with progression of human gastric cancer (Myung et al., 2000). The function of cdk inhibitors of the CIP family (p21, p27, and p57) is more complex. They display mainly inhibitory activity towards the cyclin E/cdk2 complex which is required for correct E2F activity, and thus act to block S phase entry. However, in the absence of INK4 proteins, they can also bind cyclin D/cdk4 complexes which effectively sequester them, facilitating cyclin E/cdk2 function. Thus, the reduction of cell cycle commitment observed in the presence of TFF1 results from the upregulation of both INK4 and CIP cdk inhibitors (Fig. 8). Similarly, TGF β was shown to inhibit G1 cdks through the cooperative action of INK4 and CIP cdk inhibitors (Reynisdottir et al., 1995).

TFF1 exerts an antiapoptotic function

We observed that TFF1 does not induce apoptosis, as would be expected for a tumor suppressor gene, but in contrast, TFF1 was able to reduce induced apoptosis in gastrointestinal cells. In vivo, various apoptotic pathways are activated depending on the external stimuli. Thus, three types of apoptosis were tested. Apoptosis was first induced using butyrate treatment, whose action has already been reported in colon cancer cells (Ruemmele et al., 1999). The short-chain fatty acid (SCFA) butyrate is normally present in the gastrointestinal tract, as it is produced in the lumen of the colon by bacterial fermentation of carbohydrates (Mariadason et al., 2001). Because epithelial cells require matrix attachment for their growth, the second type of induced apoptosis was evaluated by maintaining the cells in anchorage-free conditions. This type of apoptosis, termed anoikis, causes the release of cytochrome c from mitochondria and is a strong activator of caspases (Rytomaa et al., 2000). Finally, we induced apoptosis by expressing Bad (Jan et al., 1999), a member of the Bcl-2 family that acts at the mitochondrial level and leads to the activation of procaspase-9. In all cases, TFF1 protected cells from induced apoptosis. Thus, the antiapoptotic function of TFF1 is independent of the nature of the apoptotic stimulus, suggesting that TFF1 signalling targets a common process.

TFF1 targets the active form of caspase-9

Caspases belong to two main subgroups: the initiator caspases (caspase-2, -8, -9, and -10) that activate procaspases, and the effector caspases (caspase-3, -6, and -7) in-

volved in the cleavage of various substrates essential for the life of the cell (Nunez et al., 1998). Caspase-1, -4, and -5 are peculiar, as they mainly mediate inflammatory signals. In our apoptosis-induced systems, TFF1 was found to partially or completely block caspase-3, -6, -8, and -9 activities, but had no effect on caspase-1, -2, and -4/5 activities. This indicates that TFF1 specifically targets only some caspases. A study of the kinetics of caspase synthesis during the anoikis process induced in normal human intestinal epithelial cells showed that the alteration of caspase-9 alone may lead to the inactivation of caspase-3, -6, and -8 (Grossmann et al., 2001). This scheme calls into question the number and the nature of caspases inhibited by TFF1. In any case, our data indicate that caspase-9, which is upstream in the caspase cascade (Slee et al., 1999), is a target of TFF1 signalling. Moreover, we demonstrated that TFF1 does not inhibit pro-caspase-9 processing (Fig. 8). Thus, it is tempting to speculate that the TFF1 pathway might target specific inhibitor(s) of the active form of caspase-9, such as inhibitors of apoptosis (IAP) and related proteins (Datta et al., 2000).

TFF1, a factor for gastrointestinal cell differentiation

Together, our results demonstrate that TFF1 has a complex role in that it is not only a potent inhibitor of proliferation, but also an efficient inhibitor of apoptosis. Similar dual and paradoxical functions have already been reported for tumor suppressor genes involved in cell differentiation. In this context, pRb, which is essential for correct retina ontogenesis, has been shown to block cells in G1 of the cell cycle and inhibit subsequently induced apoptosis signals (Herwig and Strauss, 1997; Ross et al., 1999; Zheng and Lee, 2001). The gastrointestinal mucosa is a tissue that has a high rate of cell turnover (Karam, 1999). Therefore, TFF1 represents an important player in regulating the balance between gastrointestinal cell proliferation, death, and differentiation. Consistently, in HT29-MTX and Caco2 models of differentiation, TFF1 expression occurs when cells reach confluency and start to differentiate (Gouyer et al., 2001; unpublished data). Interestingly, Caco2 cell differentiation was reported to involve a relative G1/S block associated with the suppression of cdk2 and cdk4 activities (Ding et al., 1998), increased pRb and decreased E2F activities; p21 and p27 cdk inhibitors are also directly implicated (Deschenes et al., 2001). Finally, confluent Caco2 cells become butyrate insensitive and apoptosis levels are stable (Mariadason et al., 2001). A TFF1 function as a differentiation factor is also consistent with *in vivo* observations. Actually, TFF1 is crucial for correct gastric ontogenesis, and is expressed in differentiated gastrointestinal cells (Ribieras et al., 1998). Moreover, its deficiency is associated with human gastric cancer (Park et al., 2000a, 2000b), and leads in the mouse to the overproduction of undifferentiated gastric cells (Lefebvre et al., 1996).

TFF1, an orphan ligand

TFF1 is a secreted peptide detected in gastric juice and urine, suggesting possible paracrine, autocrine, and/or endocrine functions for this protein. In the present study, we consistently observed that the addition of exogenous recombinant TFF1, or cellular synthesis of TFF1, leads to similar results, indicating that TFF1 signalling is active at both the paracrine and autocrine levels. We sometimes observed a lower efficiency of

TFF1 activity under autocrine conditions, which was probably due to the need for additional secretory steps. Moreover, doxycycline induction of TFF1 also requires the synthesis of the transactivator (Gossen et al., 1995). Our results call into question the mode of transduction of extracellular TFF1 signals to the interior of the cell. TFF1 internalization via the endosomal pathway has been discarded as a mechanism in MCF7 human breast cancer cells (Laurent-Matha et al., 1998), but remains to be studied in the context of gastrointestinal cells. For several years, it has been hypothesized that TFF1 could act by binding to specific receptors. The present study supports this hypothesis. Nevertheless, despite numerous studies, to date no canonical receptors have been identified (Thim, 1997; Ribieras et al., 1998; Hoffmann et al., 2001). However, it has been shown that TFF1 directly binds to mucins that constitute the mucus layer lining the gastrointestinal tract (Newton et al., 2000; Tomasetto et al., 2000). It remains to be seen if mucins can transduce TFF1 signalling.

Materials and methods

Preparation of recombinant human TFF1

Human TFF1 was produced in yeast *Pichia pastoris* and purified as previously described (Kannan et al., 2001). *P. pastoris* were cultured in synthetic Kapeli's medium.

Cell cultures

The human HCT116 (colon), HeLa (cervix), AGS (stomach), and Jurkat (T cell) cancer cell lines, and the rat normal intestinal epithelial cell IEC18 were obtained from the American Type Culture Collection. Cells were grown in DME, Ham's F12 50/50 mix, 10% FCS (AGS cells), DME supplemented with 0.1 U of bovine insulin per ml, 1 mM sodium pyruvate, and either 5 or 7.5% FCS (IEC18 and HCT116 cells, respectively), or a combination of 2.5% calf serum and 2.5% FCS (HeLa cells). Jurkat cells were grown in RPMI (Sigma-Aldrich) supplemented with 2 mM glutamine and 10% FCS. All the cells were grown in 5% CO₂ at 37°C.

Stable transfection of HCT116 cells

To produce stable cell lines expressing human TFF1, two plasmids were designed. BamHI and EcoRI/BamHI fragments (~280 bp) corresponding to the open reading frame of the human TFF1 cDNA (Accession no. x00474) were inserted into the BamHI site of the pCMV-neoconstitutive expression vector (Baker et al., 1990) and the EcoRI/BamHI sites of pUHD 10-3 doxycycline inducible expression vector respectively, thus generating the pCMV-hTFF1 and the pUHD-hTFF1 plasmids.

HCT116 cells were transfected by calcium-phosphate coprecipitation with the Hind III-linearized pCMV-hTFF1 or the Pvu I-linearized pUHD-hTFF1 vector. The pUHD172-1neo that encodes for the transactivator and neomycin resistance (Gossen et al., 1995) was cotransfected with the pUHD-hTFF1 vector. The pCMV and pUHD parental vectors were used as negative controls. The culture medium was changed after 24 h and, on the following day, the selection was initiated by addition of G418 (800 µg/ml) (Life Technologies, Inc.). After 2 wk, G418-resistant clones were subcloned in 24-well plates. The TFF1 synthesis was assessed after 48 h of incubation in serum-deprived medium (pCMV-hTFF1- and pUHD-hTFF1-transfected clones) or serum-deprived medium supplemented with 1 µg/ml of doxycycline (Sigma-Aldrich) (pUHD-hTFF1-transfected clones). For each clone, 10 µl of conditioned medium was analyzed by Western blot using the p2802 anti-human TFF1 monoclonal antibody (Rio et al., 1991). Electrophoresis was done in tris-tricine high-resolution peptide separation mini-gels as described (Promega). Two clones expressing TFF1 either constitutively (HCT116/TFF1a and HCT116/TFF1b) or under doxycycline induction (HCT116/iTFF1a and HCT116/iTFF1b) were selected together with corresponding negative controls containing the vector alone (HCT116/CMVa and HCT116/CMVb, and HCT116/UHDa and HCT116/UHDb).

Cell number assay

Cells were seeded on 96-well plates (5,000 cells/well) and cultured for 48 h in the presence of different concentrations of recombinant human TFF1 (0.1–50 µM). Cell viability was measured using the MTT method by

incubation at 37°C for 2 h in medium containing 500 µg/ml of MTT reagent (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; Sigma-Aldrich). This assay is based on the cleavage of the tetrazolium salt by viable cells. The reaction produces the accumulation of a water insoluble formazan salt proportional to the number of living cells in the well (Uchino et al., 2000). The formazan crystals formed were extracted with dimethyl sulfoxide (100 µl/well) and the absorbance at 570 nm was measured by a microplate spectrophotometer (Biorad Laboratories). Cells were treated with 10 µM of an unrelated peptide (GTVERTGDTGMLPDNYVEDI) or albumin (Fraction V; Sigma-Aldrich), as negative controls.

Cell cycle analysis

HCT 116 cells were enriched in G1 phase by a 1-h treatment with 25 µM olomoucine (Calbiochem) (Park et al., 1997). Then, cells were released in fresh medium supplemented or not with 1 µM TFF1 for 1 h. The patterns of cell DNA content were assessed by incubating cells overnight at 4°C in a solution containing 10 µg propidium iodide and 1 mg/ml of RNase A in PBS.

FACS analysis

Synthesis of several proteins related to the cell cycle was studied using rabbit polyclonal or mouse monoclonal antibodies directed against human retinoblastoma protein pRb (Pharmingen), PCNA (Dako), and cyclin D1, cyclin B1, p15, p16, p21, and p27 (Santa Cruz Biotechnology). Cells, fixed overnight in 80% of cold ethanol, were centrifuged (1,200 rpm, 5 min) and resuspended in 100 µl of 1% BSA in PBS and incubated with primary antibodies at 4°C overnight. Then cells were rinsed with 1% BSA in PBS, centrifuged and incubated 30 min at room temperature in 100 µl of 1% BSA in PBS containing either FITC-conjugated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) or the AlexaFluor™ 488 goat anti-mouse IgG (H+L) (Molecular Probes Europe BV). All experiments were analyzed with a FACScan flow cytometer (Becton Dickinson). Cells with fluorescence intensity >100 were considered as positive.

Luciferase reporter assays

Parental and stably transfected HCT116 cell lines were plated on 6-well plates (5×10^5 cells/well) and transfected by calcium phosphate coprecipitation with 1 µg/well of vectors containing either a specific response element for pRb (pRb-Ta-Luc) or E2F (pE2F-Ta-Luc) (Mercury Cell Cycle Profiling System; Clontech). The pTa-Luciferase (pTa-Luc) vector devoid of response element was used as negative control. 12 h after transfection, culture medium was replaced by fresh medium containing or not 10 µM of recombinant human TFF1 and in the case of HCT116/iTFF1 cell lines the medium was supplemented with 1 µg/ml of doxycycline. After 24 h, cells were lysed and cytosolic luciferase activity was quantified on a MicroLumat LB 96P luminometer (EG&G Berthold). In all cases, an RSV-β-galactosidase reporter gene was cotransfected in order to quantify the transfection efficiency. Results were expressed as relative luciferase activities compared to the luciferase activity of the empty vectors that was set at 1, and corrected for β-galactosidase activity.

Apoptosis induction

Cells were seeded on 96-well plates at 10^3 cells/well. Apoptosis was chemically induced by incubation for 48 h in butyrate (5 mM; HCT116 and AGS cells), C2-ceramide (50 µM; IEC18 and HeLa cells), or staurosporine (1 µM; Jurkat cells).

A three-dimensional spheroid culture system was used to induce anchorage-dependent apoptosis (anoikis) in IEC18, HCT116, and AGS cells. Briefly, 5×10^4 cells per well were plated on 6-well plates coated with 1% SeaPlaque agarose to which cells can not attach. Plates were incubated in humidified atmosphere at 37°C, 5% CO₂ for 3 d. Then, cell aggregates were mechanically dissociated and replated on normal 6-well plates and allowed to attach and form colonies. After 48 h, the number of viable cells was counted.

Finally, apoptosis was induced in IEC18, HCT116, and AGS cells by transfection with 0.25 µg/well of a Bad expression plasmid (Cell Signaling Technology, Inc.) using the Fugene™ 6 Transfection Reagent (Boehringer). After 12 h of incubation, cells were fed with fresh medium.

Whatever the nature of the apoptotic stimulus, experiments were performed in the absence or presence of various TFF1 concentrations or in cells expressing TFF1. Cell viability was assessed using the MTT method (see above).

Caspase analysis

Apoptosis was assessed by measuring caspase activities with the Caspase Substrate Set IV (Calbiochem) using the 7-amino-4 (trifluoromethyl) coumarin (AFC) fluorogenic substrate. This kit allows the analysis of caspase-1, -2, -3, -4/5, -6, -8, and -9. The assays were conducted as specified

by the manufacturers. In brief, cells (1,000 cells/well; 24 wells) were incubated 5 min in ice-cold lysis buffer (50 mM Hepes, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 100 µM EDTA, 10% glycerol) and centrifuged at 10,000 g for 10 min at 4°C. The total protein concentration of the cytosolic extract (supernatant) was defined using the Bradford method (Bradford, 1976). Cell lysates were then incubated with 10 µM of the appropriate substrate for 2 h, and the fluorescence was quantified by spectrofluorometry using an excitation wavelength of 400 nm and an emission wavelength range of 505 nm. Caspase activity was expressed as pmol AFC per hour per µg of total protein.

Western blot analysis of procaspase-9 processing

Apoptosis was butyrate-induced in parental and transfected HCT116 cell lines. 48 h after TFF1 treatment (parental HCT116) or doxycycline treatment of doxycycline inducible HCT116 cell lines, cells were centrifuged (2,000 rpm), and incubated 20 min in lysis buffer (20 mM Tris, 400 mM KCl, 20% glycerol and 2 mM DTT). After three freeze/thaw cycles in liquid nitrogen, lysates were centrifuged (10,000 rpm). Supernatants (15 µg protein) corresponding to cytosolic extracts were loaded on 10% SDS-Page gels, electrophoresed, electrotransferred onto nitrocellulose membranes, and analyzed using rabbit polyclonal antibody (1:2,000), allowing the detection of the procaspase-9 (47 kD) and the caspase-9 (37 kD) (Cell Signalling Technology). After washing (PBS 1×, 0.1% Tween 20), the blots were incubated with a peroxidase-conjugated secondary antibody (1:2,000; Cell Signalling Technology), washed again, and revealed using a chemiluminescence substrate (NEN Life Science).

Statistical analysis

All assays were performed in triplicate. Data are means ± SE and are representative of at least three independent experiments (nine assays). Results were analyzed by a Student's *t* test using Microsoft Excel 2000 and were considered significantly different if **P* < 0.05 or ***P* < 0.01.

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