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Identification of NF-kappaB responsive elements in follistatin related gene (FLRG) promoter

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Abstract

Follistatin related gene (FLRG) has been previously identified from a chromosomal translocation observed in a B-cell chronic lymphocytic leukemia (B- CLL). FLRG (alternative names: follistatin-related protein, FSRP/follistatin-like-3, FSTL3) is a secreted glycoprotein highly similar to follistatin. Like follistatin, FLRG is involved in the regulation of various biological effects through its binding to members of the transforming growth factor beta (TGFβ) superfamily such as activin A and myostatin. We have previously shown that TGFβ and activin A are potent inducers of FLRG transcriptional activation through the Smad proteins. Using a biochemical approach, we investigated whether tumor necrosis factor alpha (TNFα) could regulate FLRG expression since TNFα plays a critical role in hematopoietic malignancies. We demonstrate that TNFα activates FLRG expression at the transcriptional level. This activation depends on a promoter region containing four 107–108 bp DNA repeats, which are evolutionary conserved in primates. These repeats carry a strong phylogenetic signal, which is not common among non-coding sequences. Each DNA repeat contains one TNFα responsive element (5′-GGGAGAG/TCCT-3′) able to bind nuclear factor kappaB (NF-κB) transcription factors. We also show that TGFβ, through the Smad proteins, potentiates the effect of TNFα on FLRG expression. This cooperation is unexpected since TGFβ and TNFα usually have opposite biological effects. In all, this work brings new insights in the understanding of FLRG regulation by cytokines and growth factors. It opens attractive perspectives of research that should allow us to better understand the role of FLRG during tumorigenesis.

Keywords: TGF-beta; Leukemia; FLRG; FSTL3; Smad; NF-kappaB

Abbreviations: ATCC, American type culture collection; B- CLL, B-cell chronic lymphocytic leukemia; Bp, base pair(s); Ch, chromosome; ECM, extracellular matrix; EMSA, electrophoresis mobility-shift assay; FLRG, follistatin related gene; FSRP, follistatin-related protein; FSTL3: follistatin-like-3; I-B, inhibitor of kappaB; i.e., Id est; IKK, IκB kinase; kb, kilobase(s) or 1000 bp; MAPK, mitogen-activated protein kinases; MLP, adenovirus major late promoter; NF-κB, nuclear factor kappaB; s.d., standard deviation; SBE, smad binding element; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha; TNFR, TNFα receptor.

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1. Introduction

We previously reported the molecular characterization of a t(11;19)(q13;p13) translocation in a B-cell chronic lymphocytic leukemia (B- CLL) (Hayette et al., 1998). At the breakpoint on chromosome 19, we identified a new evolutionary-conserved gene, FLRG (follistatin-related gene, also named Follistatin-related protein, FSRP/follistatin-like-3, FSTL3). It encodes a secreted glycoprotein highly similar to follistatin. As described for follistatin, FLRG physically interacts with members of the TGFβ (transforming growth
factor beta) family such as activin A and myostatin to neutralize their biological effects (Bartholin et al., 2002; Hill et al., 2002; Maguer-Satta et al., 2001; Maguer-Satta et al., 2003; Sidis et al., 2006; Tsuchida et al., 2000). In addition, TGFβ and activin A are potent inducers of FLRG and follistatin transcripational activation through the Smad proteins (Bartholin et al., 2001; Maguer-Satta et al., 2001; Maguer-Satta and Rimokh, 2004). This observation, together with the antagonistic effect of FLRG and follistatin on activin A signaling, indicates that these two secreted proteins participate in a negative feedback loop that regulates activin A function. In vivo, FLRG would be involved in the development of gonads (Xia et al., 2004), placenta (Ciaramela et al., 2003) and virtually everywhere activin A has a role. More specifically, FLRG has been involved in the regulation of human hematopoiesis (Maguer-Satta and Rimokh, 2004). The physical interaction between FLRG and fibronectin regulates the adhesion properties of hematopoietic primary cells, and in particular, increases the adhesion of functional hematopoietic progenitors (Maguer-Satta et al., 2003; Maguer-Satta et al., 2006). In addition, FLRG protein may contribute to bone formation by inhibiting osteoclast differentiation from hematopoietic precursors (Bartholin et al., 2005).

Tumor necrosis factor alpha (TNFα) has been characterized as a serum factor causing necrosis of tumors (Carswell et al., 1975; Green et al., 1976). TNFα has been involved in numerous biological processes such as apoptosis, inflammation, viral replication and blood cells homeostasis (Aggarwal, 2003; MacEwan, 2002). TNFα binds TNFR1 and TNFR2 (TNFα receptor one and two) to activate various signaling pathways such as NF-κB (nuclear factor kappaB), MAPK (mitogen-activated protein kinases) or caspases pathways. The first characterized NF-κB member, NF-κB1 (p50; p105), is a nuclear protein initially found as a kappa immunoglobulin enhancer interacting protein in cells that transcribed immunoglobulin light chain genes (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b). Other NF-κB family members have been isolated such as Rel-A (p65), NF-κB2 (p52; p100), c-Rel and Rel-B. These factors are structurally related by an amino-terminal motif of about 300 amino acids, the Rel-homology domain. In absence of ligand (TNFα or interleukin-1, for instance), the inhibitor of kappaB (IκB) sequesters the Rel factors in the cytoplasm. Activation of TNFα receptors stimulates IκB kinases (IKK1/α and IKK2/β). Activated IKKs then phosphorylate IκB to trigger its degradation. In the absence of IκB, released Rel family members migrate into the nucleus to activate transcription.

Using a biochemical approach, we investigated whether TNFα and NF-κB could regulate FLRG expression. We demonstrate that FLRG is activated at the transcriptional level by TNFα and NF-κB. This activation depends on a promoter region that contains four 107–108 bp evolutionary conserved repeats. Each repeat contains one functionally active NF-κB binding element able to interact with NF-κB1 (p50) and Rel-A(p65) after TNFα stimulation. We also demonstate that TGFβ cooperates with TNFα to activate FLRG expression.

2. Materials and methods

2.1. Cell culture

The human hepatocellular carcinoma cell line HepG2, and the mink lung epithelial cell line Mv1Lu were obtained from the American Type Culture Collection (ATCC), and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 0.03% L-glutamine, 100 mg/ml penicillin and 100 mg/ ml streptomycin sulfate.

2.2. Plasmids and deletion constructs

Various FLRG promoter fragments were cloned into the luciferase reporter plasmid pGL3-MLP containing a minimal promoter consisting of a TATA box and the initiator sequence of the adenovirus major late promoter (MLP) provided by JM Gauthier (Glaxo, Wellcome laboratory, Les Ulis, France) as described in (Bartholin et al., 2001). FLRG promoter fragments specifically used in this study, (−3293/+6)Δ(κBx4), (κBx4) and (κBx1), were generated by PCR using pGL3-FLRG(−3293/+6)-MLP as a template and further cloned into pGL3-MLP. Δ(κBx4) deletion encompasses nucleotides −1925 to −1492 and κBx4 promoter fragment covers nucleotides −1940 to −1470. κBx1 fragment covers FLRG promoter from nucleotides −1599 to −1706 (H3 repeat, Fig. 3A). In the κBx1-mut fragment, the κB site was mutated as described in paragraph 2.5. Mammalian expression vectors for Smad3 and Smad4 were provided by P ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden). Mammalian expression vectors encoding NF-κB1(p50) and Rel-A(p65) were provided by P Jalinot (Ecole Normale Supérieure, Lyon, France). IκB(A32-A36) expression vector was a gift from A Israel (Institut Pasteur, Paris, France). Expression vector encoding CA-IKK2 was obtained from B Baumann (Ulm University, Ulm, Germany).

2.3. Transient transfections and reporter assays

HepG2 cells were cotransfected (Exgen 500, Euromedex, France) with the appropriate firefly luciferase reporter and pRL-CMV (Promega) as previously described (Bartholin et al., 2001). TNFα (10–20 ng/ml, R&D Systems) and TGFβ (10 ng/ ml, R&D Systems) were added 24 h after transfection and luciferase activity was assayed 48 h after transfection (“Dual luciferase assay kit”, Promega). The same amount of plasmid DNA was kept constant by the addition of “empty” vectors. All the experiments were performed in triplicate, and the luciferase activity was normalized with regard to the renilla luciferase activity expressed by the pRL-CMV vector.

2.4. Northern blots

Total cellular RNA was purified by the acid guanidium thiocyanate–phenol–chloroform method. For northern blot analysis, 10 mg of total RNA were size-fractionated in formaldehyde-1.2% agarose gels and transferred onto nylon filters. The probes were radiolabeled (32. P-dCTP) using the Rediprime labeling kit
(Amersham Pharmacia Biotech) and hybridized according to standard procedures. RNA integrity and equal loading was assessed by ethidium bromide staining of ribosomal 18S and 28S RNAs.

2.5. Electrophoresis mobility-shift assay (EMSA)

Nuclear proteins from Mv1Lu cells were treated for 30 min in the presence of TNFα (20 ng/ml) and/or TGFβ (10 ng/ml) and gel-shift assays were performed as previously described (Bartholin et al., 2001). Specifically, the wild type (wt-κBx1) and mutant (mut-κBx1) double-stranded DNA probes cover FLRG promoter from nucleotides −1599 to −1706 (H3 repeat, Fig. 3A). In mutant-κBx1 probe, the κB site was mutated (USE Mutagenesis kit, Amersham Pharmacia Biotech) into a SpeI restriction enzyme site (wt 5′-GGGGAGATTC-3′ to mut 5′-ACTAGT3-′) to facilitate further identification of the mutant clones. The SBE (smad binding element) probe (5′-GGTCAGAGCCAGACAAAAAGCAGACAGATTAGGCA- TCTGCCGGAAGGCCGGGGA-3′) contains three Smad Binding Elements (underlined) (Dennler et al., 1998). For supershifts analysis, we used commercial rabbit polyclonal antibodies (Santa Cruz Biotechnology) directed against NF-κB(350) (#sc-114x), Rel-A(p65) (#sc-109x), Smad4 (#sc-7154x) and Oct1 (#sc-232x).

2.6. Sequence alignment and phylogenetic tree estimation

The four human repeats were first aligned using Clustal W (Thompson et al., 1994). Each repeat was then used as query in a BLAST search (Altschul et al., 1990) to identify similar sequences among complete genomes. Three matches were found among Pan troglodytes (chimpanzee) genome and one among Macaca mulatta (rhesus macaque) genome. The eight sequences (+ in human 3 in chimpanzee 1 in macaque) were then aligned using Clustal W. A phylogenetic tree was estimated from this alignment using a Bayesian approach (software: MrBayes (Huelsenbeck and Ronquist, 2001) and a maximum likelihood approach (software: PHYML (Guindon and Gascuel, 2003)).

3. Results

3.1. TNFα up-regulates FLRG expression at the transcriptional level

To investigate the ability of TNFα to modulate FLRG expression, we performed a northern blot analysis using total RNA prepared from HepG2 cells treated or not with TNFα for 24 h. This approach revealed a large increase in the level of the two FLRG RNA transcripts (2.5 and 1.2 kb) in response to TNFα (Fig. 1A). We previously reported the cloning of a reporter construct containing FLRG promoter elements located between nucleotides −3293 and +6 (plasmid pGL3-FLRG(−3293/+6)-MLP) and driving the expression of a luciferase reporter gene (Bartholin et al., 2001). To test whether increased FLRG expression in the presence of TNFα resulted from increased transcription, we transfected HepG2 cells with the reporter construct pGL3-FLRG (−3293/+6)-MLP. TNFα treatment produces a 4-fold induction of luciferase activity, indicating an increase in de novo transcription of the FLRG promoter (Fig. 1B). In contrast, the empty vector (pGL3-MLP) is not sensitive to TNFα (Fig. 1B). In all, these results demonstrate that TNFα up-regulates FLRG expression at the transcriptional level.

3.2. Identification of TNFα-responsive elements in FLRG promoter

Identification of TNFα-responsive elements was allowed by the cloning of various FLRG promoter fragments into the pGL3-MLP reporter plasmid as previously described (Bartholin et al., 2001). TNFα sensitivity was assayed into HepG2 cells. Deletions up to position −1920 do not significantly change the induction of luciferase activity after TNFα treatment. In contrast, deletion up to nucleotides −1523 completely abrogates this response (Fig. 2A). This result suggests the existence of TNFα responsive elements between nucleotides −1920 and −1523. To validate the presence of such elements in this region, we deleted nucleotides −1925 to −1492 (pGL3-FLRG(−3293/+6)Δ(κBx4)-MLP reporter construct). This deletion abolishes TNFα-mediated FLRG transcriptional activation (Fig. 2B). To explore whether this ~400 bp region was sufficient to drive TNFα activation, we cloned the promoter region located between nucleotides −1940 and −1470 (pGL3-FLRG(κBx4)-MLP reporter construct). With this FLRG promoter fragment, we observe a 4-fold induction of luciferase activity (Fig. 2B). These results are consistent with the presence of TNFα-responsive elements between nucleotides −1920 and −1523 inside FLRG promoter.

Fig. 1. TNFα activates FLRG transcription. (A) Total RNA from untreated and TNFα-treated cells (24 h treatment) was subjected to northern blot. The filter was assayed for hybridization to radiolabeled FLRG cDNA (upper panel). Equal amounts of RNA were visualized by ethidium bromide staining of 28S and 18S ribosomal RNA (lower panel). (B) HepG2 cells were cotransfected either with the pGL3-MLP empty vector or the FLRG promoter-luciferase construct pGL3-FLRG(−3293/+6)-MLP and cultured in the presence of TNFα. The luciferase activity normalized with renilla luciferase activity expressed from a PRL-CMV cotransfected vector (internal control), is given as the mean±s.d. (standard deviation) of a representative experiment performed in triplicate. MLP, adenovirus major late promoter.
3.3. Evolutionary history of the TNFα-responsive elements found within FLRG promoter

Computer assisted sequence analysis revealed that the FLRG promoter region (nucleotides 1920–1523) sensitive to TNFα, was composed of four highly conserved 107–108 bp tandem repeats (Human genome, build 36.1, chrom 19: 625,498 to 625,924) (Fig. 3A). Very interestingly, these four conserved domains contain short sequences known to bind NF-κβ transcription factors (5'-GGGAGG/TTCC-3'). This observation strongly suggests that FLRG expression could be activated by NF-κβ factors in response to TNFα. Before testing this hypothesis, we attempted to shed light on the evolutionary origin of these four 107–108 bp tandem repeats. We first searched for homologous sequences among a wide range of genomes. Each of the four human repeats was used as query in a BLAST search against various complete genomes (Altschul et al., 1990). Highly similar sequences were only found among P. troglodytes (chimpanzee) and M. mulatta (rhesus macaque) genomes. Macaque’s genome displays a single repeat located within telomeric regions of chromosome 19. Chimpanzee’s genome contains three repeats, also located on chromosome 19 (Fig. 3B). The alignment of the FLRG promoter sequences from human, chimpanzee and macaque, suggests that early primate genomes had only one repeat while unequal recombination increased the number of repeats during the course of evolution (Fig. 3B). We next aligned these sequences and estimated a phylogenetic tree in order to better understand the origin of the four human repeats. Two phylogenetic estimation methods that rely on sound statistical basis converged to the same tree, which is compatible with the well-known phylogeny of these primates (Fig. 3C). The analysis of this tree suggests that three duplication events (d1, d2 and d3) occurred after the first speciation event (s1: macaque vs. (human, chimpanzee)) and before the second speciation event (s2: human vs. chimpanzee). The position of C4 and C5 in the tree indicates that these two repeats appeared after s2 (i.e., C4 and C5 are paralogous and both are orthologous to H4). C2 and C3 are absent in chimpanzee. According to the estimated phylogeny, these sequences were lost after s1. Intriguingly, this phylogeny is also compatible with the positions of the human and chimpanzee repeats on their chromosomes, i.e., the position of the tips in the tree is the same as the positions of the corresponding sequences on the genomes. Hence, while the method that is used to build the phylogeny ignores the position of the repeats on the chromosome, this information can be recovered from the phylogenetic signal carried by these sequences. The chance that a random phylogeny with the same number of taxa displays such features is small. In all, it is very likely that the proposed phylogeny accurately describes the succession of duplications and speciation events that governed the evolution of these sequences.

3.4. Involvement of NF-κB transcription factors NF-κBI(p50) and Rel-A(p65) in TNFα-mediated FLRG activation

Having determined that FLRG promoter sensitivity to TNFα depended on a genomic DNA region containing four NF-κB binding sites, we then asked whether NF-κβ transcription factors

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Fig. 2. Identification of TNFα-responsive elements within FLRG promoter. (A) and (B). HepG2 cells cultured in the presence or the absence of TNFα were transiently cotransfected with the indicated FLRG promoter reporter constructs. The luciferase activity normalized with renilla luciferase activity expressed from a pRL-CMV cotransfected vector (internal control), is given as a fold induction (mean±s.d. of three experiments) in the presence of TNFα. MLP, adenovirus major late promoter.
NF-κB1(p50) and Rel-A(p65) could regulate FLRG transcription. To this end, we cotransfected HepG2 cells with pGL3-FLRG (−3293/+6)-MLP reporter construct along with NF-κB1(p50) and Rel-A(p65) expression vectors. Co-expression of NF-κB1(p50) and Rel-A(p65) induces a 2-fold induction of luciferase activity (Fig. 4A). Further evidence for the involvement of NF-κB transcription factors in the TNFα-mediated activation of FLRG promoter was obtained by the use of an IκB mutant named IκB (A32-A36). This mutant cannot be degraded by the proteasome due to the lack of induced phosphorylation on serines 32 and 36. IκB (A32-A36) is considered as a “super-repressor” since interaction with NF-κB transcription factors cannot be released after TNFα treatment, thereby preventing nuclear accumulation of NF-κB and transcriptional activation. Transfection of IκB(A32-A36) expression vector significantly reduces the TNFα-mediated induction of luciferase activity from pGL3-FLRG(−3293/+6)-MLP (Fig. 4B). In order to mimic NF-κB-mediated transcriptional activation, we used a constitutively active IKK2 mutant (CA-IKK2) able to stimulate NF-κB transcription factors in the absence of ligand (Azotei et al., 2005). We cotransfected HepG2 cells with CA-IKK2 along with luciferase reporters consisting of the H3 domain containing either a wild type or a point mutated NF-κB binding site (pGL3-FLRG(κBx1)-MLP and pGL3-FLRG(κBx1-mut)-MLP). Expression of CA-IKK2 is sufficient to produce a 5-fold induction of luciferase activity from the pGL3-FLRG(κBx1)-MLP strengthening the crucial role of NF-κB in FLRG transcriptional activation (Fig. 4C). Interestingly, CA-IKK2 fails to activate pGL3-FLRG (κBx1-mut)-MLP reporter (Fig. 4C). This latter result proves the functional relevance of one NF-κB binding site identified inside FLRG promoter.

Fig. 3. Sequence alignment and phylogenetic tree estimation of the TNFα sensitive region present within FLRG promoter. (A) TNFα sensitive region present in FLRG promoter is composed of four tandem repeats of a 107–108 bp motif. The four repeats were aligned using a standard procedure. Each repeat contains one NF-κB binding site (κB site, underlined). Bp, base pairs; κB, NF-kappaB binding site; MLP, adenovirus major late promoter. (B) Schematic view of the alignment of macaque, chimpanzee and human FLRG promoter sequences. Position of the human repeats on chromosome 19 is annotated (build 36.1). Bases that are identical are shaded black. The dotted lines correspond to gaps in the sequences. Ch, chromosome. (C) Phylogenetic tree estimated from the alignment of the eight conserved domains. ‘s1’ corresponds to the speciation event that separates macaque from human and chimpanzee. ‘s2’ is the speciation event that separates human and chimpanzee. ‘d1’, ‘d2’, ‘d3’ and ‘d4’ designate duplication events.
Taken together, our results demonstrate that NF-κB transcription factors, NF-κB1(p50) and Rel-A(p65), are involved in the activation of FLRG promoter after TNFα treatment.

3.5. Binding of NF-κB1(p50) and Rel-A(p65) to FLRG promoter

We next investigated whether TNFα induced the binding of nuclear proteins to the NF-κB binding sites found within FLRG promoter. We performed a gel-shift assay using nuclear proteins prepared from cells treated or not with TNFα. We used radiolabeled DNA probes containing either wild-type (wt-κBx1) or mutated (mut-κBx1) NF-κB binding sites. These probes correspond to the H3 repeat (covering promoter region from nucleotides −1706 to −1599) (Fig. 3). The migration of the wt-κBx1 probe appears to be shifted in the presence of nuclear proteins prepared from TNFα-treated cells (Fig. 5A, lines 1 and 2). Intensity of this shifted band is dramatically reduced if we use the mutant probe (mut-κBx1) (Fig. 5A, lines 3 and 4). This result shows that NF-κB binding sites found within FLRG promoter bind nuclear proteins after TNFα treatment. To further identify these nuclear proteins, we performed gel-supershift assays (Fig. 5B). Antibodies against NF-κB1(p50) and Rel-A(p65) were added to the nuclear proteins prior incubation with the radiolabeled wt-κBx1 probe. Gel separation revealed that NF-κB1(p50) and Rel-A(p65) antibodies respectively abrogates (Fig. 5B, line 5) and supershifts (Fig. 5B, line 6) the DNA-protein complex that is normally observed after TNFα treatment (Fig. 5B, line 2). In contrast, antibodies against Smad4 and Oct1 used as negative controls do not impair the formation of this complex (Fig. 5B, lines 3 and 4). These results indicate that TNFα controls the formation of a DNA-protein complex containing endogenous NF-κB transcription factors NF-κB1(p50) and Rel-A(p65).
3.6. TNFα cooperates with TGFβ to up-regulate FLRG expression

In a previous work, we demonstrated that TGFβ was a potent inducer of FLRG expression via the Smad proteins (Bartholin et al., 2001). Here, we examined whether TGFβ and TNFα had a synergistic effect on FLRG expression. Northern-blot analysis reveals that accumulation of FLRG transcripts after TGFβ + TNFα treatment is more pronounced than the accumulation observed in cells treated with TGFβ or TNFα alone (Fig. 6A). Reporter tests reveal that TNFα and TGFβ alone produce a 5-fold induction of luciferase activity, whereas in combination, these two cytokines induce a 15-fold induction of luciferase activity (Fig. 6B). These results indicate that TGFβ and TNFα act in synergy to activate FLRG transcription. We next assessed a possible functional interaction between NF-κB and Smads proteins (Fig. 6C). Luciferase assays showed that overexpression of Smads (Smad3 + Smad4) and NF-κB (p50 + p65) respectively induce a 10-fold and a 2.5-fold induction of FLRG promoter. Interestingly, combined overexpression of Smad and NF-κB (Smad3 + Smad4 + p50 + p65) proteins induces a 15-fold induction of FLRG promoter. This observation suggests that NF-κB and Smads act in cooperation to activate FLRG transcription. To explore the molecular mechanism supporting this cooperation, we performed gel-shift assays by using the wt-κBx1 probe and nuclear proteins prepared from cells cultured with TGFβ and/or TNFα (Fig. 6D, left panel). Our results indicate that TGFβ neither induces the formation of a DNA-protein complex nor disrupts the DNA-protein complex that is normally observed after TNFα treatment. Another EMSA was performed in the same conditions with a probe containing three canonical Smad Binding Element or SBE (Fig. 6D, right panel). As expected, TGFβ induces the formation of a DNA-protein complex on the SBE probe. This complex is not disrupted by TNFα. All together, these gel-shift experiments indicate that 1) TGFβ and TNFα induce the formation of DNA-protein complexes only on their respective responsive elements, 2) TGFβ does not modulate the TNFα-mediated recruitment of NF-κB onto the κB sites found within FLRG promoter and 3) reciprocally, that TNFα does not modulate the TGFβ-mediated recruitment of Smad proteins onto canonical SBE sites.

4. Discussion

In this study, we developed a biochemical approach to determine whether FLRG expression was regulated by TNFα. We clearly demonstrate that FLRG transcription is activated after TNFα treatment through the NF-κB transcription factors. This activation relies on a promoter region that contains four 107–108 bp repeats (H1, H2, H3 and H4), which are evolutionary conserved in primates. Each repeat contains one NF-κB binding element that interacts with NF-κB transcription factors after TNFα stimulation. We have previously shown that TGFβ induced transcriptional activation of FLRG through the Smad proteins. Here, we demonstrate that TNFα and TGFβ cooperate to activate FLRG expression.

NF-κB transcription factors (Rel-A (p65), NF-κB1 (p50), NF-κB2 (p52), c-Rel and Rel-B) bind DNA consensus site (5'-GGGRNNYYCC-3', N = any base, R = G or A, and Y = C or T) (Hayden and Ghosh, 2004). The three κB sites (5'-GGGA-GATTC-3') respectively found within the H2, H3 and H4 repeats perfectly match with the consensus sequence described.
above whereas the κB site found within the H1 repeat (5′-GGGAGAGTCC-3′, degenerated base is underlined) contains one degenerated base (Fig. 3A). Ancestors of these four human repeats are only found among chimpanzee and macaque genomes. These repeats carry a strong phylogenetic signal, which is not very common among non-coding sequences. This signal helped us to decipher the evolutionary origin of the human FLRG promoter. The presence of tandem repeats suggests that the structure of FLRG promoter is likely to be subject to frequent rearrangements (most probably due to unequal recombination events). The phylogenetic analysis of these primate sequences seems to confirm this hypothesis: losses and duplications observed in closely related primates clearly suggest fast rates of evolution. At the functional level, it will be of particular interest to investigate if there is a correlation between the number of tandem repeats and sensitivity to TNFα, and if we can observe polymorphisms affecting the number of tandem repeats in patients with malignant blood diseases.

FLRG and follistatin are similar in many respects. Secreted FLRG and follistatin glycoproteins both interact with members of the TGFβ family such as activin A to neutralize their biological effects. FLRG and follistatin also share common regulation. Indeed, both genes are transcriptionally activated by TGFβ and activin A through the Smad proteins (Bartholin et al., 2001; Bartholin et al., 2002). Northern blots and reporter tests realized with a 2 kb follistatin promoter fragment revealed that follistatin was not activated by TNFα (data not shown). The absence of tandem repeats and NF-κB binding motifs within the follistatin promoter probably explain this observation. The functional significance of FLRG activation by TNFα is still unknown. Interestingly, activation of NF-κB factors has been reported in various hematological malignancies (Braun et al., 2006). More specifically, it has been shown that B cells from B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit higher levels of NF-κB activity than B cells from healthy donors (Cuni et al., 2004; Furman et al., 2000). Increased NF-κB

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**Fig. 6.** TNFα and TGFβ cooperate to activate FLRG expression. (A) HepG2 cells were treated with TNFα and/or TGFβ for 24 h. Total RNA from untreated cells and TNFα-treated cells were subjected to northern blot. The filter was assayed for hybridization to a radiolabeled FLRG cDNA probe (upper panel). Equal amounts of RNA were checked by ethidium bromide staining of 28S and 18S ribosomal RNAs (lower panel). (B) HepG2 cells were transfected with pGL3-FLRG(−3293/+6)-MLP reporter construct and cultured for 24 h with or without TGFβ and TNFα before luciferase assaying. (C) HepG2 cells were cotransfected with pGL3-FLRG(−3293/+6)-MLP and expression vectors for NF-κB(x1)(p50), Rcl-A(p65), Smad3 and Smad4 in different combination 48 h before luciferase assaying. In B and C, the luciferase activity normalized with renilla luciferase activity, is given as the mean ± s.d. of representative experiments performed in triplicate. MLP, adenovirus major late promoter. (D) EMSAs were performed using a labeled probe corresponding to the H3 repeat containing either a NF-κB binding element (wt-κBx1, left panel) or a labeled probe containing 3 Smad Binding Element (SBE, right panel) and nuclear proteins prepared from cells treated with TNFα or TGFβ as indicated.
activity in malignant cells up-regulates anti-apoptotic genes to promote their survival (c-FLIP, Bel-2, Bel-XL...). In that context, NF-κB inhibitors are efficient anti-cancer drugs (Braun et al., 2006). FLRG has been identified in a t(11;19) (q13: p13) translocation in a B-CLL (Hayette et al., 1998). The chromosomal breakpoint on chromosome 19 is located about 7 kb upstream of FLRG suggesting that this rearrangement could impair the regulation of FLRG expression (FLRG coding region is maintained on the derivative chromosome 19). FLRG was highly expressed in B cells from this patient. Normally, FLRG expression is not detected in the B cell compartment. These observations suggest that FLRG up-regulation in B-CLL cells could result from the constitutively high NF-κB activity in these malignant cells. However, whether FLRG expression in these malignant B-cells is directly involved in the tumor formation remains to be clarified. To address this question carefully, additional work is ongoing in our lab, in particular in blood cells. Besides, FLRG locus was found to be rearranged in one case of non-Hodgkin’s lymphoma B-cells (Hayette et al., 1998). This observation strengthens the idea that FLRG locus rearrangements are probably not random events in malignant blood disorders. The presence of tandem repeats suggests that the region of the chromosome 19, containing FLRG promoter may experience frequent recombination events. The relation between such instability of the FLRG promoter and the progression of certain cancers needs to be scrutinized more thoroughly. Finally, activin A is known to block the proliferation of hematopoietic cells (Zipori and Barda-Saad, 2001). So, it is tempting to speculate that in some malignancies, increased NF-κB activity would result in FLRG up-regulation to block the anti-proliferative effect of activin A.

We showed in a previous work that FLRG was a direct target gene of the Smad proteins in response to TGFβ (Bartholin et al., 2001). Here, we demonstrate that TGFβ cooperates with TNFα to activate FLRG transcription. The molecular mechanism of this cooperation is still unclear. Our results suggest that it neither involves the formation of a Smad/NF-κB protein complex at the NF-κB binding sites nor an increased DNA recruitment of NF-κB transcription factors. Indeed, in EMSA experiments, TGFβ does not impair the TNFα-induced complex that forms at the κB sites. However, we cannot rule out the possibility that other NF-κB members (p52, c-Rel, Rel-B) may be involved in a Smad/NF-κB protein complex. It is also possible that NF-κB/Smad interaction complex would occur through the previously characterized Smad binding element (SBE) present within FLRG promoter (Bartholin et al., 2001). Further experiments will address these questions as well as the possible indirect mechanisms that could explain the synergetic effect of TNFα and TGFβ to activate FLRG expression. From literature, several hypotheses can be proposed. The simplest explanation would be to consider that TGFβ and TNFα activate two very distinct signaling pathways (respectively Smad and NF-κB pathways) without a real biochemical connection. In contrast, Smad and NF-κB proteins, bound to their respective and distinct responsive elements, could physically interact at the chromatin level through the recruitment of shared transcriptional co-activators such as p300 (Kon et al., 1999). Alternatively, TGFβ could stimulate post-translational modifications of NF-κB factors to potentate their transcriptional activity (Schmitz et al., 2001). TGFβ and TNFα usually have opposite effects. Antagonistic properties of TGFβ and TNFα are commonly observed in various biological processes such as inflammation, immunosuppression or extracellular matrix (ECM) remodeling. TNFα and TGFβ inverse functions result from the transcriptional activation of genetic programs with opposite outcomes. TNFα inhibits fibrosis by reducing ECM deposition whereas TGFβ activates fibrosis by increasing ECM deposition. At the molecular level, in contrast to TNFα, TGFβ down-regulates the transcription of collagenases (involved in ECM degradation) and up-regulates the transcription of type I collagen (a major structural component of the ECM) (Verrecchia and Mauniel, 2004). The cooperative effect of TGFβ and TNFα to activate FLRG transcription appears in contradiction with these observations. Interestingly, other genes have also been reported to be co-activated by TNFα and TGFβ such as type VII collagen (Kon et al., 1999) and PAI-1 (Hou et al., 2004). Unfortunately, the functional significance of the co-activation of these genes by TGFβ and TNFα remains unclear as well.

In conclusion, this work brings new insights in the understanding of the modulation of FLRG expression by cytokines and growth factors. It opens attractive perspectives of research that should allow us to better understand the role of FLRG during tumorigenesis.

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