c-Myb modulates transcription of the α-smooth muscle actin gene in activated hepatic stellate cells

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STELLATE CELLS PLAY a key role in the pathogenesis of hepatic fibrosis (14, 36). Although we (5) and others (13, 15) have reported that quiescent stellate cells produce little collagen type I, activated (myofibroblastic) stellate cells display a high level of collagen α1(I) gene expression (13, 14, 36). Therefore, stellate cell activation is a critical step in hepatic fibrogenesis. Studies with primary cultures of adult rat stellate cells have provided evidence that cell type-specific growth regulatory mechanisms exist (41), but the cell-specific factors regulating stellate cell activation have only been partially identified (9). We found that oxidative stress is a common and indispensable step in the cascade of molecular events initiated by collagen type I matrix or transforming growth factor-α (TGF-α), resulting in stellate cell activation (31).

Although little is known about the mechanisms that modulate c-Myb activity, it has been suggested that oxidation of Cys13 could function as a molecular sensor for the redox state of the cell by affecting the DNA-binding affinity of c-Myb (38). In agreement with this hypothesis, we found that addition of purified redox protein Ref-1 (52, 53) to nuclear extracts from activated stellate cells inhibits their binding (presumably c-Myb) to the α-SMA-proximal E box (32). In this study, we show that c-Myb plays a major role in the transcription of the α-SMA gene in activated stellate cells. Moreover, c-Myb antisense RNA blocked the development of the α-SMA-expressing phenotype in quiescent stellate cells, whereas transfection of c-Myb-stimulated α-SMA expression in quiescent stellate cells.

METHODS

Cell cultures. Stellate cells were prepared from male Sprague-Dawley rats (400–500 g) by situ perfusion and single-step density Nycodenz gradient (Accurate Chemical & Scientific, Westbury, NY), as described previously (5, 11, 22, 23, 25). Cells were plated on collagen type I or EHS matrix (Matrigel; Collaborative Biomedical Products, Bedford, MA) tissue culture dishes, according to the experimental design, with the initial seeding of fat-storing cells at a density of 2 × 10^6/cm². Matrigel's major components are laminin, collagen IV, proteoglycans, entactin, and nidogen. It also contains TGF-β, fibroblast growth factor, and tissue plasminogen activator. Cells were cultured under an atmosphere of 5% CO2/95% air in tissue culture dishes using DMEM containing 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 10% FBS (GIBCO BRL, Gaithersburg, MD) and 10% FCS (Omega, Tarzana, CA). Medium was changed every 48 h for all conditions. Stellate cells were identified by their typical
c-Myb and α-SM smooth muscle gene expression

autofluorescence at 328-nm excitation wavelength, staining of lipid droplets by Oil Red, and immunohistochemistry with a monoclonal antibody against desmin (2). Greater than 95% of the cells were stellate cells. Freshly isolated stellate cells were transfected with the mammalian vectors expressing the protein or reporter of interest using lipofectin (GIBCO BRL), as described by the manufacturer. To increase the transfectability of activated cells, a transfection-enhancing reagent (Life Technologies, Gaithersburg, MD) was added in conjunction with lipofectamine as recommended by the manufacturer. The efficiency of transfection was determined using the pRSV-b-galactosidase vector (7).

The rat α-SMA/luciferase (LUC) chimeric reporter genes contained −724/+46 bp (−724 α-SMA/LUC), −271/+46 bp (−271 α-SMA/LUC), −271/+46 bp [−232/−231 mut] (−271 [−232−231 mut] α-SMA/LUC), −271/+46 bp [−219/−217 mut] (−271 [−219/−217 mut] α-SMA/LUC), −230/+46 bp (−230 α-SMA/LUC), −271/+230/46 bp (−271/−230 α-SMA/LUC), −224/−46 bp (−224 α-SMA/LUC), or −191/−46 bp (−191 α-SMA/LUC) from the 5′ region of the rat α-SMA gene (4) inserted into pGL3-Basic (Promega, Madison, WI), as described previously (23). The wild-type c-Myb and c-Myb Asp33 mutant clones (38) were inserted into the pcDNA3.1 mammalian expression vector (Invitrogen, San Diego, CA). The c-Myb antisense was derived by reversing the wild-type c-Myb insert. The total amount of transfected DNA was 2 μg. The transfection efficiency was 23 ± 5% for day 0 quiescent cells and 28 ± 8% for activated cells growing on a collagen type I matrix as described previously (11, 23, 25). Cells were either harvested for LUC assays at 24–48 h (23) or fixed at 72–96 h after transfection according to the experimental design.

Immunohistochemistry. Cells fixed with acetone and methanol (60:40) at −20°C for 20 min were immunostained as described previously (6, 7, 23). Monoclonal α-SMA and anti-rabbit β-galactosidase antibodies were obtained from Sigma (St. Louis, MO) and Cappel (Durham, NC). Oregon green and Texas red secondary fluorochromes were obtained from Molecular Probes (Eugene, OR). Fluorescent labels were visualized using a triple-channel Nikon microscope as described previously (6, 7, 23, 25). The number of SMA-positive cells was expressed as a percentage of total transfected cells. At least 100 transfected cells were analyzed per experimental point, and a minimum of two observers analyzed each immunohistochemical experiment independently, as described previously (7, 25). Negative control samples were processed in parallel under the same conditions but with omission of the first antibody. Hoechst 33342 was used as a nuclear counterstain.

Statistical analysis. All results are expressed as means ± SE. Student’s t-test was used to evaluate differences of the means between groups, with P < 0.05 considered significant.

RESULTS

To assess the role of c-Myb on the transcription from the α-SMA gene, we first characterized the cis-regulatory region within the α-SMA gene 5′ flanking sequences that is necessary for high expression of the α-SMA in activated stellate cells. Primary rat stellate cells cultured on a collagen type I matrix became activated (23, 25, 31) and were transfected on day 4 with α-SMA chimeric reporter minigenes containing −724 bp, −271 bp, −230 bp, −271/−230 bp, −224 bp, or −191 bp of the rat α-SMA promoter (4) and expressing LUC (Fig. 1A). To obtain optimal reporter expression, cells were harvested 48 h after transfection. In these activated stellate cells, transcription from α-SMA/LUC reporter genes was very high when the chimeric gene contained −724 bp or −271 bp of the 5′ flanking region of the α-SMA promoter (including both E boxes and the TGGTTTAC motif (distal to −224 bp); Fig. 1B). Truncation of the region containing the distal α-SMA E box to −224 bp (including the proximal E box) or −191 bp (including the CARG A and B boxes) eliminated the transcription inducibility of the α-SMA gene (Fig. 1B), which is characteristic of the early phases of stellate cell activation (9, 43). The core binding for c-Myb, CATAAGCA (−223/−216), which is distal to the proximal E box, is disrupted with the truncation at −224 bp. However, conservation of the c-Myb (and other cooperative transcription factors) cognate DNA with the truncation at −230 bp (only an additional 6 bp) leads to a much higher α-SMA/LUC expression (Fig. 1B). Moreover, mutation of the c-Myb binding site (A−219 → T; C−217 → T) (Fig. 1A) markedly inhibited transcription from the −271 α-SMA/LUC reporter gene (Fig. 1B). Furthermore, a −271 bp/−230 bp cis element (271/230 α-SMA/LUC), containing the distal E box but neither the c-Myb-binding region nor the proximal E box, had only background LUC expression. In addition to a role for the c-Myb cis element in the transcription from the α-SMA promoter, the TGGTTTAC motif (−233 to −226), immediately distal to the c-Myb binding site, also contributes to the α-SMA transcription in activated stellate cells. Mutation of this motif (TACTTATC) (−271 bp [232/231 mut] α-SMA/LUC) markedly decreases the expression of α-SMA/LUC (Fig. 1B).

Because c-Myb expression is induced during the early stages of stellate cell activation and c-Myb binds with high affinity to an oligonucleotide including the proximal E box (GCAGCT −218 to −213 bp) of the α-SMA promoter (5′-GATCATAAGCAGCTGAACCTACG-3′) (32), we investigated whether c-Myb is capable of stimulating transcription from the α-SMA promoter in quiescent stellate cells. Day 4 primary rat stellate cells, growing on an EHS matrix to prevent their spontaneous activation and the expression of the endogenous c-Myb (11, 25, 31), were transfected with a vector expressing wild-type c-Myb. Nuclear expression of c-Myb as determined by immunofluorescence (31) (data not shown) was sufficient to increase the basal transcription from the cotransfected −724 α-SMA/LUC reporter gene by 10.2 ± 1.3 (850 ± 52 vs. 2,376 ± 850 U/mg protein; P < 0.05) (Fig. 2). In addition, a c-Myb basic domain mutant (Cys31 → Asp) that binds cognate DNA with reduced affinity (38) behaved as a dominant negative when expressed in activated stellate cells (growing on a collagen type I matrix), markedly decreasing the −724 α-SMA/LUC reporter expression (2,670 ± 29 vs. 320 ± 130 U/mg protein; P < 0.05) (Fig. 3A).

These results suggest that c-Myb is both sufficient and necessary to stimulate, perhaps in concert with other transcription activators (21, 28, 31), a high level of transcription from the α-SMA promoter in activated stellate cells. We have previously reported that c-Myb antisense oligonucleotides inhibited the activation of stellate cells induced by TGF-α in a conditioned me-
dium (31). However, Burgess et al. (8) suggested that the antiproliferative activity of antisense c-myb-specific oligonucleotides, at least in smooth muscle cells (SMC), is not due to a hybridization-dependent antisense mechanism. Therefore, to circumvent this confounding issue (8), we transfected day 4 activated primary rat stellate cells, growing on a collagen type I matrix, with a vector expressing antisense c-myb RNA and assessed the expression of the 2724 bp α-SMA/LUC reporter. As we reported previously for c-myb antisense oligonucleotides (31), c-myb antisense RNA also blocked c-Myb expression. In agreement with the results obtained by expressing a dominant-negative c-myb mutant (Fig. 3A), expression of the antisense, but not sense, c-myb RNA inhibited by approximately fivefold the 2724 bp α-SMA/LUC reporter activity (Fig. 3A) and by approximately threefold expression from the 224 bp α-SMA/LUC reporter (data not shown). These experiments strongly support the notion that c-Myb is required for optimal expression of the α-SMA gene. Furthermore, these effects were selective for the 2724 bp and 271 bp 5' flanking sequences, since c-myb antisense RNA did not modify the (already modest) expression of the 2191 bp α-SMA/LUC reporter (data not shown). As expected, overexpression of c-myb in activated stellate cells, stimulated (approximately threefold) transcription from the 271 bp α-SMA/LUC minigene but not from the same construct with point mutations of the c-Myb binding site within the proximal E box (271 [232/231 mut] α-SMA/LUC) (Fig. 3B).

In addition, we assessed whether expression of the c-myb antisense RNA could also inhibit expression of the α-SMA/LUC minigene in quiescent stellate cells. Primary rat hepatic stellate cells were freshly isolated and cultured on an EHS matrix for 6 days as described in METHODS (11, 23, 25). Luciferase assay was performed as described by kit manufacturer and corrected for cellular protein content (11, 23). Results are means ± SE of quadruplicate samples and representative of 4 independent experiments. The 2724 bp α-SMA/LUC and 271 bp α-SMA were highly expressed compared with control (pcDNA), −271/−230 bp α-SMA/LUC, −271/219/217 mut) α-SMA/LUC, −224 bp α-SMA/LUC, and −191 bp α-SMA/LUC, P < 0.01 for 2724 α-SMA/LUC and 271 α-SMA/LUC; P < 0.05 for 271 [232/231 mut] α-SMA/LUC and 230 α-SMA/LUC.

Fig. 2. c-Myb stimulates expression of α-SMA/LUC minigene in quiescent stellate cells. Primary rat hepatic stellate cells were freshly isolated and cultured on an EHS matrix for 6 days as described in METHODS (11, 23, 25, 31). On day 4, cells were transfected with 2724 bp α-SMA/LUC and vectors expressing pcDNA (control) or c-Myb as described previously (11, 23, 25). Values are means ± SE for quadruplicate samples and representative of 4 independent experiments. P < 0.05 for c-Myb.
the endogenous α-SMA gene in stellate cells and, therefore, their activation (9, 13, 43). Day 0 stellate cells growing on a collagen type I matrix were transfected with vectors expressing β-galactosidase with either pcDNA (control) or c-myb antisense RNA. On day 4, cells were fixed and analyzed in a triple-channel microscope by immunohistochemistry with specific antibodies against β-galactosidase and α-SMA, as described previously (23, 31, 32). Nuclei were stained in blue with Hoechst 33342. As expected, control stellate cells transfected with pcDNA (together with the transfection indicator β-galactosidase; in red) became activated, judged by their myofibroblastic phenotype and expression of α-SMA (in green) (Fig. 4A, β-galactosidase). The transfectant β-galactosidase is shown in yellow because of the superimposition of the green α-SMA over the red β-galactosidase. Approximately 70% of the control cells transfected with pcDNA adopted an activated phenotype and expressed α-SMA (Fig. 4B). In contrast, the majority of cells expressing the c-myb antisense RNA (together with the cotransfected β-galactosidase) had the phenotype of quiescent stellate cells (nonmyofibroblastic; in red) (Fig. 4A). More importantly, expression of c-myb antisense RNA was sufficient to block the induction of the endogenous α-SMA gene, which is expected in day 4 primary stellate cells growing on a collagen type I matrix (13, 22, 25, 31, 32). Only <10% of the cells transfected with c-myb antisense RNA expressed α-SMA and had the activated phenotype (Fig. 4B). Omission of the first antibody resulted in negative immunofluorescence for both α-SMA- and β-galactosidase-positive samples, as described previously (6, 7, 23, 25, 31).

In addition, day 1 quiescent stellate cells growing on EHS were transfected with the indicator green fluorescent protein (in green) and either control β-galactosidase or c-myb. On day 4, cells were stained for α-SMA (in red). Although only ~2% of control cells expressed α-SMA (Fig. 5), ~40% of cells transfected with c-myb were α-SMA-positive (Fig. 5). However, unlike activated stellate cells growing on collagen type I (Fig. 4B), c-myb-transfected cells growing on EHS displayed a diffuse rather than a fibrillar α-SMA phenotype.

DISCUSSION

Expression of α-SMA and a myofibroblastic phenotype defines stellate cell activation (43). In this study, we have characterized some of the molecular mechanisms involved in the activation of stellate cells, an important step in hepatic fibrogenesis (13, 14).

We have previously reported that quiescent primary rat stellate cells, cultured on an EHS matrix, are activated by the generation of free radicals using ascorbic acid/FeSO₄ as well as by malondialdehyde, a product of lipid peroxidation (31). In addition, enhanced hepatic oxidative stress in animals treated with CCl₄ (31, 32) or in patients with chronic hepatitis C (26) was associated with stellate cell activation. Complementary results supporting the role of oxidative stress in
stellate cell activation include the finding that stellate cell activation induced by collagen type I matrix or TGF-β can be blocked by antioxidants, such as D-α-tocopherol or butylated hydroxytoluene (31). Furthermore, a pilot study suggests that D-α-tocopherol can prevent stellate cell activation in patients with chronic hepatitis C (26).

Several studies indicate that c-Myb plays an important role in cell differentiation and proliferation (34, 35). For example, regulation of c-Myb expression is critical for the growth and differentiation of the progeny of hematopoietic cells (1, 17, 50). c-Myb protein binds to a consensus cognate DNA (16) through three homeo domain-like regions (44) and activates the transcription of target genes (3, 29, 51). The molecular mechanism responsible for stellate cell α-SMA expression and activation in primary cultures growing on collagen type I (25, 31) in animals treated with CCl₄ (22, 31) and in patients with chronic hepatitis C (26) seems to be associated with increased c-Myb expression (26, 31, 32) and binding of nuclear proteins to the proximal α-SMA E box (31, 32).

The appropriate expression of the α-SMA gene requires the interaction of cell type-specific sequences within the promoter and transcriptional factors (18–20, 37, 45, 49). Although in SMC –125 bp of the 5′ flanking region are sufficient to confer high expression of the α-SMA gene, at least –271 bp are required in skeletal myotubes (45), which also express α-SMA. Critical cis-acting elements within the –125 bp of the α-SMA gene in SMC include the CArG boxes, which bind serum-response factors (45) and are necessary for ANG II inducibility (18). A mesenchymal transcription factor, MHox, mediates the ANG II stimulation of α-SMA expression (20). In addition, stimulation of α-SMA gene expression by TGF-β in SMC requires the...
interaction of the CArG A (−62 bp) and B (−112 bp) boxes, together with the TGF-β control element (−42 bp) (19).

Here we demonstrated that, unlike in SMC, high-level transcription of α-SMA/LUC chimeric genes in activated stellate cells requires more than −224 bp within its 5′ flanking region. This −271 bp 5′ region includes the E boxes, the TGTTTATC motif, and a c-Myb binding site (37, 45). In contrast, the presence of −224 bp (including the proximal E box as well as the CArG boxes) is not sufficient for efficient transcription from the α-SMA promoter in activated stellate cells. Studies including truncations, deletions, and mutations suggest which are the relevant cis elements within the −724 bp of the 5′ region of the α-SMA gene in activated stellate cells. Most likely, the −224 bp truncation disrupts the c-Myb binding site (−223/−216) because addition of only 6 bp (−230 α-SMA/LUC) restores, to a substantial extent, transcription from the α-SMA promoter. Indeed, mutation of the c-Myb binding site (−219/−217 bp mut), within the proximal E box, markedly inhibits expression from the −271 α-SMA/LUC reporter gene in activated stellate cells. We found that the TGTTTATC motif also plays a role in α-SMA transcription in activated primary rat stellate cells because a mutation of this site (TACTTATC) impairs expression of the α-SMA/LUC. Constructs displaying the distal E box and adjacent cis elements (−271/−230 α-SMA/LUC) had only a background level of expression.

Transfection of a vector expressing wild-type c-Myb in quiescent stellate cells, which express negligible quantities of nuclear c-Myb (31), was sufficient to induce a ~10-fold stimulation of the −724 α-SMA/LUC reporter gene. As expected, transfection of c-myc stimulated expression of the −271 α-SMA/LUC but not of the −271 [−219/−217 mut] α-SMA/LUC. Conversely, expression of a c-myc antisense RNA in activated stellate cells prevented, to a substantial extent, transcription from the −724 α-SMA/LUC and −271 α-SMA/LUC chimeric genes. These studies indicate that c-Myb plays an important role in the transcription of the α-SMA gene in stellate cells. In chicken, α-SMA gene expression also involves a conserved sequence motif at −225 to −233 bp (TGTTTATC) (37), which is included in the −724 α-SMA/LUC and −271 α-SMA/LUC constructs that are fully expressed in activated stellate cells. Therefore, it is conceivable that the c-Myb binding site (−223/−216) interacts with the adjacent TGTTTATC motif through the formation of a transcriptional unit involving c-Myb. Indeed c-Myb is able to cooperate with other transcription factors, including CCAAT/enhancer binding protein-β (28), which transactivates the collagen α1(1) enhancer (21).

As reported for SMC and myotubes (45), α-SMA-expressing cells, a construct containing more than −547 bp of the α-SMA promoter was also transcriptionally active in myofibroblastic stellate cells growing on collagen type I matrix. However, the −724 α-SMA/LUC construct was inactive in quiescent stellate cells growing on an EHS matrix. It remains to be determined whether repression of the α-SMA gene occurs in quiescent stellate cells through the MCAT motif as reported for other cell types (49).

Although little is known about the mechanisms that modulate c-myc expression, it has been suggested that oxidation of CysG could function as a molecular sensor for the redox state of the cell by affecting the DNA-binding affinity of c-Myb (38). The modulation of AP-1 proteins involving oxidative stress is mediated by the nuclear redox factor Ref-1 (52), which also functions as a DNA repair enzyme (53). Ref-1 stimulates DNA-binding activity of several transcription factors, including c-Myb, and may itself be under a posttranslational control that is sensitive to the redox state of the cell (53). The redox activity of Ref-1 is mediated through a conserved Cys amino acid motif (KCR) that is present in Fos, c-Jun, and related proteins. In c-Myb, redox changes probably affect the motif KQCR (which includes CysG) within the DNA binding domain. In agreement with this novel hypothesis (38, 53), we reported that oxidative stress affects the DNA-binding activity and expression of c-Myb (31). Although the molecular mechanisms remain to be elucidated, the increased expression of c-Myb could be achieved, for instance, by positive autoregulation of c-myc (39) through the redox modulation of c-Myb protein (53). We found that both the reducing agent dithiothreitol and the redox enzyme Ref-1 prevent the binding of c-myc in nuclear extracts of activated stellate cells to the α-SMA-proximal E box (31, 32), suggesting a redox mechanism that may involve c-Myb, as proposed in cell-free systems (38, 52, 53).

Because of the previous suggestion that oxidative stress may modulate α-SMA-proximal E box-binding activities in activated stellate cells through c-myc (32), we assessed whether the c-Myb basic domain (including CysG) is a molecular target necessary for activation. Day 0 stellate cells were cotransfected with a c-Myb AspG mutant and the α-SMA/LUC reporter gene. Expression of c-Myb AspG, lacking the redox sensor CysG (38), did markedly reduce transcription from the −724 α-SMA/LUC in activated stellate cells. These results suggest that modification of CysG within the DNA-binding domain of c-Myb is critical in stimulating α-SMA gene expression from its promoter. The signal-transduction pathway targeting c-Myb CysG is likely to involve either an oxidative modification, such as an aldehyde adduct (6, 10, 24, 42), or a nitrosylation of CysG (12, 48), because the CysG mutation would be refractory to either pathway. In this context, oxidative stress pathways are known to stimulate, at least in skeletal muscle (6), liver, and brain (M. Buck, unpublished observations), nitric oxide synthase expression and activity resulting in the synthesis of NO, which interacts with superoxide to generate peroxynitrite (33, 48), a compound highly reactive with sulfur-containing amino acids, such as Cys (12). However, NO, apparently in the absence of oxidative stress, downregulates, at least in rat lung fibroblasts, α-SMA expression (54).

What is the physiological relevance of c-Myb activity in the expression of the endogenous α-SMA gene during
stellate cell activation? We have previously suggested that c-Myb plays a critical role in the expression of α-SMA, on the basis of experiments in which c-myb antisense phosphorothioate oligonucleotides prevented α-SMA expression and the activation of stellate cells induced by TGF-α (31). However, this argument may not be valid because the antiproliferative activity of c-myb-specific oligonucleotides, at least in SMC, is not due to hybridization-dependent antisense mechanisms (8). Rather, a stretch of four contiguous guanosine residues, which is present in the antisense c-myb used by us (31) and others (46, 47), may be responsible for the sequence-specific but nonantisense antiproliferative effects of these oligonucleotides.

Because proliferation and activation of stellate cells are usually linked (23, 25, 31, 32) and c-Myb stimulates both the proliferation and activation of these cells (31), we studied the effects of c-myb antisense RNA on stellate cells. Our results indicate that expression of c-myb antisense RNA markedly inhibits stellate cell activation (and presumably proliferation), whereas transfection of c-myb into quiescent stellate cells stimulates α-SMA expression. It is conceivable that the antagonistic effects of phosphorylated Ser133 CREB and oxidatively modified c-Myb Cys43 on the stellate cell cycle (25, 31) are mediated through cyclin/cdk repression/derepression. For example, CREB-binding protein (27) is known to bind preferentially to the oncoprotein c-Myb or to CREB, depending on the cell cycle state of the cell (30, 40).

In summary, this study, in conjunction with our previous results (26, 31, 32), strongly suggests that c-Myb is a key transcriptional activator of the α-SMA gene in activated stellate cells by interacting with the proximal E box (and possibly other distal cis elements) and behaving as a redox sensor through its DNA-binding domain. These findings may facilitate development of therapeutic approaches to prevent stellate cell activation in chronic liver diseases.

We are indebted to Drs. G. K. Owens and C. A. McNamara for providing the genomic rat α-SMA DNA, −271 α-SMA, and −271 [232/231 mut] α-SMA and to Dr. O. S. Gabrielsen for the c-Myb and c-Myb Asp43 mutant constructs. We thank Tao Li for technical assistance and Amy King for the preparation of this manuscript.

This study was supported by the National Institutes of Health Grants DK-38652, DK-46971, and GM-47165 and by grants from the Department of Veterans Affairs. D. J. Kim was supported by a grant from II Song Foundation (South Korea), and M. Buck was supported by fellowships from the National Cancer Institute and the American Liver Foundation.

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Received 3 March 1999; accepted in final form 3 November 1999.

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