TC1 (C8orf4) is involved in ERK1/2 pathway-regulated G₁- to S-phase transition

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INTRODUCTION

Although previous studies have implicated a role for TC1 (C8orf4) in cancer cell proliferation, the molecular mechanism of its action is still largely unclear. In this study, we showed, for the first time, that the mRNA levels of TC1 were upregulated by mitogens (FBS/thrombin) and at least partially, through the ERK1/2 signaling pathway. Interestingly, the over-expression of TC1 promoted the G₁- to S-phase transition of the cell cycle, which was delayed by the deficiency of ERK1/2 signaling in fibroblast cells. Furthermore, the luciferase reporter assay indicated that the over-expression of TC1 significantly increased Cyclin D1 promoter-driven luciferase activity. Taken together, our findings revealed that TC1 was involved in the mitogen-activated ERK1/2 signaling pathway and positively regulated G₁- to S-phase transition of the cell cycle. Our results may provide a novel mechanism of the role of TC1 in the regulation of cell proliferation. [BMB reports 2008; 41(10): 733-738]

INTRODUCTION

TC1 (C8orf4), with the highest conservation across species focused on the ORF, was first identified in papillary thyroid carcinoma and encodes a nucleus-localized protein (1, 2). The structural characterization of the TC1 protein indicates that it belongs to an increasingly large group of proteins known to be "natively unfolded" and which are believed to perform pivotal functions in the areas of cell cycle control as well as transcriptional and translational regulation (3, 4). An increasing number of studies have demonstrated that TC1 is correlated with the proliferation of normal and cancer cells (4-7). Of these, one of the earliest transcriptional regulatory elements that connect ERK1/2 signaling to the transcription of Cyclin D1 remain to be fully defined.

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Fig. 1. The effects of FBS/thrombin on the transcription of TC1. NIH3T3, HEK293T and Hela cells were arrested in the G0-phase by a 24 hour-incubation in serum-free DMEM and stimulated with 1 U/ml thrombin or 10% FBS. The mRNA levels of TC1 were checked by Semiquantitative RT-PCR after 0, 6, 12 or 24 hours for HEK293T and Hela, and after 0, 1, 6, 12 hours for NIH3T3 cells. GAPDH was used as a loading control. The grayscale of the DNA band is annotated on the left of each gel image.

Fig. 2. The effects of ERK1/2 inhibitor (PD98059) on FBS/thrombin-induced upregulation of TC1. NIH3T3 cells were rendered quiescent (G0 arrested) by a 24 hour-incubation in serum-free DMEM. PD98059 (50 uM) was added into the culture 90 minutes before stimulating with 10% FBS or 1 U/ml thrombin. After a 12-hour stimulation period, the cells were harvested by trypsinization. Three quarters of the total number of cells were used for extraction of total protein and the levels of p-ERK1/2 were detected by Western blot analysis (A). The other cells were used to extract total RNA and the mRNA levels of TC1 were analyzed by real time PCR (B).

our data indicated that TC1 may be involved in the ERK1/2 pathway-regulated G0-phase progression through the regulation of the transcription of Cyclin D1.

RESULTS

TC1 levels were induced by mitogens
In order to investigate the effect of mitogens on the transcription of TC1, NIH3T3, HEK293T and HeLa cells were treated with 10% FBS and 1 U/ml thrombin, respectively, as described in the materials and methods section. The expression of the mRNA levels of TC1 was found to be upregulated in all 3 cell lines studied, under the stimulation of FBS/thrombin. However, the pattern and degree of upregulation varied depending on the cell lines (Fig. 1). These results suggested that mitogens were capable of inducing the transcription of TC1 in normal and cancer cells.

ERK1/2 signaling pathway partially mediated mitogen-induced upregulation of TC1 levels
It is well established that in most cell types, mitogenic signals are relayed from the cytoplasm to the nucleus by the nuclear translocation of the ubiquitously expressed p-ERK1 and p-ERK2 MAPK isoforms, after which they become involved in the regulation of the transcription of targeted genes (17, 18). In order to investigate whether mitogens upregulating TC1 levels utilized the same mechanism, PD98059, an inhibitor of ERK1/2 activation, was employed. After FBS/thrombin-stimulation, the levels of phosphorylated ERK1/2 were detected by Western blot analysis, using beta-actin as a loading control in this system. Accordingly, real-time PCR was used to precisely evaluate the transcription levels of TC1. As illustrated in Fig. 2, FBS/thrombin stimulated the phosphorylation of ERK1/2 and remarkably elevated the transcription of TC1 levels. However, when PD98059 was added into the system in conjunction with FBS/thrombin, a significant reduction in the levels of both phosphorylated ERK1/2 (Fig. 2A) and TC1 mRNA (Fig. 2B) was observed. These data indicated that mitogen induced the transcription of TC1 at least partially through the ERK1/2 signaling pathway.

TC1 was involved in cell cycle progression from G1- to S-phase
The ERK1/2 signaling pathway plays a pivotal role in the control of cell cycle progression (13). The continuous ERK1/2 activation in response to mitogenic signals induces positive regu-
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Fig. 3. The effects of TC1 on G0-phase progression. NIH3T3 cells were synchronized at G0-phase and stimulated with 10% FBS and/or transiently transfected with plasmid pcDNA3.1-TC1. To determine the effects of TC1 on ERK1/2-regulated G0 to S-phase transition, PD98059 (50 μM) was employed to inhibit the activation of ERK1/2. Twenty-four hours after transfection, the cells were harvested by trypsinization and fixed with ice-cold 70% ethanol overnight. The cell cycle phase distribution was checked by flow cytometric analyses (A). The statistical cell fractions in the G0/G1 phase (B) and S phase (C) are shown in the figure. The numbers represent the means ± S.E.M. of 3 independent experiments. Two asterisks indicate a significant difference at P < 0.001.

Fig. 4. The effects of TC1 on Cyclin D1 promoter-driven luciferase activity. pcDNA3.1-TC1 and CCD1-luc were transiently co-transfected into 293T cells and pRL-TK was transfected simultaneously as the internal control. Luciferase activity was measured 24 hours post-transfection, normalized to that of renilla and compared to the control. Experiments were repeated in triplicate and the results were expressed as the mean ± SD. Two asterisks indicated a significant difference at P < 0.001.

The over-expression of TC1 increased the transcriptional activity of Cyclin D1 promoter
Cyclin D1 is one of the most important molecules induced by ERK1/2 in the G1-phase progression (11, 20). However, the mechanism by which sustained ERK1/2 activation regulated the transcription of Cyclin D1 remains unknown. Interestingly, recent studies have demonstrated that the over-expression of TC1 was capable of increasing the mRNA levels of Cyclin D1 in cancer cells (2, 4, 6). To determine whether TC1 was a positive transcriptional regulator of Cyclin D1, we used the luciferase assay system to detect the effects of TC1 on the transcriptional activity of Cyclin D1 promoter by over-expression of TC1 in HEK293T cells. A schematic diagram of the Cyclin D1 promoter-driven luciferase activity is shown in Fig. 4A. The
results showed that the over-expression of TC1 increased the luciferase activity significantly (Fig. 4). This result implied that TC1 was able to upregulate the transcriptional activity of the Cyclin D1 promoter.

**DISCUSSION**

We have shown that the transcription of TC1 was upregulated by mitogens (FBS/thrombin) and that this upregulation was mediated at least partially by the ERK1/2 signaling pathway (Fig. 1, 2). It is already well established that the ERK1/2 signaling pathway is one of the key transduction pathways responsible for mitogenic signals and for relaying of information to the cell cycle regulatory system (21). Persistent ERK1/2 activation throughout the G1-phase in response to mitogenic signals is required for S-phase entry. Even when the continuous activation of ERK1/2 is inhibited in late G1-phase, the cells cease to enter the S-phase (19). Furthermore, several lines of evidence have demonstrated that sustained ERK1/2 activation plays a pivotal role in the induction of positive regulators of the cell cycle and inactivation of anti-proliferative genes (22). Accordingly, the upregulation of TC1 by mitogen-activated ERK1/2 signaling suggested that it may play a role in the G1-phase progression as a novel downstream target of ERK1/2. Interestingly, our data demonstrated that the over-expression of TC1 promoted the G1- to S-phase transition which was delayed by the suppression of ERK1/2 activation (Fig. 3). This further supported the role of TC1 in the ERK1/2 pathway-regulated G1-phase progression. It is well-known that abnormal cell proliferation is essentially attributable to aberrant control of the cell cycle and that G1- to S-phase transition is the first restriction point in cell cycle progression (12). These results suggested, therefore, that TC1 may regulate cell proliferation through the modulation of the G1-phase progression.

Previous studies showed that phosphorylated ERK1/2 was able to activate nuclear targets such as Elk-1 which upregulated immediate-early genes such as c-fos, and then this resulted in the regulation of subsequent induction of delayed early genes (22, 23). One of the most important delayed early genes is D-type cyclins which result in the synthesis of proteins required for S-phase entry into the cell cycle (22). Additionally, the over-expression of Cyclin D1 is one of the most commonly observed genetic aberration in the passage from G1- to S-phase in human cancers (24). In agreement with this, transgenic animal models with parathyroid-targeted over-expression of Cyclin D1 have further confirmed the role of Cyclin D1 in mammalian cells, pDNA3.1-TC1 was constructed by inserting the full cDNA of TC1 into pcDNA3.1 (+) (invitrogen) vector at BamHI and Apal sites. The Cyclin D1 reporter constructs used for luciferase assays termed CCD1-luc, contained the human Cyclin D1 promoter from −958 to +154 cloned upstream of the luciferase gene of the pGL3-Basic (Promega) reporter construct at KpnI and BglII sites as described previously (15).

**Real-time and semiquantitative RT-PCR**

The mRNA expression levels of TC1 after stimulation by mitogens were tested after 0, 6, 12 and 24 hours in HEK293T and HeLa cells, and after 0, 1, 6 and 12 hours in NIH3T3 mouse fibroblast cells. Total RNA was extracted using Trizol reagent (invitrogen) and cDNA was synthesized using primeScript RT reagent kit. Real-time PCR was performed using the following primers: hTC1, 5'-CAA GCC ATC ATC TTG TCTAC-3' and 5'-GAG CCC ACT GGC TTT CTT-3'; mTC1, 5'-ACC AGC ATG TCC TCG TCT-3' and 5'-GGT GAG TCC AGC GAC-3'; mGAPDH, 5'-GAC CAC AGT CAC ATG TCC AC-3' and 5'-GGT GCC ACC GAC GCC TTT CTT AC-3'; mGAPDH, 5'-GAC CAC AGT CCA TGC CAT CAT-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'; hGAPDH, 5'-ATT CAA CGG CAC AGT CTA GTG ATG TTG CTT CT-3'; hGAPDH, 5'-GAC CAC AGT CCA TGC CAT CAT-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'; mGAPDH, 5'-ATT CAA CGG CAC AGT CTA GTG ATG TG AAG TGG-3'. NIH3T3 cells were rendered quiescent (G0 arrested) by a 24 hour-incubation in serum-free DMEM. PD98059 (50 μM) was added to the culture 90 minutes before being stimulated with 10% FBS or 1 U/ml.
thrombin. The expression levels of TC1 were measured using real-time PCR analysis after 10% FBS treatment for 12 hours. Quantitative PCR was performed using a continuous fluorescence detecting thermal cycler ABI PRISM 7000 Sequence Detection System (ABI, Foster city, CA), and a SYBR Green real time PCR master mix (TAKARA) as described previously (26). Measurements were carried out in triplicate using the beta-actin gene as an endogenous control.

Western blotting
The cells were harvested, washed with PBS and lysed using RIPA buffer with protease inhibitors for 30 minutes on ice. Total protein (20 μg) was run on a 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in tris-buffered saline tween-20 (TBS-T) with 5% BSA (bovine serum albumin) for 1 hour at RT (room temperature). It was then incubated with the primary antibodies overnight at 4°C. The membrane was finally incubated with the goat-anti rabbit HRP (horseradish peroxidase)-conjugated secondary antibodies for 1 hour at RT. Immunoreactive bands were identified using the SuperSingal west pico chemiluminescent substrate (Pierce) and exposed to X-rays films (Kodak) as described previously (27).

Flow cytometric analyses of DNA content
NIH3T3 cells were plated onto 6-well dishes and incubated to 90% confluency. Subsequently, the medium was replaced with FBS-free medium and cultured for 24 hours to synchronize the cell cycle at the G0-phase. The cells were then transiently transfected with 4 μg plasmid pcDNA3.1-TC1 or corresponding vehicle. This was followed by the application of 50 μM pD98059 90 minutes before the addition of 10% FBS. After 24 hours, the treated cells were removed from culture dishes by trypsinization, collected by centrifugation, and washed with PBS twice. Equivalent number of cells (1 × 10^6) from each sample were fixed with ice-cold 70% ethanol and incubated on ice for at least 30 minutes. The cell cycle distribution of G0/G1- and S-phase was analyzed by flow cytometric as described previously (28).

Luciferase assay
For the CCD1-luc expression assays, HEK293T cells were seeded at a density of 1 × 10^5 cells per well in 6-well plates and co-transfected with 1 μg of reporter CCD1-luc together with 3 μg of the relevant expression vector, or the corresponding empty vector. For experiments conducted in the exponentially growing cells, luciferase activity was measured 24 hours after transfection. Normalization was achieved by co-transfecting 0.1 μg of pRL-TK, a renilla reporter construct as an internal control for the transfection efficiency. Luciferase and renilla activities were measured according to the Promega protocol. The reporter luciferase activities were normalized against the renilla activity.

Statistics
The results were presented as means ± SD or means ± S.E.M. Differences were tested by T-test in statistical software SPSS11.0 and P < 0.05 was taken as the level of significance.

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