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Repression of CCSP Expression by KLF4

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Clara cell secretory protein (CCSP) plays an important role in protecting the lungs from inflammation. This research focuses on identifying the cis-element for binding the repressor of CCSP gene expression. A DNase I footprinting experiment revealed three protected regions between -812 and -768 bp (45 bp) of the mCCSP promoter. One motif (D3: GCCTGGGAA) was 100% conserved across rat, hamster, and human. The addition of excess amounts of the D3 motif exhibited high competition within that 45 bp range in an electrophoretic mobility shift assay. However, when mutated D3 (GAATGTTAA) was used, the competition was significantly reduced. This demonstrates that the D3 motif within that 45 bp region of the mCCSP promoter is an important site for the protein-DNA interaction. Transient transfection assays with -756 Luc resulted in highly decreased expression of CCSP than those with -812 Luc, suggesting that the 45 bp could function as a binding site for the repressor. Co-transfection of KLF4 exhibited significant repression of the -812 Luc but not the -768 Luc which clearly shows that KLF4 might function as a repressor for the CCSP gene and also suggests that the D3 motif is strongly involved in the binding of KLF4. In addition, when anti-KLF4 antibody was added, super-shifted bands were observed. This result demonstrates that KLF4 could function as a repressor by binding to this 45 bp region of the CCSP promoter and that the D3 motif might be involved in the specific binding of KLF4.

Key words : Clara cells, CCSP, KLF4, lung, repression

Introduction

Airway epithelium of the lung plays a role in protecting lung against external stimuli and in regulating airway inflammation. The Clara cell is one of the epithelial cell types whose function is to protect the lung from the inflammation [9, 15]. The Clara cells are domed shaped and non-ciliated secretory cell of the bronchiolar epithelium [2]. The Clara cells contain many types of secretory granules and they produce many secretory proteins such as surfactant proteins and Clara cell secretory protein (CCSP) [9, 12]. The CCSP is the major secretory protein of the Clara cells and play anti-inflammatory role to protect bronchiolar epithelium from the outside environment [10]. The gene expression of CCSP is only observed in Clara cell and the promoter region of CCSP gene might be responsible for this restriction. Many promoter analysis of on the CCSP expression have indicated that several transcription factors are involved in the regulation of the CCSP expression [9, 11, 13]. Most of researches on the promoter analysis of the CCSP gene have focused on the positive regulator (activator) of the CCSP expression. However, functional studies on the negative regulator (repressor) of the CCSP gene have not thoroughly investigated. This research has focused on the identification of a negative cis-element of the distal promoter of the CCSP gene. Furthermore, transcriptional repressor which could bind this cis-element will be investigated on the promoter of the CCSP.

Kruppel-like factor 4 (KLF4), also known as gut-enriched Kruppel-like factor is belong to a family of transcription factors containing zinc fingers [1, 4, 8]. Interestingly, KLF4 can function as activator or repressor in transcription of several genes [6, 14]. KLF4 can also function as a tumor suppressor in some cancers including lung cancer [5, 7]. In this study, the functional role of KLF4 on the gene expression of CCSP in Clara cells will be investigated.

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Materials and Methods

Dnase I in vitro footprint and Sequence analyses of CCSP promoter

The mouse CCSP promoter sequences, spanning bp -812 to -768 was PCR amplified and PCR product was ligated

to the TA cloning vector (PCRII, Invitrogen) and DNA fragment was isolated by digesting with *Hind*III and *Kpn*I. The end labeling was performed with Klenow and [^{32}P] dATP. The DNase I reaction was followed by the manufacture's protocol (Promega, Footprinting system). Nuclear extracts were obtained from mouse transformed Clara cells (mtCC) and were carried out by incubating the end-labeled probe with increasing amounts of nuclear extract from mtCC. After digestion with DNase I, reaction products were electrophoresed and sequencing reaction was performed in parallel in the same gel for the sequence identification of the protected regions. Genomic sequences of the CCSP genes from four different species of animals were obtained from Gen Bank database (mouse: L24372.1, rat: X51318.1, hamster: Y13765.1 and human: X59875.1).

EMSA and super-shift assay

Nuclear extracts from mtCC cells were prepared by the procedure described earlier [10, 11] and 5mg of nuclear extracts were used for the electrophoretic mobility shift assay (EMSA). Synthetic probes for EMSA were prepared by heating complementary oligonucleotides to 95℃ for 5min and then cooled and labeled with ^{32}P-dCTP using a Sequenase reaction kit (USB Corporation). The EMSA was performed by incubating the labeled probes for 15min with a binding buffer (Promega). To confirm whether the observed protein-DNA complexes were specific binding, 100- fold molar excess of un-labelled probes was added to reactions as a competitor. For super-shift assay, goat polyclonal KLF4 antibody was added to the binding reaction at 4℃ for 15 min before the addition of labeled probe. The protein-DNA reaction complexes were separated from unbound DNA by polyacrylamide gel electrophoresis and dried onto filter paper and exposed to auto-radiographic film.

Cell culture and transient transfection assays

MtCC were cultured at 37℃ in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. DMEM and FBS were purchased from Gibco/BRL. Trypsin and antibiotics were obtained from Sigma. The -812-Luc construct [10, 12], which contains 812 bp of mCCSP promoter linked to the luciferase reporter (Luc) (Promega), was used as a template to generate -768-Luc construct. The PCR amplified DNA fragment which remove the 45 bp spanning between -812 and -768 bp was cloned into pGL3-Basic Luc (Promega). For transient transfection assays, cells were transfected with a mixture of reporter vector and Superfect (Qiagen) reagent. For co-transfection assays with KLF4, the cells were transfected with a fixed or various amounts of expression vector(s) and empty vector was used as a control. Transfected cells were incubated for 4 hr and then washed with DMEM and incubated for 24 hr at 37℃. The cells were harvested and re-suspended in cell-lysis buffer (Promega) and protein concentration was measured using BCA protein assay kit (Pierce). Luciferase (Luc) activities were measured using kit from Promega.

Results

Protected 45 bp region by DNase I footprinting assay

When DNase I foot printing assay was conducted to determine *cis*-elements within the promoter region of the mCCSP, three protected regions were identified between -812 and -768 bp of the promoter. Protected region was localized in the distal region of mCCSP promoter and named GGCCAAGAG as D1, GGAGACTAA as D2 and GCCTGGG AA as D3 (Fig. 1). These three regions exhibited the expected protection patterns to the increasing amounts of nuclear extracts from mtCC. To further study *cis*-elements within distal promoter region, DNA sequences of CCSP promoter from different species including rat, hamster, and human were compared. When the 45 bp (Fig. 2A) of the mouse CCSP promoter was aligned with other animals, a 39 bp region

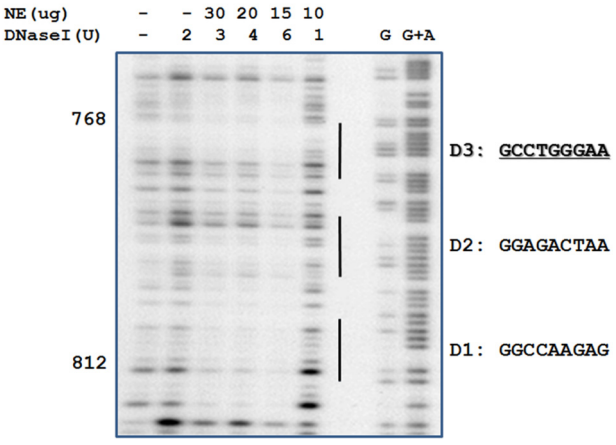

Fig. 1. DNase I foot printing assay of distal promoter region the mCCSP promoter. (A) Three protected regions between bp -812 and -768 of the mCCSP are shown. The amount of nuclear extract NE (ug) and DNase I (Unit) were marked. The sequences of the mCCSP promoter in parallel and the sequences three protected regions are marked (D1, D2 and D3).

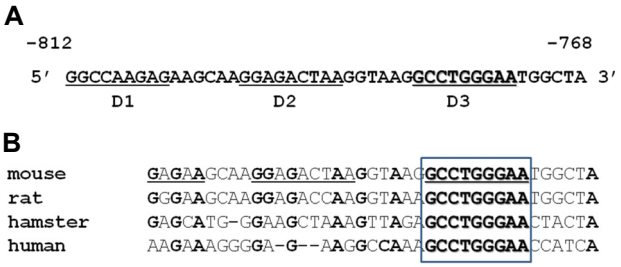

Fig. 2. The sequences of 45 bp (from -812 to-768 bp) of the distal promoter region. (A) The sequences of three protected regions are underlined (D1, D2 and D3). (B) Genomic sequences alignment of 45 bp of the distal promoter region the mCCSP promoter (GeneBank # of mouse: L24372.1 rat: X51318.1, hamster: Y13765.1 and human: X59875.1). The sequences conserved in all animals were bald. The sequence of conserved D3 motif (GCCTGGG AA) was boxed and underlined.

displayed some degree of homology between four species (Fig. 2B). Both D1 and D2 motif showed a lower homology with rat, hamster and human. However, D3 motif (GCCT GGGAA) exhibited 100% homology with other three animals. This finding suggests that highly conserved D3 motif might be an important *cis*-element for the CCSP promoter. Further examination of D3 motif (GCCTGGGAA) revealed that D3 motif contains high GC rich sequences, which is well known as binding site for the SP1 family transcription factor [1, 6].

EMSA with 45bp

Electrophoretic mobility shift assay (EMSA) was performed with 45bp which contain D1, D2 and D3 motif and excess (100 X) amount of D1, D2 or D3 motif was added as a cold competitor (Fig. 3). When D1 or D2 motif was added as cold competitor, they both exhibited lower degree of competition with 45 bp (Fig. 3A). Interestingly, when D3 motif (GCCTGGGAA) was added as cold competitor, it exhibited a very high competition with 45bp. However, when mutated D3 motif (GAATTGTTAA) was added as cold competitor (mD3 in Fig. 3A), it showed a much less completion than wild type D3 motif. It suggests that high GC rich contents in D3 motif are important for the binding of *trans*-element in the CCSP gene expression.

45 bp as KLF4 biding site by transient transfection assays

Previous researches on the promoter region of the CCSP gene revealed several transcription factors were responsible for the specific expression of this gene [3, 12, 13]. However,

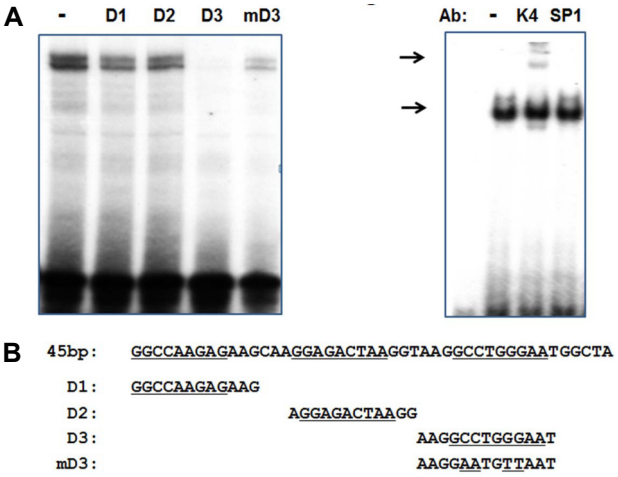

Fig. 3. Electrophoretic Mobility Shift Assay (EMSA) with 45 bp. (A) 5 ug of nuclear extracts from mtCC cells were used and 100- fold molar excess of un-labelled probes was added. (B) The sequences of synthetic probes used for competitor. Corresponding sequences of D1, D2 D3 and mutated D3 (mD3) were underlined. (C) Gel super-shift assay using antibodies including KLF4 (K4) and SP1.

researches on the negative *cis*-element of the promoter of CCSP gene are limited. In order to confirm 45 bp is functional region in CCSP promoter, a truncated construct lacking 45bp was generated (-768-Luc in Fig. 4A). Transient transfection assays with both -812- and -768-Luc constructs were performed in mtCC. As shown in Fig. 4B, the expression of the -812-Luc is much greater than that for the -768-Luc. It demonstrates that sequences between -812 bp

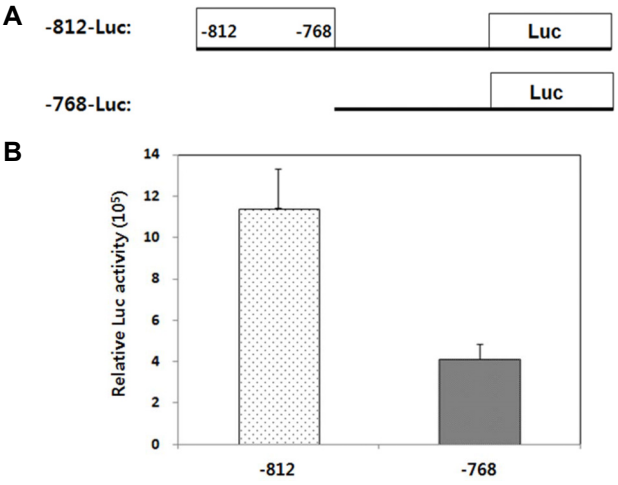

Fig. 4. Transfection analysis with deletion constructs of mCCSP gene. (A) -812 and -768 bp of promoter region fused to the luciferase (Luc) reporter vector. (B) -812- and -768 -Luc constructs of mCCSP promoter were transfected into mtCC cells.

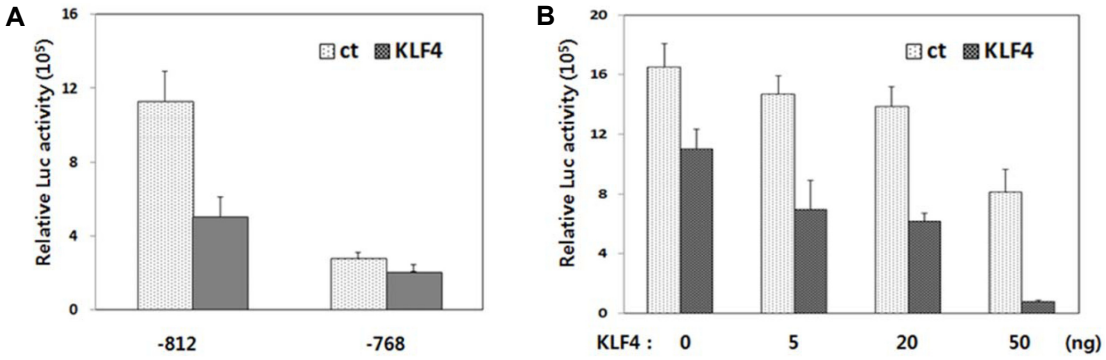

Fig. 5. Repression of mCCSP expression by KLF4. (A) -812-Luc and -768-Luc were co-transfected with KLF4 in mtCC cells. (B) Increasing amounts of KLF4 were used for co-transfection with -812-Luc in mtCC cells.

to -768 bp of the mCCSP promoter are responsible for dramatic reduction of CCSP reporter gene expression. It strongly suggests that a negative *cis*-element could be localized within this 45 bp for the binding of the repressor. In order to verify the 45 bp region is responsible for binding of a transcriptional repressor, several transcription factors were examined with co-transfection.

Interestingly, when a transcription factor KLF4 was co-transfected with -812-Luc, a significant repression of the CCSP gene expression was observed (Fig. 5A). However, the co-transfection of KLF4 in the 768-Luc construct showed less significant repression (Fig. 5A). In addition, the repression of the -812-Luc by increasing amount the KLF4 exhibited does-dependent decrease of the CCSP gene expression (Fig. 5B). These finding demonstrates that 45 bp of CCSP promoter is required for the biding of KLF4 and KLF4 function as repressor in CCSP gene expression. The localization of the KLF4 responsive *cis*-element in the mCCSP promoter suggests that highly conserved D3 motif (GCCTGGGAA) could act as important *cis*- element as a biding site for KLF4 for the repression of CCSP gene expression.

Super-shift by anti-KLF4 Ab

To confirm the binding of 45 bp with KLF4, super-shift assay was performed using antibodies including KLF4. The addition of the anti-KLF4 antibody in the reaction resulted in the super-shift of protein-DNA complexes with a labeled probe corresponding to the 45 bp (K4 in Fig. 3C). However, when the anti-SP1 antibody was added, significant band shift was not observed (SP in Fig. 3C). It demonstrates that super-shift band is specific for KLF4 and nuclear extracts of mtCC are responsible for the binding with KLF4 antibody.

Discussion

The results from transient transfection assays indicated that 45 bp region of the distal promoter of mCCSP gene was responsible for the repression of CCSP gene expression (Fig. 4). The D3 motif (GCCTGGGAA) within 45 bp region was highly conserved and could be responsible for the binding of a repressor (Fig. 2). The results in this research clearly showed that KLF4 could function as a repressor for the expression of CCSP gene in mtCC (Fig. 5). It suggests that the D3 motif could be a potential candidate for the repressor KLF4 binding. The results of EMSA (Fig. 3A) and band super shift assay (Fig. 3C) exhibited that this GCCTGGGAA motif might function as binding site for KLF4. Although, the biological role of KLF4 in CCSP expression is unknown, KLF4 could repress the CCSP gene expression in mtCC.

The functional roles of the KLF4 as transcriptional repressor were studied in several researches. KLF4 could inhibit Sp1-dependent transactivation the CYP1A1 by interaction with Sp1 [16]. KLF4 also could compete with other transcription factors for the binding with the coactivator [6, 17]. Several evidences suggest that KLF4 play important role in cancer. KLF4 could function as a tumor suppressor in in colorectal cancer [17] and in lung cancer [7] by the up-regulation of p21 expression in cell-cycle and then suppressed tumor growth [7, 17]. The role of KLF4 as transcriptional repressor of the CCSP gene expression and the finding of 45 bp is negative *cis*-element for binding of KLF4 could provide important information for the understanding of CCSP and Clara cells in the lung.

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초록 : KLF4에 의한 CCSP 발현 억제

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클라라 세포에 의해 생산되는 클라라 세포 분비 단백질(CCSP)은 폐를 염증으로부터 보호하는데 중요한 역할을 한다. 이 연구는 CCSP 유전자 발현에 관여하는 프로모터 부위에서 repressor에 결합할 수 있는 cis-element를 밝히는데 있다. DNaseI footprinting법을 사용하여 mCCSP 프로모터의 -812에서 -768 bp (45 bp) 사이에서 3 개의 보호된 motif를 찾았고, 그 중 하나인 D3 모티프(GCCTGGGAA)는 다른 3 가지 동물들과 염기서열이 100% 일치하였다. 45 bp를 사용한 EMSA 분석에서 D3 모티프(GGCCTGGGAA)는 45 bp에 높은 경쟁을 보였으나, 변이된 D3 모티프가 (GGAATGTTAA)를 사용되었을 때, 경쟁은 상당히 감소되었다. 이는 mCCSP 프로모터의 45 bp의 D3 모티프가 단백질과 DNA 상호 작용을 위한 중요한 element임을 시사한다. -756-Luc과 -812-Luc을 이용한 transient transfection 분석 결과, -756-Luc은 -812-Luc보다 CCSP의 발현이 현저하게 감소되었다. 이는 mCCSP 프로모터의 45 bp부위가 repressor의 결합 부위로서 기능을 할 수 있음을 의미한다. -812-Luc에 KLF4를 co-transfection 한 결과, KLF4는 CCSP 발현을 현저하게 저해(repression)함을 밝혔다. 그러나 -768-Luc이 사용되었을 때 KLF4에 의한 repression은 관찰되지 않았다. 이것은 KLF4가 CCSP 유전자의45 bp에 결합할 수 있고, 전사 억제자 역할을 하여 mCCSP 발현을 억제 할 수 있음을 명확히 보여 준다. 또한 이는 45 bp 중, D3 모티프가 KLF4의 결합에 강하게 관여 함을 시사한다. 이 반응에 KLF4에 대한 항체가 첨가되었을 때는 super-shifted 밴드가 관찰되었으나, SP1에 대한 항체가 사용되었을 때는 관찰되지 않았다. 이는 KLF4가 CCSP 프로모터의 45 bp 영역에 결합하여 repressor기능 할 수 있고, D3 모티프가 KLF4의 특이적 결합에 관여 할 수 있음을 시사한다.