Title: Analysis of transcriptional activity of human Mucin5AC gene under the influence of *V.cholerae* GbpA protein.

Abstract

GlcNAc binding protein A (GbpA) is a secretory protein of *Vibrio.cholerae* that facilitates its adherence to human intestine. Binding of *V.cholerae* to intestinal mucin by GbpA results in increased mucus secretion which attracts more bacteria for better colonization. However, the molecular insights of GbpA induced mucin production in host cells is yet to be investigated. Based on this knowledge gap the objective of this study was set to elucidate the role of GbpA in inducing Mucin5AC gene in HT-29 cell line and to identify the transcription factor binding sites of Mucin5AC gene involved in the activation process. To study the transcriptional activity of the Mucin5AC gene under the influence of GbpA, Luciferase-reporter gene assay was performed at varying conditions. The upregulation of Mucin5AC promoter activity was found dose-dependent and interestingly the upstream region between -324 bp to -64 bp of Mucin5AC was prerequisite for the GbpA-induced trans-activation. Thus, this study provide an insight into the GbpA induced mucin secretion at a molecular level which ultimately reinforce the concept of successful intestinal colonization and pathogenesis by *V.cholerae* that could lead to therapeutic approaches for blocking bacterially-induced mucin overproduction.

Keywords: N-acetylglucosamine, *Vibrio cholerae*, Mucin, Luciferase-reporter assay.

V. cholerae produces several chitinases and chitin-binding protein (4). GbpA is required for efficient colonization of human epithelial cells as well as attachment to the exoskeleton of marine organisms (3). GbpA consists of four domains and promotes bacterial attachment by interactions with GlcNAc residues present in both mucin and chitin. Binding studies have shown that GbpA interacts with several forms of chitin through its N-terminal (GbpA-D1) and C-terminal domain (GbpA-D4), including amorphous forms of chitin as well as chito-oligosaccharides (6). Additionally, it has been demonstrated that GbpA-D1 interacts with the GlcNAc residues of intestinal mucin (3,4). GbpA and mucin appear to upregulate each other in a cooperative manner, facilitating the process of pathogenesis to host cells (1). Mucin 5AC was found to be the predominant one upregulated by GbpA among other mucin tested (1). In light of prior research demonstrating the GbpA induced Mucin 5AC secretion in the intestinal cells, there is a need of investigation to specify the regulatory region present on the upstream of Mucin 5AC gene. This study aims to shed light on the molecular mechanism underlying the induction of Mucin 5AC gene by *V.cholerae* GbpA protein that could be useful for potential therapeutic measures to inhibit the bacterially-induced mucin overproduction.

Materials and Methods

Expression and purification of rGbpA

Recombinant GbpA was expressed in *E.coli* C43 cells harbouring the pET22b vector encoding the gbpA gene (VCA0811) and purified from the bacterial lysate following the previously described method (6).

Cell culture, construction of deletion promoters and transfection

Human colonic epithelial cell line HT-29 was cultured in complete McCoy's 5A (Sigma, St Louis, USA) supplemented with 10% Fetal Calf Serum (Eurobio, Paris, France), Non-essential amino acids and Penicillin-Streptomycin (MP Biomedicals, USA). HT-29 cell line was cultured and maintained in DMEM supplemented with 10% Fetal Calf Serum (Eurobio, France), Non-essential amino acids, Penicillin-Streptomycin (MP Biomedicals, USA) and 100 μg ml^{-1} blasticidin (MP Biomedicals, USA). Confluent monolayers were starved in incomplete medium specific for the cell line (with 0.5% FCS) for overnight before treatment with GbpA.

Transfection of the cells was performed using lipofectamine (Invitrogen, Massachusetts, USA) according to the manufacturer's protocol. Briefly, pGL3-Basic vector (Promega, Madison, Wisconsin, USA) was used to clone a putative promoter region of the Mucin 5AC gene. This putative promoter region was identified and selected using the software programmes Genomatix and Alibaba 2.1.

In the present study, the 5' sequence of the human Mucin 5AC gene with a 1,348-bp DNA sequence was amplified from HT-29 cells genomic DNA by polymerase chain reaction (PCR), and the product of the PCR was analysed by agarose gel electrophoresis. For promoter deletion analysis, three truncated promoter segments of different lengths were amplified by PCR.

The pGL3-Basic vector was cut using two restriction endonucleases, Mlu1 and BglII. The selected portions of the Mucin 5AC promoter (–1300 to +48) were cloned into the pGL3-Basic vector using different sets of primers (IDT, India). These pGL3-Mucin 5AC promoter-luciferase constructs were transfected in HT-29 cells along with pRLTK (Rennila luciferase construct). Briefly, 6 µg each of the promoter constructs and 0.15 µg of pRLTK were transfected into 10^5 HT-29 cells using lipofectamine following the manufacturer's protocol.

Primers Used

Sequence of oligonucleotides used in PCR are listed in Table1.

Cell treatment and Luciferase assay

The luciferase assay was performed using a kit from Promega, following the protocol provided. In brief, HT-29 cells transiently transfected with promoter constructs were cultured in 12-well cell culture plates. Cells transfected with full length promoter construct were treated with varying dose of GbpA (0 to 1000 ng /mL) and cells containing deletion constructs were treated with 250 ng /mL of GbpA for 8 hours. The treated and untreated cells were scraped with ice cold 1× passive lysis buffer and were subjected to several rapid freeze-thaw cycles to lyse the cells completely. Total protein from the cells was isolated, an equal amount of the protein from each of the samples was mixed with 100 µL of luciferase assay reagent II, and the inducible firefly luciferase activity was measured using a luminometer (Berthelot). Then, 100 µL of Stop & Glo reagent was added to stop the luminescence of firefly luciferase and simultaneously the intensity of Renilla luciferase was measured.

Statistical analysis

Where applicable, the results presented in this manuscript are the mean ± standard error (SE) of at least three separate experiments. Statistical differences were analyzed by ANOVA with the level of significance being set at 5% ($p < 0.05$).

Results

Construction of full length and truncated versions of Mucin 5AC gene promoter

The construction of full length and three truncated version of Mucin 5AC gene promoter and the cloning into the luciferase-reporter vector was successfully done. The DNA fragments of truncated and full length promoter were visualized upon agarose gel electrophoresis [Fig. 1].

The Mucin 5AC gene promoter was found responsive to GbpA in a dose-dependent manner.

The activity of the Mucin 5AC gene promoter was up regulated in a dose dependent fashion upon GbpA treatment on transfected HT-29 cells [Fig.2]. A fivefold increase of promoter activity over no treatment control was monitored.

The region between -324 bp to -64 bp of Mucin 5AC promoter is prerequisite for GbpA mediated induction

A twofold reduction in the promoter activity was recorded for the shortest promoter construct (-324 bp to -64 bp) [Fig.3]. The luciferase reporter assay with deletion constructs revealed that the region between -324 bp to -64 bp is required for the GbpA mediated activation of Mucin 5AC gene.

Discussion

In order to understand the mechanism of GbpA induced mucin secretion in intestinal cells, we constructed the luciferase-reporter gene fused with Mucin 5AC gene promoter and performed luciferase assay under varying conditions. The GbpA-mediated activation of Mucin 5AC gene promoter could increase the expression of Mucin 5AC gene in a dose-dependent manner as evident from the luciferase assay results. The results obtained from the deletion –promoter constructs suggest that the region between -324 bp to -64 bp is important for the GbpA induced trans-activation of Mucin 5AC gene. Interestingly, the region contains transcription factor binding sites for SP-1 and NF-kB. The involvement of NF-kB in the GbpA-induced cell signaling cascades is well-established in previous studies (1,2,5) . The current study further provides insights into the NF-kB mediated cellular response in relation to GbpA induced Mucin 5AC gene expression. However, the mutational analysis of transcription factor binding sites would be helpful to understand the molecular mechanism in deep is lacking in this study.

Conclusion

This short study certainly provide an insight into the GbpA induced mucin secretion at a molecular level which ultimately reinforce the concept of successful intestinal colonization and pathogenesis by *V.cholerae* that could lead to therapeutic approaches for blocking bacterially-induced mucin overproduction. Moreover, the findings of this study generate a picture where it is able to demonstrate that how a bacterial colonization-factor protein is involved in the modulation of host cell response during the host-microbe interaction.

Acknowledgements

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Conflict of Interest

The author hereby declares no conflict of interest.

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Table: List of primers used in the construction of Mucin 5AC promoter constructs.

1	MUC5ACF	AGGGTACCAGAGCTTGGGACGGGTCC
2	MUC5ACR	CCGCTCGAGTGTGTGGACGGCGGGGAAGA
3	MUC5AC-689F	AGGGTACCTGAGGGACGCCTTGGCTC
4	MUC5AC-689R	CCGCTCGAGTGTGTGGACGGCGGGGAAGA
5	MUC5AC-324F	AGGGTACCCCTTAAGGCTGTTCTGACCA
6	MUC5AC-324R	CCG CTCGAGTGTGTGGACGGCGGGGAAGA
7	MUC5AC-64F	AGGGTACCCCGTGAAGCACGGGGCTGGA
8	MUC5AC-64R	CCGCTCGAGTGTGTGGACGGCGGGGAAGA

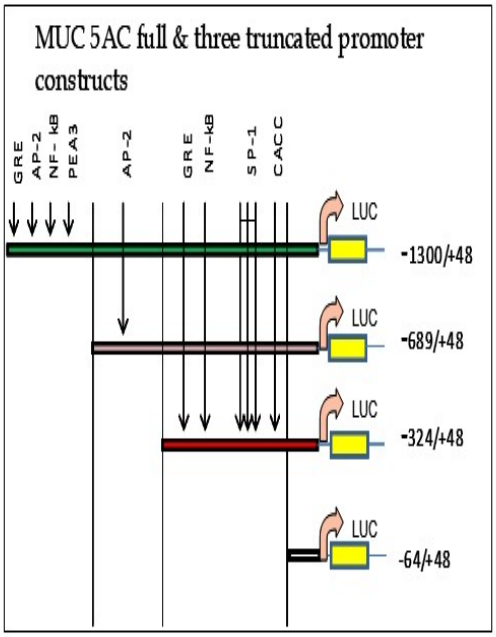
Figure Legends

Fig.1. (a) Mucin 5AC Full length & its three deletions construct showing different transcription factor binding sites. (b) A schematic diagram showing the principle of Luciferase assay used to analyze the promoter activity. (c) Mucin 5AC promoter fragments cloned into upstream of the luciferase reporter gene of pGL3-Basic vector. (d) Agarose gel electrophoresis of the Mucin 5AC promoter fragments obtained after PCR.

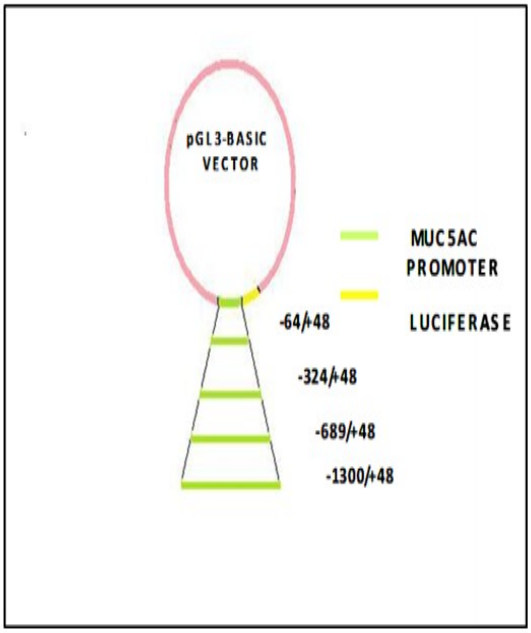
Fig.2. Dose dependent expression of Mucin 5AC- luciferase reporter gene in transfected HT-29 cells induced by GbpA.

Fig.3. Expression of Mucin 5AC- luciferase reporter gene in four different Mucin 5AC promoter constructs.

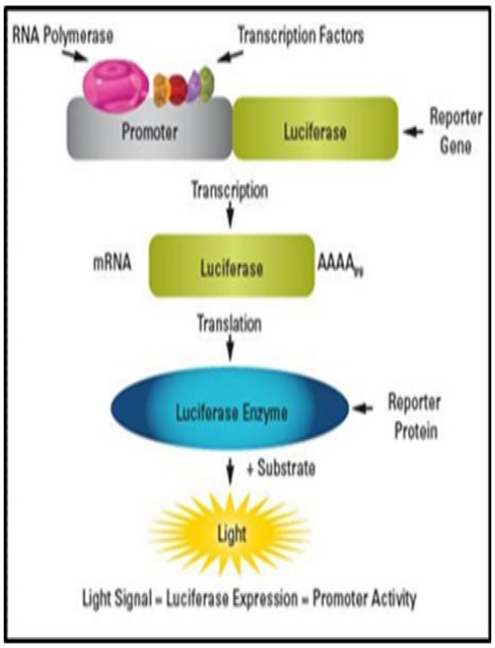
Fig.1 (a)


MUC 5AC full & three truncated promoter constructs
GRE
AP-2
NF- kB
PEA3
AP-2
GRE
NF-kB
SP-1
CACC
LUC
-1300/+48
LUC
-689/+48
LUC
-324/+48
LUC
-64/+48

(c)


pGL3-BASIC VECTOR
MUC5AC PROMOTER
LUCIFERASE
-64/+48
-324/+48
-689/+48
-1300/+48

(b)


RNA Polymerase
Transcription Factors
Promoter
Luciferase
Reporter Gene
Transcription
mRNA
Luciferase
AAAA
Translation
Luciferase Enzyme
Reporter Protein
+ Substrate
Light
Light Signal = Luciferase Expression = Promoter Activity

(d)

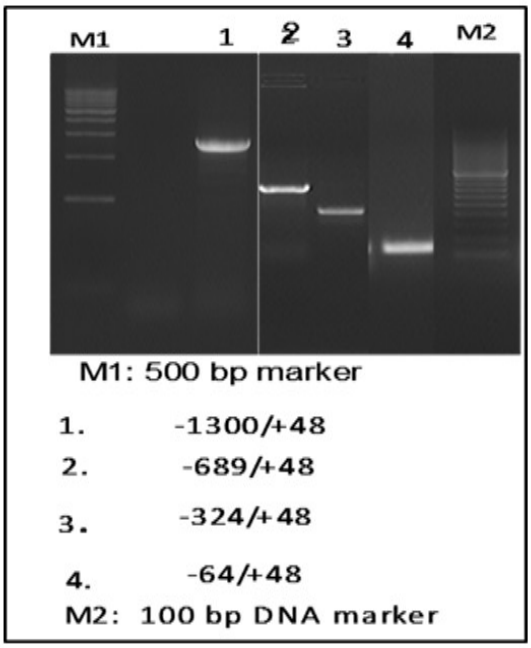
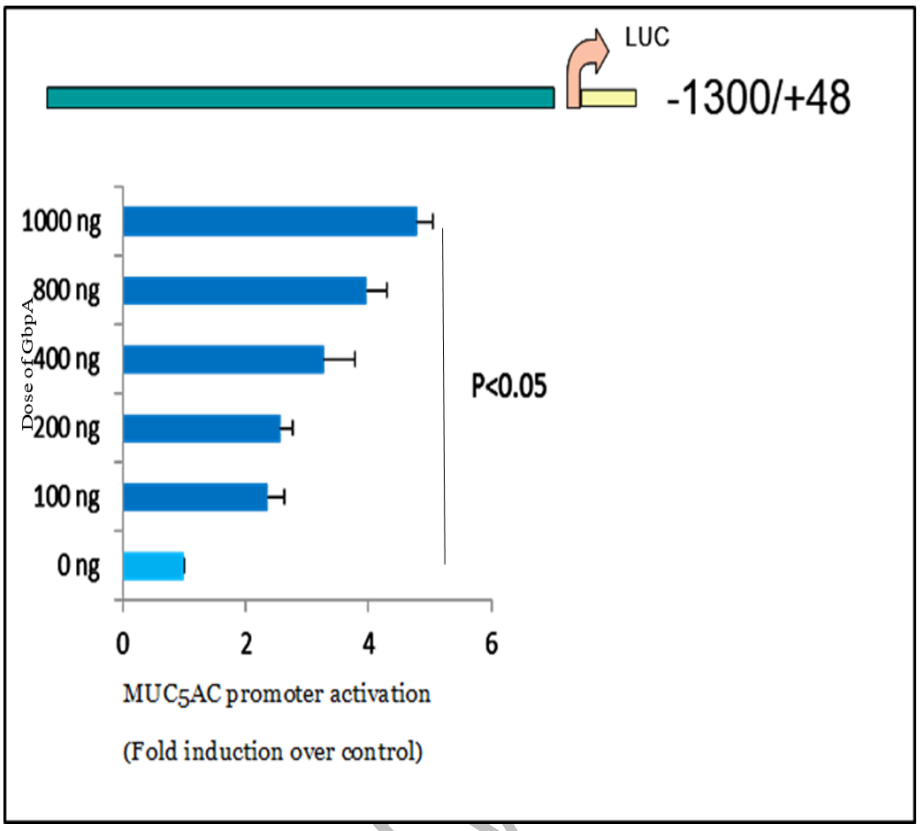

M1
1
2
3
4
M2
M1: 500 bp marker
1. -1300/+48
2. -689/+48
3. -324/+48
4. -64/+48
M2: 100 bp DNA marker

Fig.2



153
154
155
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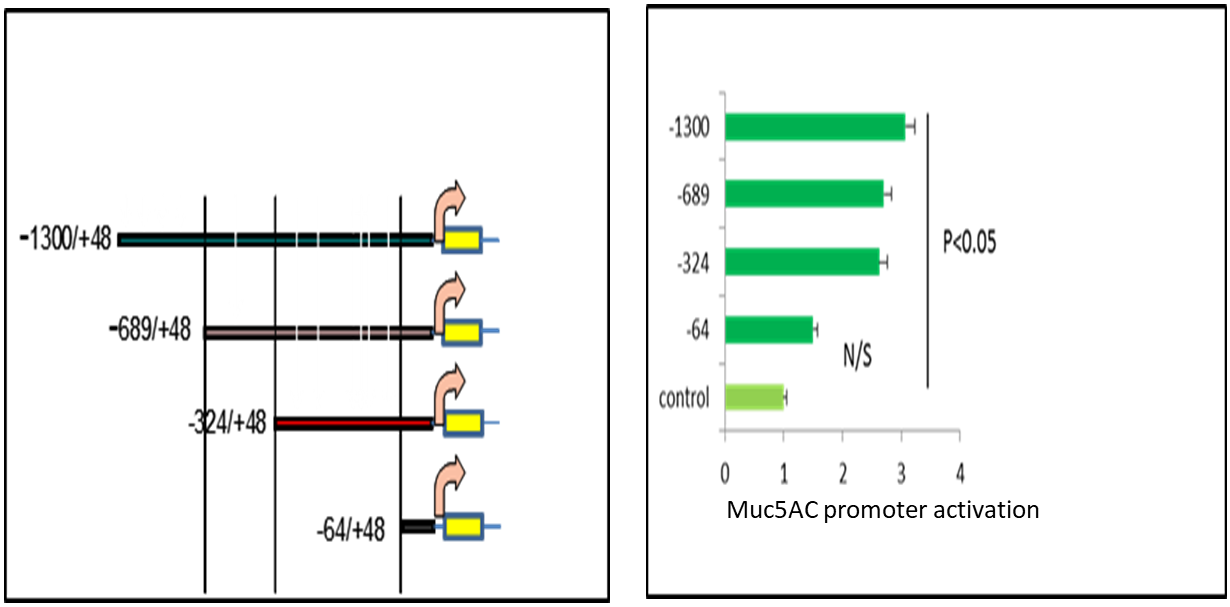
Fig.3



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