Translational fusion of heat labile enterotoxin chain B and β-subunit of human chorionic gonadotropin: periplasmic expression in Escherichia coli and its immunogenicity

Devika Pillai\textsuperscript{a}, Aparna Dixit\textsuperscript{a}, Deoraj Alok\textsuperscript{b,\textsuperscript{**}}, Lalit C. Garg\textsuperscript{b,\textsuperscript{*}}

\textsuperscript{a}Department of Zoology, Delhi University, Delhi-110 007, India
\textsuperscript{b}National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110 067, India

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Abstract A fusion gene was constructed consisting of heat labile enterotoxin chain B (LTB) of \textit{E. coli} genetically linked at its C-terminus to the β-subunit of human chorionic gonadotropin in translational fusion, under the control of tac promoter and LTB signal sequence. Expression of the fusion gene (about 5 μg/ml) in \textit{E. coli} was confirmed by immunoblot analysis using both anti-LTB and anti-hCG polyclonal antibodies. The fusion protein was efficiently processed and exported to the periplasmic space. LTB in the fusion protein retained its ability to bind to GM1 ganglioside receptor. Mice immunized with the fusion protein produce antibodies that recognize recombinant hCG and the native hCG suggesting its potential use as a contraceptive vaccine.

Key words: Periplasmic expression; Fusion protein; hCG; Signal sequence; Immunogenicity

1. Introduction

Human chorionic gonadotropin (hCG), a heterologous mammalian placental protein, has been the first choice as a target for immunocontraception [1]. A member of the family of glycoproteins consisting of two dissimilar subunits, α and β, it is produced by the trophoblast cells during the first trimester of pregnancy. The α-subunit is similar to that of other members of the family but the β-subunit has a distinctive amino acid sequence that confers the hormonal specificity. To avoid cross-reaction with other members of the glycoprotein family, the β-subunit has been used in contraceptive vaccine preparations. However, being a self protein, hCG is not immunogenic by itself and is, therefore, conjugated to a non-self carrier protein. In all the hCG based vaccines that have entered clinical trials, conjugation has been achieved through chemical conjugation. Gene fusion offers a practical alternative to chemical conjugation as it allows the protein to be conjugated to a carrier protein through the recombinant route. The fusion protein thus obtained would have a consistent composition unlike that produced by chemical conjugation. Cholera toxin chain B (CTB) and heat labile enterotoxin chain B (LTB) of \textit{E. coli} have gained interest as fusion partners in recent years. Oral administration of these proteins has gained interest as fusion part-

2. Materials and methods

2.1. Reagents

Enzymes were purchased from New England Biolabs, USA. Bacteriophage lambda, Bacto-agar, Bacto-yeast extract, tryptone, Bacto-agar and Bacto-yeast extract, were from Difco Laboratories, USA. Ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), Freund's complete (CFA) and incomplete adjuvant (IFA) were obtained from Sigma Chemicals, USA. Recombinant protein A was a kind gift from Dr. S.K. Gupta.

2.2. Bacterial strains and plasmids

E. coli DH5α and HB101 cells were used as bacterial hosts. The clone pKGhCG [8], having hCG structural gene under the control of tac promoter and LTB signal sequence, and plasmid pBSLTB (constructed in our laboratory) containing the LTB gene were used as the parent clones. It is to be noted that as a result of the cloning strategy of LTB in pBSLTB, the termination codon of LTB was deleted and a Sac1 site was introduced at that position. In order to generate the fusion construct, the plasmid pBSLTB was digested with Sac1 and the LTB structural gene fragment thus released was cloned into Sac1 digested pKGhCG to give a N-terminal translational fusion of LTB to hCG. The resulting construct is designated pLTBhCG. The resulting constructs were designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designated pLTBhCG. The resulting construct was designed

2.3. Induction of recombinant clones and immunoblot analysis of fusion protein

Cells harboring the recombinant plasmid pLTBhCG, having the LTB insert either in the right or in the reverse orientation, were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml). Freshly subcultured 0.5 A\textsubscript{600} cell/ml unit cultures were induced with 1 mM IPTG for 8 h. Uninduced cultures served as controls. Cells were harvested by centrifugation and total cell extract was prepared by resuspending the cell pellet of 1 ml culture in 50 μl reducing sample buffer. Extracts thus prepared were analyzed by 12% SDS-PAGE and immunoblot analysis [10] was carried out using both anti-LTB and anti-hCG polyclonal antibodies to check for expression of the fusion protein delivered orally, not only for infectious disease control but also to control mucosal physiological processes such as fertility [7].

In the present study, LTB was cloned at the N-terminus of hCG to generate a translational fusion gene under the control of tac promoter and LTB signal sequence. We report here the expression, efficient processing and export of the fusion protein to the periplasm in \textit{E. coli}. Our studies also indicate that the fusion protein could bind to GM1 ganglioside receptor, the receptor for LTB, in an in vitro receptor binding assay and was found to be immunogenic in mice.

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protein. To determine the localization of the fusion protein in E. coli, total cell extract, periplasmic fraction [11] and culture supernatant of induced and uninduced cells were analyzed by Western immunoblot using anti-βhCG antibodies.

2.4. Receptor binding activity

The receptor binding capacity of LTB in the fusion protein was determined in vitro using a sandwich ELISA. 100 µl of GM1 ganglioside (10 µg/ml) was coated onto ELISA plates and incubated at 37°C for 1 h followed by overnight incubation at 4°C. Non-specific sites were blocked with 1% lactogen in PBS-Tween followed by successive incubations with 100 µl each of fusion protein, anti-CTB goat antiserum (1:1000) and anti-goat IgG-HRP conjugate (1:2000), respectively. All incubations were carried out at 37°C for 1 h each followed by thorough washes with 50 mM PBS containing 0.5% Tween-20. Subsequent to the final incubation and washes, 0.05% ortho-phenylene diamine (OPD) in citrate phosphate buffer (pH 5.0) and 1 µl/ml of hydrogen peroxide were added to develop color. The reaction was terminated using 50 µl of 5 N H₂SO₄ and the absorbance was read at 490 nm.

2.5. Immunization

To determine whether the recombinant fusion protein is immunogenic, 100 µl of emulsion of the periplasmic fraction containing the fusion protein with CFA was administered to 6-8 week old BALB/c inbred mice (n=10) at fortnightly intervals. Primary immunization with CFA was given subcutaneously and the subsequent boosters with IFA were given intramuscularly. The mice were bled retro-orbitally a week after the third booster. Antibody titers were assayed by ELISA and were expressed as the last dilution at which the absorbance at 490 nm was above the background level.

2.6. Colloidal immunogold assay

The serum samples were pooled and analyzed for the presence of antibodies by colloidal immunogold assay [12]. Colloidal gold solution was prepared as described [12] and 1 µl of this solution was mixed with 17 µl of 1% K₂CO₃. To this mixture, 6 µg of purified native hCG was added and vortexed for a few seconds. After incubation at room temperature (RT) for 5 min, 6 µl of 20% BSA was added and the contents were centrifuged at 5000 rpm for 5 min at RT. The pellet was resuspended in 50 µl of the supernatant containing the colloidal gold mixture, discarding the remainder. Nitrocellulose strips (BA 85) were dotted with 5 µg of recombinant protein A and dried at RT. 40 µl of the pooled serum, 10 µl of 50 mM PBS pH 7.2, 10 µl of hCG-colloidal gold conjugate and finally, 10 µl of 10% alkali-treated casein were added in the wells of a microtiter plate. The contents were thoroughly mixed and nitrocellulose strips (NCS) were placed in the

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**Fig. 1.** Construction of the fusion plasmid pLTBJhCG. The 309 bp LTB fragment lacking the termination codon released by Sac1 digestion of pBSLTB was cloned into Sac1 linearized pKGBhCG. Fusion gene LTBjhCG is thus under the control of LTB signal sequence and tac promoter.

**Fig. 2.** Analysis of fusion protein expression. Total cell extract from cells grown in the presence or absence of IPTG were prepared by suspending 0.5 A₅₅₀/ml unit cells in reducing sample buffer and were analyzed by Western blotting. (A) Samples were immunoblotted with anti-βhCG polyclonal antibodies. Lanes 6 and 7 represent uninduced and induced samples of the clones containing the plasmid in the right orientation while lanes 8 and 9 correspond to the uninduced and induced cells of the reverse orientation clones, respectively. Lanes 1 and 2 are the uninduced and induced cells of the parent clone pKGBhCG expressing βhCG, lanes 3 and 4 are the induced and uninduced control cells which lack the recombinant plasmid. Lane 5 corresponds to the molecular weight markers. The arrowhead on the right points to the fusion protein and the arrowhead on the left points to βhCG. (B) Samples were immunoblotted with anti-LTB polyclonal antibodies. Lane 4 is the positive control corresponding to pure LTB. Lanes 5 and 6 correspond to uninduced and induced cells harboring the pLTBJhCG plasmid in right orientation, respectively. Lanes 7 and 8 correspond to uninduced and induced cells harboring the pLTBBhCG plasmid in reverse orientation. Lanes 9 and 10 are the negative controls consisting of uninduced and induced cells which lack the recombinant plasmid whereas lanes 1 and 2 correspond to uninduced and induced cells harboring pMMB68 plasmid expressing LTB. Lane 3 is the molecular weight marker showing a band at 30 kDa. The arrowhead on the right points to the fusion protein and the arrowhead on the left points to LTB.
periplasmic space but not secreted further into the extracellular environment. A single band corresponding to the expected size of fusion protein was detected in induced cells harboring the plasmid pLTBPhCG. The fusion protein was confirmed by Western blot analysis using anti-DhCG antibodies. A 30 kDa band was observed in induced cells but not in uninduced cells. This band was not detected in the culture supernatant of induced cells harboring pLTBPhCG only (Fig. 2A, lane 7; Fig. 2B, lane 6). A faint band at a slightly higher molecular weight was also observed when the samples were blotted with anti-LTB antibodies. This faint band was not picked up when these samples were blotted with anti-DhCG antibodies. The fusion protein could not be detected in uninduced cells suggesting a stringent control on inducibility.

In order to check the localization of the expressed product, different cellular fractions of induced and uninduced cells harboring the plasmid pLTBPhCG were analyzed. The fusion protein could be detected as a single band in the total cell extract and periplasmic fraction of induced cells (Fig. 3, lanes 2 and 3) but not in the culture supernatant (lane 4). The fusion protein could not be detected in the corresponding fractions of uninduced cells. The fusion protein was used for the test.

The strategy for the construction of the fusion gene plasmid pLTBPhCG is shown in Fig. 1. The fusion gene pLTBPhCG is thus under the control of Tat promoter and LTB signal sequence. E. coli cells harboring the recombinant plasmid pLTBPhCG with the insert in either the right or the reverse orientation were induced with IPTG and an expression level of 3-5 μg/ml of fusion protein was observed. Authenticity of the fusion protein was confirmed by Western blot analysis using both anti-DhCG (Fig. 2A) and anti-LTB (Fig. 2B) polyclonal antibodies. A 30 kDa band corresponding to the expected size of fusion protein was detected with both antibodies in induced cells harboring pLTBPhCG only (Fig. 2A, lane 7; Fig. 2B, lane 6). A faint band at a slightly higher molecular weight was also observed when the samples were blotted with anti-LTB antibody (Fig. 2B, lanes 5-8). This is probably a non-specific band as it could be observed in the uninduced and induced cells harboring the plasmid in reverse orientation. However, this band was not picked up when these samples were blotted with anti-DhCG antibody (Fig. 2A). The fusion protein could not be detected in uninduced cells suggesting a stringent control on inducibility.

In order to check the localization of the expressed product, different cellular fractions of induced and uninduced cells harboring the plasmid pLTBPhCG were analyzed. The fusion protein could be detected as a single band in the total cell extract and periplasmic fraction of induced cells (Fig. 3, lanes 2 and 3) but not in the culture supernatant (lane 4). The fusion protein could not be detected in the corresponding fractions of uninduced cells. The detection of the fusion protein in the periplasmic fraction of induced cells indicated that the fusion protein was exported across the cytoplasm, into the periplasmic space but not secreted further into the extracellular environment. A single band corresponding to the expected size of fusion protein was detected in induced cells harboring pLTBPhCG. The fusion protein was confirmed by Western blot analysis using anti-DhCG antibodies. A 30 kDa band was observed in induced cells but not in uninduced cells. This band was not picked up when these samples were blotted with anti-DhCG antibodies. The fusion protein could not be detected in uninduced cells suggesting a stringent control on inducibility.

The expression of the fusion protein improved with increasing concentration of IPTG until 0.5 mM beyond which no detectable increase in expression was observed (data not shown). The time course of fusion protein expression revealed that the level of expression increased with time attaining its maximum at 8 h and plateaued at later time points (data not shown).

3. Results and discussion

3.1. Analysis of the expression of pLTBPhCG

The periplasmic fraction of E. coli cells harboring pLTBPhCG or HB101 cells were tested for receptor binding activity of LTB in the fusion protein using GM1 sandwich ELISA. Pure LTB was maintained as the positive control. The periplasmic fraction of the fusion protein was used for the test. E. coli HB101 cells and the reactants which were devoid of GM1 ganglioside and the fusion protein were used as negative controls.
coli cells containing pLTBbhCG (Fig. 4). However, its binding affinity for GM1 was reduced compared to standard LTB which was used as the positive control (Fig. 4). The binding affinity of the fusion protein to GM1 ganglioside compared to LTB, observed in the present study, supports the earlier finding [13] that fusion of CTB to a relatively large protein could alter its affinity for GM1. Background levels of absorbance observed in HB101 cells or control samples that lacked either fusion protein or GM1 demonstrate the specificity of the reaction. These data clearly suggest that fusion protein is biologically active in terms of GM1 receptor binding assay.

3.3. Immunogenicity of the fusion protein

To establish the immunogenicity of the recombinant fusion protein, mice were immunized with fusion protein as described in Section 2. Anti-LTB and anti-bhCG antibodies were assayed by ELISA in serum samples immunized with fusion protein. Antibody titers against each component of the fusion protein, i.e. LTB and bhCG, were found to be high, however, titers against LTB were 1:10000 and against bhCG were 1:5000. The serum samples from immunized mice were also tested for its reactivity to recombinant bhCG and native hCG by colloidal immunogold assay. The antisera raised against the fusion protein could bind to recombinant bhCG and native hCG (data not shown). These data suggest that the fusion protein is immunogenic and has a potential to be used as a contraceptive vaccine.

An expression cassette such as described here could be used to clone other genes of interest, using LTB as a fusion partner, thus bypassing the need for chemical conjugation of proteins, a process which is time consuming and cumbersome.

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