Efficient expression, processing and secretion of a biologically active mammalian protein by *Vibrio cholerae*

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Abstract The use of *Vibrio cholerae* as a secretory expression system for the expression of a mammalian protein, namely human growth hormone, under the control of the heat labile enterotoxin chain B signal sequence is reported. The protein is efficiently expressed and processed. The mature protein is exported to the periplasm after which it is secreted to the extracellular milieu. The expressed and secreted hGH actively binds to its receptor as established by its receptor binding activity. The biological activity of the protein is demonstrated in vitro in a Nb2 proliferation assay.

Key words: Human growth hormone; Secretory expression; Signal sequence; Nb2 cell bioassay

1. Introduction

A variety of prokaryotic systems have been used for the expression of genetically engineered proteins. In *E. coli*, the prototype prokaryotic organism, a protein can either accumulate in the cytoplasm in a soluble form [1], aggregate into insoluble cytoplasmic aggregates [2] or be exported across the membrane to the periplasmic space [3–5]. Hence, the development of a prokaryotic secretory expression system in which a heterologous recombinant protein is secreted utilizing the natural secretory pathway of the host is of great interest. *Vibrio cholerae*, an organism closely related to *E. coli*, secretes some of its natural toxins to the medium [6]. The cholera toxin chain B (CTB) and the heat labile enterotoxin chain B (LTB) of *E. coli* origin are expressed and efficiently secreted in *Vibrio cholerae* [6] whereas they are retained in the periplasmic space when expressed in *E. coli*.

Expression of CTB in a mutant of *Vibrio cholerae* leads to an accumulation of the CTB in the periplasm; the mutation maps outside the toxin genes indicating that the secretory pathway of the host system is being used for the secretion [7]. When LT is expressed in such a strain, it is not secreted [8]. The potential of this system for secretory expression has been limited to the expression of homologous proteins of prokaryotic origin. The system has also been used for secretory expression of a decapeptide homologous to the *E. coli* heat labile enterotoxin A, fused to cholera toxin B chain [9]. However, the secretory capacity of the *Vibrio cholerae* export apparatus has not yet been exploited for the expression of heterologous mammalian proteins.

In the present investigation, we demonstrate the use of *Vibrio cholerae* for efficient expression and secretion of a heterologous mammalian protein – the human growth hormone (hGH). This is the first report dealing with expression of a mammalian protein in the *Vibrio cholerae* system. The expressed protein is transported to the extracellular environment via a periplasmic intermediate and the biological activity of the recombinant protein is demonstrated in an in vitro Nb2 cell proliferation assay.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Ampicillin, polymyxin B and kanamycin were purchased from Sigma Chemicals, USA, Gibco Laboratories, USA and Boehringer Mannheim, Germany, respectively. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were from Difco Laboratories, USA. All DNA restriction and modification enzymes were procured from New England BioLabs, USA and used as per their recommendations. Isopropyl-β-D-thio-galactopyranoside (IPTG) was obtained from Boehringer Mannheim. *E. coli* strain HB101 was used for all transformation experiments. El Tor *Vibrio cholerae* JBK70 (A^-B^-) cells were kindly provided by Dr. M. Lewin. The plasmid ptrpcGH was a gift from Dr. M. Ikehara. *E. coli* cells harboring plasmid pMMB68 and plasmid pRK2013 were gifts from Dr. J. Holmgren and Dr. H. Das, respectively. Commercially available plasmid pUC18 was used.

2.2. Construction of plasmid pGGhGH-E

Convenient cloning sites were generated by PCR amplification using primers harboring *Sac*I and *Hin*dIII sites at the 5′ and the 3′ ends of the cGH fragment, respectively. The construction of the plasmid pGGhGH-E (Fig. 1) has been reported earlier [10]. The first amino acid of LTB was retained at the junction of the signal sequence and mature protein. The +1 site in the mature protein is thus occupied by an Ala residue. *E. coli* HB101 cells were transformed and plasmid DNA was isolated according to standard protocols as described [11].

2.3. Conjugal transfer of the construct to El Tor Vibrio cholerae JBK70 cells

E. coli pGGhGH-E, JBK70 and *E. coli* pRK2013 cells were used as donor, recipient and helper strains, respectively. The donor, recipient and helper strains were grown for 12–14 h in Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin, 50 units/ml polymyxin B and 50 μg/ml kanamycin, respectively. The donor was subcultured in ampicillin free LB and grown for 3 h with gentle shaking. 100 μl of each culture was mixed in an Eppendorf tube and centrifuged at 10000 rpm for 2 min. The pellet, consisting of all three different types of cells, was resuspended in 100 μl of fresh LB and deposited on an LB plate in a very small area. The plate was incubated at 37°C for 10 h. The cells were scraped off and resuspended in 5 ml sterile normal saline. Serial dilutions were made and plated on LB plates supplemented with 50 μg/ml ampicillin and 50 units/ml polymyxin B. An elaborate set of positive and negative controls was plated including a neg-

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Abbreviations: hGH, human growth hormone; VhGH-E, *V. cholerae* cells harboring the plasmid encoding hGH; LTB, heat-labile enterotoxin chain B; pGGhGH-E, plasmid harboring the hGH gene; IPTG, isopropyl-β-D-thio-galactopyranoside

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ative control for conjugation in the absence of the helper strain. The transconjugant *V. cholerae* cells harboring the plasmid coding for hGH were called VhGH-E cells.

2.4. Induction of hGH and preparation of cellular fractions

VhGH-E cells were grown for 10–12 h in LB medium with 50 μg/ml ampicillin and 50 units/ml polymyxin B. Freshly subcultured 0.5 A_{600}/ml unit cells were induced with 1 mM IPTG solution for different time intervals. Periplasmic and cytoplasmic fractions were separated by osmotic shock [12].

Cell supernatant collected from harvested induced cells was treated with TCA at a resultant concentration of 25% for 5 min on ice followed by centrifugation at 6000 rpm at 4°C in a Sorvall SS34 rotor. The pellet thus obtained was washed twice with acetone, resuspended in 50 mM phosphate buffer (pH 7.2) and analyzed for the presence of hGH.

2.5. Western immunoblot analysis of hGH expression

The cellular fractions and the supernatant samples, processed as described, were electrophoresed on a 15% SDS-PAGE. The resolved proteins were electrotransferred onto a nitrocellulose membrane for 10 h at 30 mA. The nonspecific sites were saturated by incubating the membrane in a 1% nonfat milk solution prepared in 50 mM PBS (pH 7.2) for 1 h. The blot was then incubated with a goat anti-hGH polyclonal antiserum followed by an incubation with an anti-goat IgG HRPO conjugate. The membrane was washed thoroughly with PBS containing 0.05% Tween-20 between successive incubations. The immunoreactive bands were visualized using 4-chloro-1-naphthol.

2.6. Receptor binding activity and radioimmunoassay of recombinant hGH

A rat liver tissue membrane preparation was used as a source of hGH receptor. Crude plasma membrane fraction was prepared by the method of Yeko et al. [13] with minor modifications. hGH was iodinated as described by Fraker and Speek [14]. The receptor binding activity of recombinant hGH was analyzed in the radio receptor assay (RRA) by the method described earlier by Yeko et al. [13] with minor modifications. Radioimmunoassay (RIA) of recombinant hGH was performed by the method described by Sambrook et al. [11] with minor modifications.

2.7. Biological activity of secretory hGH

The Nb2 cell proliferation assay [15] was used to check the biological activity of recombinant hGH. The proliferation was monitored by using an MTT cytotoxic assay [16].

3. Results and discussion

3.1. Expression of hGH in Vibrio cholerae

The transconjugant clones were selected and the presence of the plasmid pGGhGH-E was confirmed by plasmid DNA isolation and analysis. VhGH-E cells were induced for 6 h for the expression of hGH as described. The immunoblot analysis of the cellular fractions and cell supernatant (Fig. 2) indicates that the El Tor *Vibrio cholerae* cells expressed hGH, exported it to the periplasmic fraction after processing and further secreted it into the extracellular milieu. To our knowledge, this is the first report of expression of any mammalian protein in *Vibrio cholerae*.

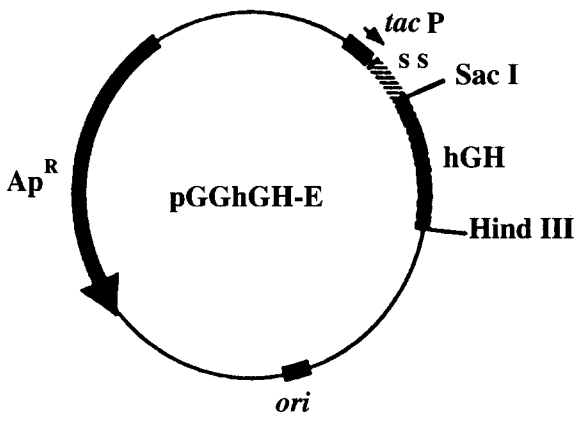

Fig. 1. Structure of plasmid pGGhGH-E. Plasmid pGGhGH-E consisting of human growth hormone gene (hGH) under the control of tac promoter (tac P) and heat-labile enterotoxin chain B signal sequence (ss).

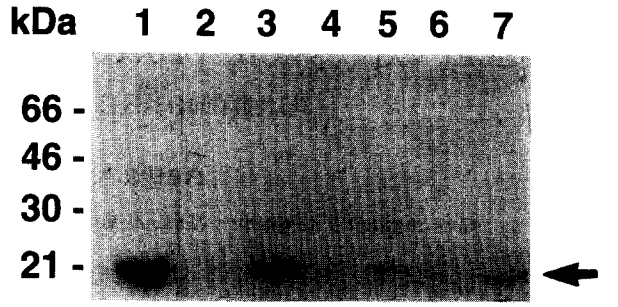

Fig. 2. Analysis of hGH expression. Cell supernatant, periplasm, and total cell extracts of VhGH-E cells grown in the presence and the absence of IPTG were boiled with SDS-PAGE reducing sample buffer, electrophoresed on a 15% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was immunoblotted with anti-hGH polyclonal antiserum. Lanes 3, 5 and 7 are supernatant, periplasmic and total cell extract of induced VhGH-E cells, respectively. Lanes 2, 4 and 6 are the corresponding fractions of uninduced VhGH-E cells. Lane 1 corresponds to standard pituitary hGH preparations. The arrow on the right points towards the migration of mature hGH. The migration of molecular weight markers (kDa) is shown on the left.

The total cell extract of the induced cells showed the presence of processed hGH. No band corresponding to the unprocessed protein (hGH along with the signal peptide) can be seen in the total cell extract (Fig. 2, lane 7). The antibody used for the detection of the recombinant protein is capable of recognizing the unprocessed protein (data not shown) indicating that the processing of the protein is very efficient.

It is observed that a fraction of the expressed protein was retained intracellularly in the periplasm (Fig. 2, lane 5) of the cells in addition to the secretory product (Fig. 2, lane 3). This indicates that the protein is probably secreted from a periplasmic pool. A similar phenomenon has been reported in the case of secretory expression of proteins in *Bacillus subtilis* and also in the case of expression of LTB in *Vibrio cholerae* [6,17,18]. Proteins that are secreted with the help of the signal sequences follow the type II pathway of secretion and involve a periplasmic intermediate [19]. The protein is probably held transiently in the periplasm. In *Vibrio cholerae* the secretion of the processed protein can be detected in the cell supernatant. The construct, pGGhGH-E, when induced for the expression of the protein in *E. coli*, however, can only export the protein to the periplasmic space and no protein can be detected in the supernatant in *E. coli* [10]. This shows that the heat labile enterotoxin chain B signal sequence can mediate secretion of the protein in the medium only in *V. cholerae* and not in *E. coli*. It also suggests that *Vibrio cholerae* has a secretory apparatus which is absent in *E. coli* and probably it is this secretory apparatus that mediates the secretion of the heterologous proteins.

3.2. Kinetics of hGH expression

VhGH-E cells were induced with IPTG and samples were

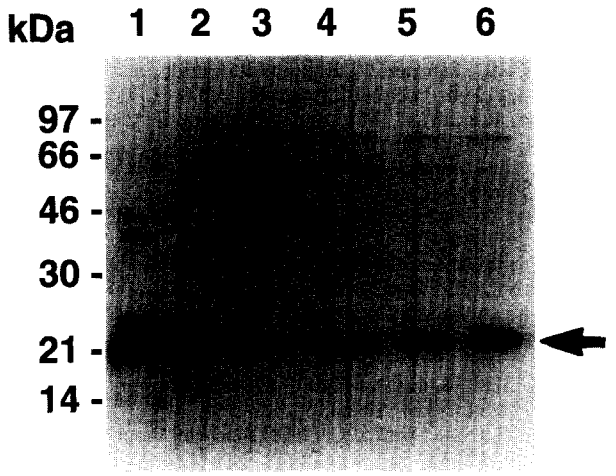

Fig. 3. Time course of hGH expression. The cell supernatant of the VhGH-E cells induced with 1 mM IPTG was harvested and the proteins were precipitated with TCA as described. Equal volumes of supernatant were mixed with reducing SDS-PAGE sample buffer and analyzed as discussed. Lanes 2–6 represent cells withdrawn at 0, 0.5, 2, 4 and 8 h, respectively. Lane 1 represents standard pituitary hGH preparation. The arrow on the right indicates the migration of mature hGH. The migration of the molecular weight markers (kDa) is shown on the left.

drawn at time points 0–8 h. The supernatants were analyzed for the expression of hGH (Fig. 3) and secretory hGH could be detected by immunoblotting. The levels of secretory hGH increase with time and hGH can be detected until 8 h post induction indicating that the cells are actively secreting the recombinant protein in the late phases of growth as well. Aberrant secretion of hGH has been reported in *Bacillus subtilis* where in the early phases of secretion, a reduced mobility band corresponding to the unprocessed protein is detected in the supernatant [20]. In *Vibrio cholerae*, however, only the processed form of the protein, devoid of the signal sequence, is detected in the supernatant (Figs. 2 and 3). Absence of a band corresponding to the unprocessed protein in the cells at any time point during induction suggests that the rate of processing of the protein is equivalent to or more than the rate of induction of expression.

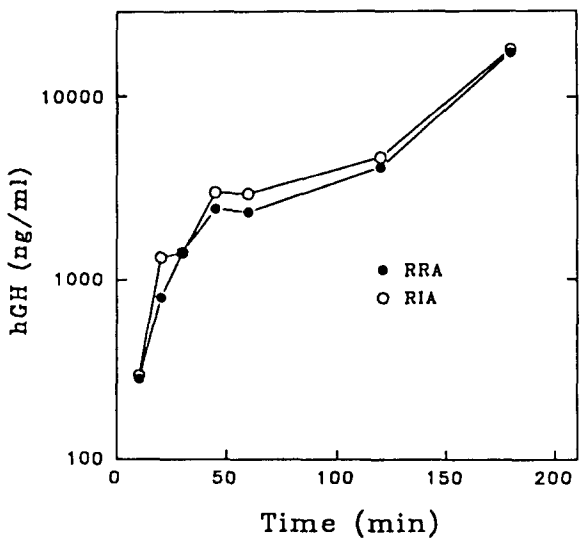

Fig. 4. Correlation between immunological activity and receptor binding activity of recombinant hGH. The periplasmic fractions of IPTG induced VhGH-E cells prepared from a constant number of cells at different time points were analyzed for a comparative quantitation in an RIA and an RRA.

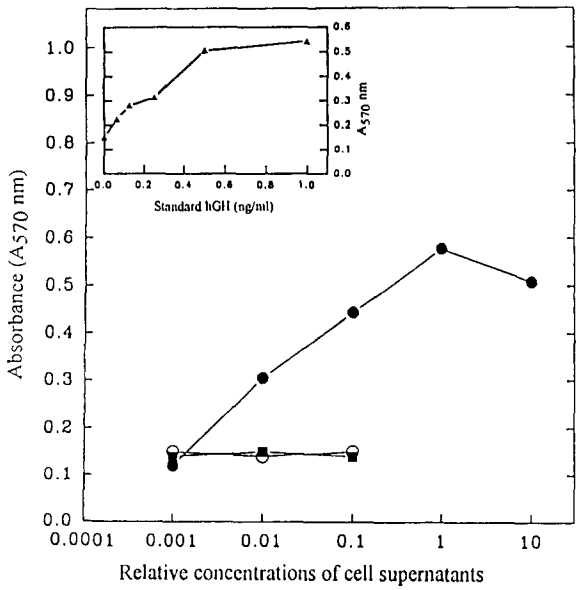

Fig. 5. Biological activity of secretory hGH. VhGH-E cells were induced with 1 mM IPTG. Cell supernatant was harvested, concentrated and used as a source of recombinant hGH. Uninduced VhGH-E and induced *V. cholerae* JBK70-68 cells were used as negative controls. Closed and open circles represent induced and uninduced VhGH-E cells, respectively. Closed squares represent induced JBK70-68 cells. The inset shows Nb2 cell proliferation in the presence of standard hGH.

Prior to induction, there is no hGH present in the cells (Fig. 2). However, the cells harvested immediately after the addition of the inducer exhibit a band corresponding to the processed protein in the supernatant fraction of cells indicating that the induction of expression is very efficient (Fig. 3, lane 2). With progressive induction, the amount of protein secreted in the medium showed a concomitant increase further confirming that the protein is being expressed, processed and secreted in the medium continuously.

3.3. Receptor binding activity of the recombinant hGH and radioimmunoassay

For the receptor binding activity and radioimmunoassay of recombinant hGH, fractions of induced *Vibrio cholerae* cells were used. The RRA and RIA were used for quantification of recombinant hGH in periplasmic fractions (Fig. 4). The levels of hGH as quantitated increase during the time course of induction. Quantitation of the levels by both the assays gave 14 μg/ml of hGH at 3 h post induction. The patterns of inhibition obtained in both the assays indicate that recombinant hGH is recognized with an affinity equal to standard hGH by both anti-hGH antibodies and the hGH receptor.

3.4. Biological activity of secretory hGH

Cell supernatant of induced VhGH-E cells was tested for its ability to induce Nb2 cell proliferation in vitro (Fig. 5). Supernatants from uninduced VhGH-E cells and induced JBK70 cells were used as controls. Supernatant from induced VhGH-E cells alone could induce cell proliferation. This indicated that the secretory hGH is biologically active. As

supernatant from uninduced cells is unable to induce proliferation, it also consolidates the observation of a stringent control on inducibility noted earlier from immunoblots.

Thus, the use of *Vibrio cholerae* as a secretory system for the expression of a heterologous mammalian protein, namely human growth hormone, is demonstrated in this report for the first time. This system has the potential to be developed as an efficient secretory system of expression for a variety of other heterologous proteins. Availability of such a system will be critical to obtain recombinant product for direct structural and functional studies.

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