High expression of human chorionic gonadotrophin β -subunit using a synthetic vaccinia virus promoter

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ABSTRACT

We have constructed a recombinant vaccinia virus to express the β -subunit of human chorionic gonadotrophin (β hCG), a secretory glycoprotein that is used as an antigen for a contraceptive vaccine. The cDNA encoding the subunit was cloned under the control of a synthetic promoter that could be recognised by a vaccinia virus RNA polymerase to direct transcription. The peak expression level of β hCG directed by a late

synthetic promoter (Psyn) was 11.5 μ g/ml, a level that was at least sixfold higher than that directed by the p7.5 early/late promoter. The expressed protein was correctly processed post-translationally such that it attained a conformation with correctly folded discontinuous epitope(s) similar to that seen in native β hCG.

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INTRODUCTION

Human chorionic gonadotrophin (hCG) is a member of a family of heterodimeric glycoprotein hormones that also includes luteinizing hormone (LH), follicle-stimulating hormone and thyroid-stimulating hormone. Each hormone consists of a common α -subunit and a hormone-specific β -subunit, encoded by separate genes. hCG is produced by the peri-implantation embryo and stimulates the corpus luteum to continue production of progesterone until the placenta assumes the role of producing this steroid. Because of the crucial role played by hCG in the establishment and maintenance of early pregnancy, it has been considered to be a target for an anti-fertility vaccine. A prototype vaccine based on the entire β -subunit of hCG (β hCG) generating hCG-neutralising antibodies (Singh *et al.* 1989, Pal *et al.* 1990) has completed clinical testing in women (Talwar *et al.* 1994), demonstrating the feasibility of the approach in preventing pregnancy.

Conventionally, β hCG is obtained by dissociating native hCG, a procedure that is cumbersome and expensive. The isolated β hCG is invariably contami-

nated with trace amounts of α -subunit, an impurity that may lead to generation of antibodies with undesirable reactivity. With a view to expressing β hCG by a recombinant method, a vaccinia virus (VV) recombinant was previously constructed. This virus (vSS1) expressed only moderate levels of β hCG however (Srinivasan *et al.* 1995). The gene for β hCG was placed under the control of the widely used VV p7.5 early/late promoter (Cochran *et al.* 1985). In the present study, we report construction of a VV recombinant using a late synthetic promoter (Psyn) (Davison & Moss 1990), which can express high levels of β hCG that is immunologically similar to the native molecule.

MATERIALS AND METHODS

Plasmids, virus and primary cell cultures

The cloning vector pBluescript KS[−] was obtained from Stratagene, La Jolla, CA, USA. The VV (WR strain) and vaccinia transfer vector pMJ602 (Davison & Moss 1990) were gifts from Dr B Moss,

National Institutes of Health, Bethesda, MD, USA. The monkey CV1 cells and human TK⁻143B cells (obtained from the National Centre for Cell Science, Pune, India) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone Labs Inc., Logan, UT, USA). Restriction enzymes were procured from Boehringer Mannheim, Mannheim, Germany; other fine chemicals, media and antibiotics were from Sigma Chemical Co., St Louis, MO, USA or Gibco-BRL, Gaithersburg, MD, USA, and radioisotopes were from Amersham International plc, Amersham, Bucks, UK. All cloning steps were performed as described by Maniatis *et al.* (1982).

Transfection and selection of recombinants

CV1 cells (1×10^6) were seeded into a T-25 culture flask and incubated overnight at 37 °C to obtain a confluent monolayer. A total of 3×10^6 cells were infected with VV (WR) at a multiplicity of infection (MOI) of 0.05 plaque-forming unit (pfu)/cell in medium containing 2.5% FBS, and incubated for 2 h with intermittent rocking. The inoculum mixture was aspirated and the cells, after washing with serum-free medium, were overlaid with 10 µg DNA complexed with lipofectin (Gibco-BRL) as per the manufacturer's instructions. Following incubation for 5 h at 37 °C in a humidified atmosphere containing 5% CO₂, the DNA-containing medium was replaced with 2 ml normal growth medium supplemented with serum. Two days later, the cells (containing the vaccinia recombinants) were scraped, centrifuged at 1800 *g* at 4 °C for 5 min, suspended in 0.5 ml DMEM with 2.5% FBS and lysed by three cycles of freeze-thawing followed by sonication.

A total of 5×10^5 TK⁻143B cells, seeded in a six-well plate and grown to confluency in DMEM supplemented with 10% FBS and 5-bromodeoxyuridine (BrDU; Boehringer Mannheim; 25 µg/ml), were infected with the cell lysate obtained after transfection. Following incubation at 37 °C for 2 h, the virus inoculum was aspirated and 1.5 ml selective agarose (low melting point agarose in DMEM with 2% FBS and 25 µg/ml BrDU) was added to each well. The agarose was allowed to solidify at 4 °C and the cells were incubated for a further 48 h at 37 °C. For visual selection of the recombinant virus, a second selective agarose overlay containing 5-bromo-4-chloro-3-inolyl- β -D-galactopyranoside (X-gal; Amresco, Solon, OH, USA) (300 µg/ml) was added. Following overnight incubation, five to six blue plaques were collected

by suction with a Pasteur pipette and transferred to 500 µl DMEM supplemented with 2.5% FBS. The virus particles were released by alternate cycles of freezing and thawing followed by indirect sonication for 30 s in an ice-water bath. After three to four rounds of purification, the recombinant plaque isolate was amplified by infection of successively larger number of cells. The selective drugs were added during the initial amplification steps after which no selection was required.

Detection and characterisation of β hCG

Amounts of β hCG expressed in culture supernatants were determined by competitive radioimmunoassay using a specific monoclonal antibody and purified native subunit as the standard (Singh & Capoor 1993). The affinity of β hCG for binding to antibodies (monoclonal and polyclonal) was determined by the cold displacement method essentially as described (Singh *et al.* 1989). Briefly, increasing concentrations of β hCG (recombinant or native) were incubated in a total volume of 0.5 ml with a limited amount of antibody in the presence of ¹²⁵I-hCG for 24 h at 4 °C. Antibody-bound fractions precipitated by polyethylene glycol molecular weight 800 (Amresco; 12.5% final concentration) were separated by centrifugation at 1500 *g* for 20 min, and counted for radioactivity in an LKB multi- γ -counter. The association constant (*K_a*) of β hCG-antibody interaction was calculated using a computer programme (Munson & Rodbard 1980). The ability of recombinant β hCG to associate with α hCG was tested in a mouse Leydig cell assay system as described (Rao *et al.* 1988). Culture supernatant containing expressed β hCG was incubated with a twofold excess of native α hCG in an end-to-end shaker for 24 h at room temperature. For Western blot, culture supernatant (harvested 72 h after infection) was fractionated on 12.5% denaturing SDS-PAGE (Laemmli 1970) and the resolved bands were transferred onto nitrocellulose membrane (Amersham International plc) in a transfer buffer containing 20 mM Tris-Cl, 150 mM glycine, pH 8.0 and 20% methanol. Non-specific sites were blocked by incubation with 2% bovine serum albumin (BSA) (in 50 mM phosphate-buffered saline, pH 7.4 and 0.05% Tween 20; PBST) for 1 h at room temperature. The filter was washed with PBST and then incubated for 2 h with the primary (β hCG-specific monoclonal) antibody. The membrane was washed again with PBST and incubated with goat anti-mouse IgG-horse radish peroxidase conjugate (Sigma) for 1 h. Following washings, reactive proteins were visualised using 3-3'-diaminobenzidine.

RESULTS

Generation of recombinant VV expressing β hCG

A recombinant VV vAO1 containing the β hCG gene under the control of the synthetic promoter, Psyn, was constructed employing standard protocols (Mackett *et al.* 1984). To achieve this, a recombinant VV transfer vector pAO6 was generated by cloning a 600 bp β hCG gene fragment at the *Hind*III site of the VV vector pMJ602 downstream of Psyn (Fig. 1). The orientation of the gene with respect to the promoter was determined by restriction analysis followed by Southern blotting and probing with 32 P-labelled β hCG (Fig. 2). The ability of the promoter to direct the expression of β hCG was checked in a transient expression system using CV1 cells. A significant amount (170 ng/ml) of β hCG was secreted into the culture supernatant but no activity was detected in the medium of cells infected with the wild-type virus. The expression cassette was inserted into the VV TK region by homologous recombination. The *tk* gene enabled a positive selection of the recombinant plaques in the presence of BrdU in a *tk*⁻ cell line. The *lacZ* gene under the control of the VV p7.5 promoter resulted in blue recombinant plaques in the presence of the chromogenic substrate X-gal.

Expression and characterisation of β hCG

Time kinetics of β hCG secretion were investigated in virus-infected cells. CV1 cells (2×10^6) were infected at an MOI of 0.5 pfu/cell. Expression of β hCG by the recombinant virus vAO1 increased as a function of time (Fig. 3). β hCG could be detected within 6 h of VV infection and the saturation level (11.5 μ g/ml) was attained at 48 h after infection. To determine the size of the expressed protein, culture supernatant of the cells infected with the recombinant virus vAO1 was subjected to Western blotting and the protein identified using a monoclonal antibody specific for β hCG. The migration pattern of recombinant β hCG was almost the same as that of the standard β hCG (Fig. 4), indicating that the size and overall extent of glycosylation of the vaccinia-expressed protein is similar to that of native β hCG.

The expressed protein was evaluated for its reactivity with a specific monoclonal antibody as well as with antibodies generated by five women immunised with the β hCG vaccine during efficacy studies. These antibodies are bionutralising in nature and recognise a discontinuous epitope on β hCG (Talwar *et al.* 1994, 1997). Analysis of cold

FIGURE 1. Construction of recombinant transfer vector pAO6 for the insertion of β hCG cDNA into the vaccinia genome. The gene for β hCG (β -hCG) was extracted from pAO1 by digestion with *Hind*III (right panel) and ligated to the VV transfer vector pMJ602 at its *Hind*III site (left panel). The recombinant clone pAO6 identified after colony hybridisation has the gene for β hCG under the control of Psyn (p-syn) and the *lacZ* gene under the control of an early portion of the p7.5 (p-7.5) promoter. TK-1 and TK-r refer to the segments of VV DNA that include the left and right portion of the thymidine kinase gene and pBR328 bacterial origin of DNA replication.

displacement curves revealed that VV-expressed recombinant β hCG bound each antibody tested with the same affinity as did native β hCG ($K_a = 2.5 \times 10^{10} \text{ M}^{-1}$ to $4.7 \times 10^{10} \text{ M}^{-1}$) (Fig. 5). These results indicate that the expressed subunit folds to recreate the conformational integrity of the

FIGURE 2. Southern blot analysis of the transfer vector pAO6 confirming the correct orientation of the β hCG gene with respect to the Psyn. The plasmid was cut with different restriction enzymes, run in 1% agarose gel (left panel), blotted onto a nitrocellulose membrane and probed with 32 P-labelled β hCG gene fragment (right panel). Lambda *Hind*III marker (lane 1), plasmid pAO6 digested with *Hind*III (lane 2), *Sma*I (lane 3), *Hind*III and *Sma*I (lane 4), *Bam*HI (lane 5) and *Eco*RI (lane 6). There is a unique internal *Sma*I site close to the 3' end of the gene for β hCG and another *Sma*I site in the multiple cloning site of the vector pMJ602 downstream of the *Hind*III site (site for insertion) with respect to the promoter. Hence, if the gene were in the right orientation with respect to the promoter, it would release a small fragment of ≈ 100 bp while the major portion of the insert would remain behind with the vector (lane 3). If the gene were in the wrong orientation, it would release almost the entire insert except for a small portion at its 3' end. Digestion with *Hind*III would release the insert of ≈ 600 bp (lane 2) irrespective of its orientation, while a double digestion with *Hind*III and *Sma*I would release a truncated gene for β hCG which would run faster than the whole gene in agarose gel (lane 4). There is only one site for each of the enzymes *Bam*HI and *Eco*RI in pAO6 so that the construct is only linearised (lanes 5 and 6) when digested with these enzymes.

native molecule. To confirm this, binding of β hCG with LH/hCG receptors was also investigated. Since isolated subunits cannot bind to receptor, recombinant β hCG was associated with the native α hCG and the potency of the resultant dimer tested in an *in vitro* bioassay. As is evident from Fig. 6, the dimer of recombinant β hCG and α hCG produced a steroidogenic response parallel to that of native dimer. On a weight basis, the recombinant dimer was observed to be nearly as potent (94%) as the native dimer, confirming that vaccinia-expressed recombinant β hCG has maintained the integrity of the molecule. As expected, medium containing individual subunits did not produce any biological activity.

DISCUSSION

We have here described the construction of a recombinant VV vAO1 which can express high levels of β hCG. The recombinant virus vAO1 expressed and secreted β hCG into culture medium in a time-dependent manner until peak levels of $11.5 \mu\text{g/ml}$ were obtained 48 h post infection. The levels of expression are at least sixfold higher than those secreted by the VV recombinant (vSS1) constructed earlier with p7.5 early/late promoter (Chakrabarti *et al.* 1989). These results are in contrast to the SV40 virus (Reddy *et al.* 1985)- and yeast (Sen Gupta & Dighe 1999)-based systems where co-expression of both α - and β -subunits was

FIGURE 3. Kinetics of β hCG secretion by cells infected with recombinant virus, vAO1. 2×10^6 CV1 cells were infected with 0.5 pfu/cell of recombinant VV vAO1 (with Psyn). Culture supernatants harvested at the time-points indicated were assayed for β hCG by competitive radioimmunoassay employing a specific monoclonal antibody.

FIGURE 4. Western blot analysis of total protein isolated from vAO1-infected CV1 cells. Culture supernatant of CV1 cells infected with the recombinant virus (lane 2) and purified native β hCG (lane 1) were fractionated in a 12.5% polyacrylamide gel under denaturing conditions. After blotting onto a nitrocellulose membrane, the proteins were identified by specific monoclonal antibody and HRPO-conjugated second antibody. mw, protein molecular weight marker lane.

FIGURE 5. Cold displacement analysis of β hCG–monoclonal antibody interaction. Slopes of the Scatchard curves with recombinant β hCG (●) and native β hCG (○) gave K_a values of $4.2 \times 10^{10} \text{ M}^{-1}$ and $4.5 \times 10^{10} \text{ M}^{-1}$ respectively.

required to obtain a reasonable yield. Since significantly high levels of expression could be achieved by VV-based system, low expression of β hCG by other expression systems may not be due to intrinsic instability of the molecule as suggested by Reddy *et al.* (1986).

β hCG consists of 145 amino acids, which include 12 cysteine residues that form six disulphide bonds. The three-dimensional structure of β hCG determined by X-ray crystallography (Lapthorn *et al.* 1994) has revealed a unique cystine knot motif (formed by three disulphide bonds) that largely determines the peptide folds of β hCG. β hCG has two N-linked and four O-linked glycosylation

FIGURE 6. Ability of recombinant β hCG (r β hCG) to associate with native α hCG and bind LH/hCG receptors. The dimer of recombinant β and native α was compared with native hCG at various concentrations in a mouse Leydig cell system and the testosterone produced was taken as an index of receptor binding.

sites. While O-linked carbohydrates are responsible for a longer circulatory half-life of the hormone, N-linked carbohydrates are required for proper folding of the molecule (Matzuk & Boime 1988). The β hCG-specific antibodies (mouse monoclonal and human polyclonal) used in this study are capable of neutralising hCG and recognise a discontinuous determinant that dominates the immune response in humans (Talwar *et al.* 1994, 1997). Discontinuous epitopes are, by their nature, highly dependent on the correct folding and conformation of the molecule. The synthesis of recombinant β hCG, with antibody-binding affinity similar to that of the native molecule, indicates that the molecule folds properly and forms the correct disulphide pairings to recreate native epitopes. Recombinant β hCG exhibited the ability to associate with native α -subunit and the resultant dimer could bind receptors to produce a steroidogenic response equal to that obtained with native hCG. On a Western blot, the recombinant β hCG migrated at a level similar to that of native β hCG. These characteristics of vaccinia-expressed protein indicated that correct post-translational processing of the molecule had occurred.

These results demonstrate that the vAO1 with Psyn expressed significantly high levels of β hCG that attains a conformation similar to that of the native molecule. β hCG, being a 'self' molecule, is

required to be linked to protein carrier molecules to render it immunogenic. Protein carriers have limitations and can be substituted by a combination of helper T-cell peptides (Mandokhot *et al.* 2000). Fusion of peptides at the C-terminal of β hCG does not affect the folding or antigenicity of β hCG (Srinivasan *et al.* 1995, Gupta *et al.* 1998). It will be of interest to express β hCG fused with helper T-cell epitopes to construct a well-defined chimeric immunogen, obviating the need for chemical linkage that invariably results in batch-to-batch variation.

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