Expression of Growth Factors in Dictyostelium discoideum

Sassan Asgari¹,³, Sharmila Arun¹, Martin B. Slade², John Marshall¹, Keith L. Williams² and John F. Wheldrake¹

¹School of Biological Sciences, Flinders University of South Australia, Bedford Park SA 5042, Australia
²Macquarie University Centre for Analytical Biotechnology (MUCAB), School of Biological Sciences, Macquarie University, NSW 2109, Australia
³Present address: Dept. of Applied and Molecular Ecology, Adelaide University, Waite Campus, Glen Osmond SA 5042, Australia

Abstract

Growth factors and their binding proteins are important proteins regulating mammalian cell proliferation and differentiation so there is considerable interest in producing them as recombinant proteins, especially in hosts that do not already produce a complex mixture of growth factors. Many growth factors require post-translational modifications making them unsuitable for production in Escherichia coli or other prokaryotes. Since several expression vector systems have been recently developed for foreign protein production in the cellular slime mould, Dictyostelium discoideum, we attempted to use two of these systems to express human insulin-like growth factor binding protein 6 (hIGFBP6) and bovine beta-cellulin (bBTC) as secreted proteins. Although both proteins were successfully produced in stably transformed amoebae, no secretion was detected in spite of several attempts to facilitate this occurring.

Introduction

In most situations the preferred expression system for the production of a protein by recombinant techniques is a bacterial one. However, that is not the case where the protein undergoes post-translational modification such as glycosylation because prokaryotes do not carry out such modifications in ways that are the same as eukaryotes. Therefore, a number of alternative systems are used in these circumstances and one that has many promising features is the cellular slime mould Dictyostelium discoideum.

This organism is a free-living amoeba feeding on soil bacteria. However, on depletion of the food source the amoebae aggregate and differentiate into a multicellular organism. This unusual life cycle has facilitated new experimental approaches to study the cellular interactions required for a multicellular organism and has also allowed the identification of promoters that are developmentally regulated and therefore of use for the controlled production of heterologous proteins. D. discoideum is also a robust eukaryotic microorganism with simple and economical growth conditions. A further advantage of D. discoideum is that it does not require serum to support its growth and hence avoids potential contamination with mammalian growth factors and infectious agents (Dittrich et al., 1994). Furthermore, various shuttle vectors have been developed that are either stably integrated into the genome or maintained extrachromosomally following transformation.

Several mammalian glycoproteins have been successfully expressed in D. discoideum, including rotavirus VP7 (Emslie et al., 1995), human muscarinic receptors m2 and m3 (Voith and Dingermann 1995, Voith et al., 1998), antithrombin III (Dingermann et al., 1991), a soluble form of mast cell IgE receptor (Wilson et al., unpublished) and choriogonadotropin (Heikoop et al., 1998). The muscarinic receptors were expressed as a membrane protein, but the others were secreted making downstream purification of a commercial product much simpler. The basic structure of N-linked glycosylation in D. discoideum is predominantly Man9GlcNAc2 similar to the mammalian high mannose structure, but completely lacking galactose, N-acetyl galactosamine or sialic acids found in complex N-linked structures (Sharkey and Kornfeld, 1991). Single, O-linked N-acetyl glucosamine residues attached to the mucin MUC1 and MUC2 repeats (Jung et al., 1998) is the only detailed characterisation of O-linked glycosylation on mammalian proteins expressed in D. discoideum. Dispite the replacement of N-acetyl galactosamine with N-acetyl glucosamine, the pattern of glycosylation was similar to that reported in mammals. The smaller, less variable glycosylation structures in D. discoideum results in recombinant glycoproteins having lower apparent molecular weights compared to proteins expressed in mammalian cells (Slade et al., 1997).

Extensive research has established the importance of insulin-like growth factors (IGFs), their binding proteins and receptors in health and disease. Insulin-like growth factors (IGF-I and IGF-II) stimulate cell division and differentiation of mammalian cells. IGFs form a complex with specific binding proteins. Six IGF binding proteins (IGFBPs) are known to regulate IGF activity and transport. Furthermore, an IGF-independent action of IGFBPs has recently been established (Reviewed in Oh et al., 1998). The binding proteins differ in their ability to bind IGF-I and/or IGF-II. Depending on the location of IGFBPs or metabolic conditions, they enhance or inhibit IGFs’ action (Ranke and Elmlinger, 1997). Here we expressed human insulin-like growth factor binding protein 6 (hIGFBP6) and bovine beta-cellulin (bBTC) in D. discoideum under both actin 15 and discoidin promoters. bBTC has recently been isolated.
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Figure 1. Partial coding region of (A) hIGFBP6 and (B) bBTC expressed in *D. discoideum*. First line, authentic nucleotide sequence; second line, modified nucleotide sequence according to the *D. discoideum* codon usage, substituted nucleotides are shown in bold; third line, amino acid sequence. The amino acid sequence of the original hIGFBP6 signal peptide was used to make construct no. 4 (see Figure 2) and the PsA signal peptide used for other constructs are shown on top. The cleavage sites are shown with an asterisk.
Protein Expression in *D. discoideum* from whey and the expression of this protein would help further investigations regarding the function of the protein. Both proteins were successfully expressed, but no secretion of the proteins was detected despite genetic modifications to the secretion signals.

**Results**

Expression of hIGFBP6 and bBTC

Initially, the coding regions of hIGFBP6 and bBTC were reconstructed (Figure 1) to favour *D. discoideum* codon usage and cloned under the control of *D. discoideum* actin 15 promoter and PsA secretion signal (Figure 2, constructs 1 and 5). The clones were sequenced in both directions to confirm the sequence. Both constructs were transformed into *D. discoideum* NP2 cells by the calcium-phosphate method. Transformed cells were starved for up to 6 hours in KK2 (see experimental procedure) and analysed for expression of recombinant proteins and mRNA. Northern blot analysis revealed that transformed cells with hIGFBP6 and bBTC genes both produced the expected size transcripts (Figure 3A). Western blots of cells demonstrated that both proteins were expressed. An increase in the level of expression of hIGFBP6 was observed over time following starvation (Figure 3B) and, as a control, antibodies against hIGFBP6 did not cross-react with a similar size protein in non-transformed cells. The size of the protein (ca. 33kDa) was found to be slightly lower than hIGFBP6 expressed in Chinese hamster ovary (CHO) cells (ca. 36kDa), but substantially larger than the 25kDa predicted from the peptide. The difference in size indicates the protein is glycosylated, but also that there are differences in the glycosylation structure compared to that found in mammalian cells. We often detected two hIGFBP6 bands of very similar molecular weight being produced by *Dictyostelium*. These were both found to be glycosylated as shown by Wheat Germ Agglutinin binding (Figure 5C) which recognizes N-acetyl glucosamine residues from whey and the expression of this protein would help further investigations regarding the function of the protein. Both proteins were successfully expressed, but no secretion of the proteins was detected despite genetic modifications to the secretion signals.

**Expression of hIGFBP6 and bBTC**

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Asgari et al. (Neumann et al., 1998). In other experiments, only one of the two bands was detected. It seems likely that two glycoforms of hIGFBP6 can be formed in *Dictyostelium* as is also seen in CHO cells expressing the protein (Neumann et al., 1998).

The expressed hIGFBP6 was found to be active with regard to ligand binding. Compared to IGF1, IGFII binds IGFBP6 with 20-100 fold more affinity (Neumann et al., 1998), therefore, we used labeled 125I-hIGFII as a ligand. In ligand blot experiments, 125I-hIGFII bound to the expressed IGFBP6 protein (Figure 4). Interestingly, the probe also bound to a *Dictyostelium*-specific protein of a lower molecular weight. Attempts to isolate and identify the protein were unsuccessful.

Antibodies against human BTC were used to probe the bBTC expressed in *D. discoideum* cells. The unmodified protein is predicted to be about 9 kDa, but seems to run as a dimer when analysed on SDS-PAGE (Shing et al., 1993). Therefore, the protein expressed in bacteria, where an 18 kDa protein was identified as the primary product, was used as a positive control (Figure 3B). However, the bBTC expressed in *D. discoideum* was found to be around 26 kDa (Figure 3B). The higher molecular weight of the protein expressed in *D. discoideum* compared to the one expressed in bacteria is attributable to post translational modifications by the amoebae. No secretion of either of the two proteins was detected (data not shown) indicating the absence of a functional secretion by constructs using a PsA signal in these cells. Since the PsA signal has been successfully used in production of secreted proteins in *D.*

Figure 5. hIGFBP6 transformed *D. discoideum* cells starved for 6 h in (A) KK2 and MES buffers containing various CaCl2 concentrations (0, 2 and 10 mM). NP2, non-transformed control cells; The amount of hIGFBP6 increases by an increase in CaCl2 concentration. (B) various concentrations of ZnSO4 and CaCl2 (0-10 mM). S1 and S2, supernatants from cells starved in 10 mM CaCl2 and ZnSO4, respectively. The protein was not detected in the supernatants indicating the lack of secretion (C) Two forms of hIGFBP6 expressed in *D. discoideum* detected by anti-hIGFBP6 antibodies (Anti-BP6) is also detected by Wheat Germ Agglutinin (WGA, arrows).
discoideum, lack of secretion of expressed hIGFBP6 and bBTC proteins is assumed to be due to differences in post-translational modifications in this organism.

Effect of Varying Induction Conditions on Protein Expression

Since the level of expression of both proteins was low, attempts were made to increase the level of expression by modifying the induction condition. Thus, hIGFBP6 transformed cells were starved under various conditions. We found temperature (20-27°C), cAMP (1 mM) and proteinase inhibitors (E64 5 µM, benzamidine 5 mM, TLCK 50 µg/ml, TPCK 100 µg/ml) had no effect on the level of expression (data not shown). However, the addition of calcium and zinc did increase the level of expression although the results were not always consistent. In one experiment, an increase in CaCl₂ concentration from 0-10 mM in KK2 and MES buffers was found to increase the expression up to about three times (Figure 5A). ZnSO₄ increased the level of expression compared to CaCl₂ (Figure 5B). However, although we increased the level of expression several fold by changing the starvation conditions we did not observe any protein secretion (Figure 5B).

Modifications of the Constructs

Since there was no secretion of the expressed hIGFBP6 and bBTC proteins with the initial constructs (Figure 2, no. 1 and 5), modifications were made to the signal sequences. In constructs 1 and 5, the C-terminus of secretion signal has the sequence YALA. We modified this sequence to the authentic PsA sequence YANA which has been successfully used for protein secretion (Dittrich et al., 1994). For this, new constructs (Figure 2, no. 2 and 3) containing the original hIGFBP6 DNA sequence (not adjusted for D. discoideum codon usage) and modified sequence were generated with the new leader sequence. The constructs were transformed into D. discoideum cells to produce a protein of the expected size range. However, no secretion of the protein was detected (data not shown). A similar construct was made for bBTC (codon usage as in construct no. 5) with the new leader sequence (Figure 2, no. 6). In this case, although the expression plasmid was detected in transformed cells, no expression of the protein was detected.

An additional construct (Figure 2, no. 4) was made containing the original hIGFBP6 leader sequence with a cleavage site before amino acid residue 4 (Arginine, R) shown in figure 1A (Neumann et al., 1998). In this case, pHVEII plasmid, a single vector system, containing the gene was transformed into AX2-2-44 cells. Putative transformed clones were tested for expression and secretion of the protein on Western blots. The protein was detected in the starved cells, but not in the supernatant indicating that there is no secretion.

When transformants containing the authentic and codon modified hIGFBP6 genes were compared for the level of expression, we found that those cells which were transformed with the codon adjusted gene expressed higher amounts of the protein compared to the human coding DNA as judged by the intensity of the bands detected in Western blots (Figure 6). In all cases, a similar number of cells were starved and sampled for analysis.

Discussion

The data presented here show that the simple eukaryote D. discoideum is able to produce biologically active hIGFBP6 and bBTC proteins. Although hIGFBP6 and bBTC were successfully expressed in transformed amoebae, no secretion of the proteins was detected. The possibility of enzymatic degradation after secretion was ruled out by using a variety of proteinase inhibitors. This failure of secretion is in contrast to other cases, where the PsA signal has also been used as a secretion signal, and the recombinant proteins have been detected in the starvation buffer indicating that both secretion and cleavage of the expressed proteins had occurred (Dittrich et al., 1994; Heikoop et al., 1998). In an effort to express human choriongonadotropin, Heikoop et al., 1998 observed a higher level of protein production when they used the protein's own leader peptide compared to the PsA signal. In a similar attempt, we replaced the PsA signal with hIGFBP6 leader peptide, but no protein secretion was observed. Attempts to modify the PsA secretion signal also failed to induce the secretion of the proteins.

Earlier work showed that calcium ions increased the stability, secretion and yields of rotavirus VP7 (Emslie et al., 1996). In this case CaCl₂ and ZnSO₄ were found to increase the amount of protein produced, but in spite of an increase in protein production no secretion was detected. However, both proteins appear to be glycosylated indicating they had been processed through part of the secretion pathway.

The retention of these proteins could be due to either an inappropriate signal used in mammalian cells to control secretion or perhaps to inappropriate glycosylation. The hIGFBP6 expressed in D. discoideum cells is slightly lower in molecular weight (about 3 kDa) compared to the protein expressed in CHO cells suggesting glycosylation may be absent or incorrect. hIGFBP6 has been shown to be O-glycosylated (Neumann et al., 1998) and bBTC has both N- and O-glycosylation (Dunbar et al., 1999). All D. discoideum recombinant proteins analysed to date have glycosylation in appropriate sites (Slade et al., 1997). However, this may not be the case for all O-linked glycosylation. D. discoideum can produce some unusual
O-linked phosphodiester structures that are not found in mammals (Gustafson and Milner 1980; Srikrishna et al., 1998). GlcNAc-1-P-Ser replaces phosphorylation of N-linked structures on lysosomal proteinases (Mehta et al., 1996) so might act as a signal for sorting proteins to the lysosomal compartment. This form of glycosylation could be disastrous for recombinant protein production. In conclusion, the data presented here reveal that in spite of successful protein synthesis, D. discoideum does not appear to be suitable for the production of insulin-like growth factor binding proteins, especially those that contain O-linked carbohydrates.

**Experimental Procedures**

**D. discoideum Strains and Culture Conditions**

Laboratory strains NP2 and AX2-2-44 (B. Wetterauer, personal communication) were maintained by weekly passage on Enterobacter aeroginosa lawns on SM agar at 21°C. Cells were also grown axenically by inoculating HLS nutrient broth with sporadic heads and weekly passage (Firtel and Lodish, 1972).

G418-resistant transformants were maintained on lawns of Micrococcus luteus strain PRFP3 (Wilczynska and Fischer, 1994) on SM agar with 1/5 of the organic nutrients (SM/5) and 10 µg/ml G418. Transformants were also maintained axenically in HL5 containing 10µg/ml G418.

**Construction and Cloning of hIGFBP6 and bBTC**

cDNA coding for hIGFBP6 and bBTC were reconstructed by PCR using long synthetic oligonucleotides as templates and primers to favour D. discoideum codon usage (Figure 1, A and B). Four versions of hIGFBP6 and two of bBTC were constructed with alterations in their secretion signals and stop codon (Figure 2). PCR amplifications were carried out in 50µl reactions containing 1-10ng plasmid or PCR product, 20 pmol oligos, 150 µM dNTPs, 5U 10PCR buffer (Boehringer) plus 2.5 mM MgCl₂. The reactions were heated to 94°C for 3 min and 1U Taq DNA polymerase (Promega) was added. The reaction used 15 cycles of 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C with a final 7 min extension at 70°C. The PCR primers contained restriction sites to allow the constructed genes to be cloned in frame into pMUW1630 (Dittrich et al., 1994) and pVEII (Blusch et al., 1992) plasmid vectors containing the D. discoideum actin 15 and discoidin promoters, respectively. All the constructs were verified by sequence analysis.

**Transformation and Selection**

A two vector expression system (Dittrich et al., 1994) which consisted of an extrachromosomal expression vector, pMUW1630, and an integrating vector, pMUW110, was used for most of transformations. 2µg of pMUW1630 incorporating the relevant insert and 10µg pMUW110 were co-transformed into 10⁷ D. discoideum cells (NP2 strain) using calcium-phosphate precipitation procedure (Nellen et al., 1984; Early and Williams, 1987). After transformation and glycerol shock, cells were incubated overnight at 21°C in HLS, before 10µg/ml G418 was added for a further 24 hour incubation. Transformant cells were resuspended, centrifuged at 500g for 5 min and spread on overnight grown lawns of M. luteus on SM/5 agar plates containing 10µg/ml G418. Plates were incubated at 21°C for 10-14 days till spor formation plaques appeared. Spore heads were streaked on lawns of M. luteus on SM5 plates containing 10µg/ml G418. Those clones that grew on subculture and formed spores were screened for protein expression.

In a case where the pVEII plasmid, a single vector system, was used, cells were isolated by plating 5×10⁶ cells on SM agar in cold KK2 buffer by centrifugation at 200g for 10 min. After the final wash, cells were resuspended in starvation buffers (KK2 or 20 mM 2- (N- morpholino)ethanesulfonic acid (MES), containing 0-10 mM CaCl₂ or 0-10 mM ZnSO₄) at a density of 1×10⁷ cells/ml. Cells and supernatants were collected at several time points after starvation.

**RNA isolation and Northern Blotting**

Total RNA was extracted from about 1×10⁷ cells as described (Chomczynski and Sacchi, 1987). 20µg RNA samples were electrophoretically separated under denaturing conditions on low-formaldehyde 1.2% agarose gels (Ausubel et al., 1993) and transferred onto a nylon membrane (Sambrook et al., 1989). The membrane was prehydrized for 1 hour in 5×SSC, 5×Denhardts solution, 0.5% SDS and 0.2 mg/ml herring sperm DNA. hIGFBP6 and bBTC cDNAs were labeled with 32P-dCTP by primer extension (Ready to Go, Pharmacia). Hybridization was carried out overnight at 65°C by adding the labeled probe to the prehybridization solution. The membrane was washed twice in 2×SSC-0.1% SDS for 20 min and twice in 0.2×SSC-0.1% SDS at 65°C under continuous rotation and then autoradiographed.

**Ligand and Immunoblotting**

Cells (ca. 1×10⁷) were collected from starvation medium by low speed centrifugation, mixed with 4×sample buffer (3.02g Tris-base, 8g SDS, 20mM glycerol, 4mg bromophenol blue, pH 6.9 in 100ml final volume) and run on 12% SDS-PAGE after heating at 65°C for 15 min. Separated proteins were transferred onto a nitrocellulose membrane by semi-dry blotting (Bio-Rad). The membranes were incubated overnight at room temperature with 125I-hIGFI (1×10⁶ cpm) in 50 ml TBST (10mM Tris-HCL, pH 8.0, 150 NaCl, 0.05% Tween 20) containing 5% BSA. Membranes were washed several times in TBST and scanned 24 h later using a phosphorimager scanner (Aga). Samples for immunoblotting were separated on a 12% SDS-PAGE gel under denaturing conditions and transferred onto a nitrocellulose membrane (Sambrook et al., 1989). Membranes were probed with rabbit anti-hIGFBP6 or anti-bBTC antibodies (1:2000). Peroxidase-conjugated anti-rabbit IgG (Sigma) was used as a secondary antibody (1:5000) which was detected by chemiluminescence using Renaissance Western Blotting Reagents (AMRAD). Peroxidase-conjugated Wheat Germ Agglutinin (WGA) was used as a probe (1:5000 TBST-5% BSA) for a blot to detect glycosylated hIGFBP6. Chemiluminescence was used for detection as above.

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**References**


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