A simple method for the production of recombinant proteins from mammalian cells

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Expression of recombinant proteins in mammalian cells is useful for obtaining products with normal post-translational modifications. We describe a simple and economical method for the production of milligram levels of proteins in murine fibroblasts. Retroviral or LIPOFECTAMINE™ (Gibco Laboratories) transduction was employed to generate stable murine-fibroblast producer cells. Confluent cultures of stable fibroblast clones were maintained for up to 1 month in 0.5% serum. Culture medium was collected every 2–3 days and polyhistidine-tagged proteins were purified by ammonium sulphate precipitation and Ni^{2+}-nitrilotriacetic acid affinity chromatography. Highly pure, active, glycosylated recombinant proteins, including human β-glucuronidase, mouse β-glucuronidase, aminopeptidase N (CD13) and a single-chain antibody-enzyme fusion protein, were obtained with yields of 3–6 mg/l of culture medium. Fc-tagged proteins were also produced and purified in a single step by Protein A affinity chromatography with yields of 6–12 mg/l. The techniques described here allow simple and economical production of recombinant mammalian proteins with post-translational modifications.

Introduction

Recombinant proteins expressed in *Escherichia coli* lack post-translational modifications and may fold incorrectly and lack activity. Mammalian expression systems, in contrast, allow the production of recombinant proteins with normal post-translational modifications, which are important for many pharmacological properties. For example, glycosylation can affect the biological activity, stability, immunogenicity and pharmacokinetics of glycoproteins [1], and phosphorylation can alter the properties such as receptor-mediated uptake [2]. Although the advantages of mammalian expression systems are clear, the relatively low levels of production and the complex media requirements of tissue cell cultures complicate product recovery and have hindered their widespread use. In the present study, we report a general, cheap and simple method for the production of pure recombinant proteins from murine fibroblasts.

Materials and methods

Cell lines

HEF fibroblasts were kindly provided by Dr Kurt von Figura (Department of Biochemistry, University of Gottingen, Gottingen, Germany). GM637 human fibroblasts and Sf21 (*Spodoptera frugiperda*) cells were gifts from Dr T.-C. Lee and Dr Y. Chern (Academia Sinica, Taipei, Taiwan). CL1-5 cells were a gift from Dr P.-C. Yang (National Taiwan University Hospital, Taipei, Taiwan). BALB/3T3 fibroblast cells, BHK-21 (baby hamster kidney) cells, WISH human amnion cells, HeLa human cervical carcinoma cells, EL4 murine lymphoma cells, J774A.1 mouse macrophage cells, L6-20-4 hybridoma (murine anti-L6) cells and UC10-4F10-11 hybridoma (hamster anti-mouse CTLA4) cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). A mouse anti-CD28 hybridoma (37.51) was kindly provided by Dr N.-S. Liao (Academia Sinica). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Sigma, St. Louis, MO, U.S.A.), supplemented with 10% (v/v) heat-inactivated bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a 5% CO_2 humidified atmosphere. All the cells were free of mycoplasma as determined by a PCR-based mycoplasma detection kit (A.T.C.C.).

Plasmid construction

The mature form of hβG (human β-glucuronidase) was PCR-amplified from pHUGP13-hβG (generously provided by Dr W. S. Sly, St. Louis University, St. Louis, MO, U.S.A.) and inserted in-frame with the GP67 leader sequence in the baculovirus transfer vector pAcGP67B (BD Biosciences, San Diego, CA, U.S.A.) to generate pAcGP67B-hβG. The mature form of hβG was also inserted into pSecTag2 (Invitrogen, San Diego, CA, U.S.A.) to generate pSecTag2-hβG with an

Key words: aminopeptidase N, ammonium sulphate, β-glucuronidase, His tag, mammalian cell, purification.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; hβG, human β-glucuronidase; L6–hβG, L6 scFv–hβG fusion protein; mβG, murine β-glucuronidase; Ni-NTA, Ni^{2+}-nitrilotriacetic acid.

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immunoglobulin κ-chain leader sequence at the 5′-end and a myc epitope and a polyhistidine tag at the 3′-end of the hβG cDNA. The *Ase*I–*Sph*I fragment of pSecTag2-hβG was blunt-end cloned into the pLHCX retroviral vector (BD Biosciences) at the unique *Hpa*I site to generate pLHCX-hβG. A cDNA fragment encoding mβG (mouse β-glucuronidase) was generated by reverse transcriptase-PCR amplification of RNA isolated from J774A.1 cells and sequentially inserted into pSecTag2 and pLHCX in an analogous manner to create pLHCX-mβG. The cDNA containing the extracellular domain of human CD13 was also sequentially cloned into pSecTag2 and pLNCX to create pLNCX-sCD13.

A single-chain mAb (monoclonal antibody) L6 was constructed from L6-20-4 hybridoma cells with a $(Gly_4Ser)_4$ linker as described in [3]. hβG cDNA was amplified by PCR to introduce a six-amino-acid linker (GSGGSG) at the 5′-end before it was inserted in-frame at the 3′-end of the L6 scFv cDNA to generate pSecTag2-L6-hβG. The entire cassette was transferred to pLHCX to generate the retroviral vector pLHCX-L6-hβG with a polyhistidine tag at the 3′-end of the fusion protein.

The γ1 domain encoding the hinge-CH_2-CH_3 domains of human IgG_1 was excised from p2C11-γ1-B7 [3] and inserted in place of the PDGFR (platelet-derived growth factor receptor) transmembrane domain in p2C11-PDGFR [3] to create p2C11-γ1. The 2C11 scFv was replaced with cDNA (A.T.C.C.) encompassing amino acids 1–161 of murine CTLA4 to generate pCTLA4-γ1. In a similar fashion, the extracellular portion of murine CD28 (amino acids 1–149) was reverse transcriptase-PCR-amplified from EL-4 cells and inserted in place of the CTLA4 fragment to generate pCD28-γ1.

Transfection

Cells were transiently transfected using LIPOFECTAMINE™ 2000 (Gibco Laboratories, Grand Island, NY, U.S.A.). βG activity in the culture medium was assayed 48 h later as described in [4]. To generate permanent cell lines, BALB/3T3 fibroblasts were infected with recombinant retroviral particles packaged in Phoenix-Eco cells (generously provided by Dr Gary Nolan, Stanford University, CA, U.S.A.). Stable BALB/3T3 cells were also prepared by LIPOFECTAMINE™ transfection with pCTLA4-γ1 or pCD28-γ1. Fibroblasts were selected in 0.5 mg/ml G418 or 0.2 mg/ml hygromycin. Stable producer cell lines were isolated by plating 0.5 cell/well in 96-well microtitre plates without antibiotic selection.

Protein production

Single clones of recombinant baculovirus were produced in Sf21 cells by transfection with pAcGP67B-hβG and linearized baculogold viral DNA as per the manufacturer's instructions (BD Biosciences). Sf21 cells were infected with recombinant virus at an MOI (multiplicity of infection, i.e. number of viral particles per cell) of 10 and cultured for 4 days at 27 °C. For protein production in mammalian cells, BALB/3T3 producer cells were cultured in 15 cm dishes in DMEM/5 % bovine serum until confluence. The medium for CTLA4-γ1 and CD28-γ1 was then changed to DMEM/0.5 % bovine serum or 0.5 % low IgG serum (Gibco Laboratories). We harvested 90 % of the culture medium every 2–3 days. Polyhistidine-tagged proteins were precipitated by adding ammonium sulphate to 40–60 % saturation. The pellets were dissolved in binding buffer (0.5 M NaCl/20 mM Tris/HCl, pH 7.9) and purified on an Ni-NTA (Ni^{2+}-nitrilotriacetic acid) column (Amersham Biosciences, Uppsala, Sweden). CD28-γ1 and CTLA4-γ1 were directly purified by Protein A affinity chromatography. The eluted proteins were desalted on Sephadex G-25, equilibrated with PBS and concentrated by ultrafiltration. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.).

Activity assays

Enzymic activities of hβG, mβG and L6–hβG (L6 scFv–hβG protein) were measured at 37 °C using *p*-nitrophenol β-D-glucuronide as the substrate [4]. The combined antigen-binding and enzymic activity of L6–hβG was determined as described in [5]. Activity of sCD13 was determined by adding 20 μl of culture medium or purified sCD13 to 160 μl of PBS containing 0.5 % BSA. Then, 20 μl of L-alanine-*p*-nitroanilide (20 mM) was added for 30 min at 37 °C before measuring the absorbance at 405 nm. Binding activities of CTLA4-γ1 and CD28-γ1 were measured by ELISA in microtitre plates coated with 1 μg/well anti-CTLA4 (4F10) or anti-CD28 (37.51) antibodies as described in [6].

sCD13 deglycosylation

A mixture (2.5 μl) containing 5 % (w/v) SDS and 10 % (v/v) 2-mercaptoethanol was added to 13 μl (5 μg) of sCD13. The mixture was boiled at 100 °C for 10 min. Then, 2.5 μl of 10 % (v/v) Nonidet P40 in 0.5 M sodium phosphate (pH 7.5), 2 μl (10 units) of peptide N-glycosidase F (New England Biolabs, Beverly, MA, U.S.A.) and 5 μl of water were added for 24 h at 37 °C.

Results

We screened several cells for high expression of hβG. A baculovirus transfer plasmid pAcGP67B-hβG was employed to transfect Sf21 insect cells, but secretion of hβG to the culture medium was low ($< 0.1\ \mu g \cdot ml^{-1} \cdot day^{-1}$; results not shown). Transient transfection of several mammalian cells (BALB/3T3, GM637, WISH, HeLa and BHK-21) with

pLHCX-hβG resulted in hβG secretion rates of 0.25–1.8 $\mu g \cdot ml^{-1} \cdot day^{-1}$ and the highest production was from BALB/3T3 fibroblasts (results not shown). Since hβG is normally targeted to lysosomes by mannose 6-phosphate receptors [2], we tested whether higher levels of hβG could be secreted by mannose 6-phosphate receptor-deficient HEF fibroblasts [7]. We infected both BALB/3T3 and HEF fibroblasts with recombinant hβG retrovirus and selected stable producer cells by limiting the dilution. Stable BALB/3T3 clones secreted higher levels of hβG compared with HEF clones (results not shown). We therefore employed BALB/3T3 cells for recombinant protein production. After they were transduced with recombinant retrovirus, selected in G418 and cloned by limiting dilution without drug selection, BALB/3T3 cells secreted 5–6 μg of hβG $\cdot ml^{-1} \cdot day^{-1}$.

Essentially, no polyhistidine-tagged hβG was recovered after passage of culture medium through an Ni-NTA column under different conditions. Batch processing allowed the recovery of up to 25% of the recombinant hβG but only when large amounts of the Ni-NTA resin were employed (20–50%, v/v). Extensive dialysis of the culture medium against the Ni-NTA-binding buffer did not improve product recovery (results not shown). In contrast, ammonium sulphate precipitation before Ni-NTA affinity chromatography increased the recovery of recombinant proteins. As it can be seen from Figure 1(A), near-quantitative recovery of hβG and sCD13 was achieved by ammonium sulphate precipitation of the culture medium. After dissolving the precipitate in Ni-NTA-binding buffer, sCD13 bound well to Ni-NTA, resulting in a sharp peak after elution with excess imidazole (Figure 1B). The final yield was 3–5 mg of purified sCD13 per litre of culture medium with an overall recovery of approx. 40%. As shown in Figure 2(A), sCD13 could not be seen after SDS/PAGE of the original culture medium, whereas the final product consisted of a major band with the expected molecular mass of approx. 150 kDa (Figure 2A). As shown in Figure 2(B), peptide N-glycosidase F treatment decreased the apparent mass of sCD13 from 150 kDa to approx. 110 kDa, which is the predicated molecular mass of the peptide backbone. This result demonstrates that recombinant sCD13 was glycosylated as expected. The simple procedure employed by us to produce and purify sCD13 gave similar yields and purities for hβG (Figure 3A) and mβG (Figure 3B). Both hβG and mβG displayed the expected optimum pH of approx. 4.5 (Figure 3C). hβG and mβG prepared in the present study displayed specific enzymic activities that were similar to published values for these enzymes, whereas our recombinant sCD13 was more active compared with previously described preparations (Table 1).

The production of sCD13 in each batch of culture medium collected every 2–3 days for 3 weeks was monitored by measuring the sCD13 activity of the culture medium (Figure 4, solid line) and the final purified protein (broken line). Stringent washing conditions (50 mM imidazole) were employed to produce a highly purified protein at the expense of decreased amount of yield. The secretion of sCD13 from BALB/3T3 producer cells was stable for at least 3 weeks with an average yield of 1 mg of purified sCD13 l^{-1} day^{-1}.

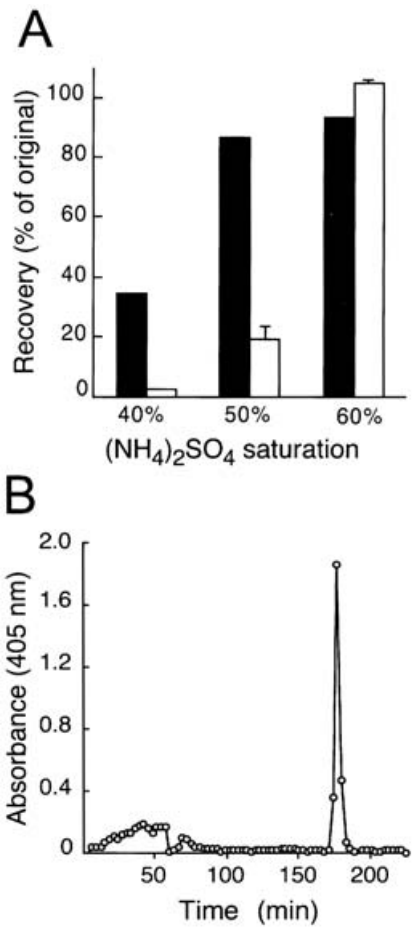

Figure 1 Purification of polyhistidine-tagged proteins

(A) Increasing amounts of ammonium sulphate were added to the culture medium of BALB/3T3 producer cells. Precipitated proteins were solubilized in PBS, and the hβG (black bar) or sCD13 activities (open bar) were measured. (B) Samples collected from a 3 ml Ni-NTA column were assayed for CD13 activity. 0–54 min, loading; 54–165 min, washing; 165–225 min, elution.

There are many difficulties in attaining high production levels of antibody–hβG fusion proteins in mammalian cells [8,9]. In contrast, production of L6–hβG in BALB/3T3 fibroblasts followed by purification on Ni-NTA resulted in the recovery of 3–5 mg/l L6–hβG. Purified L6–hβG appeared as a major band on SDS/polyacrylamide gel (Figure 5A) and it bound antigen-positive CL1-5 cells in a dose-dependent manner (Figure 5B, circles). hβG, in contrast, did

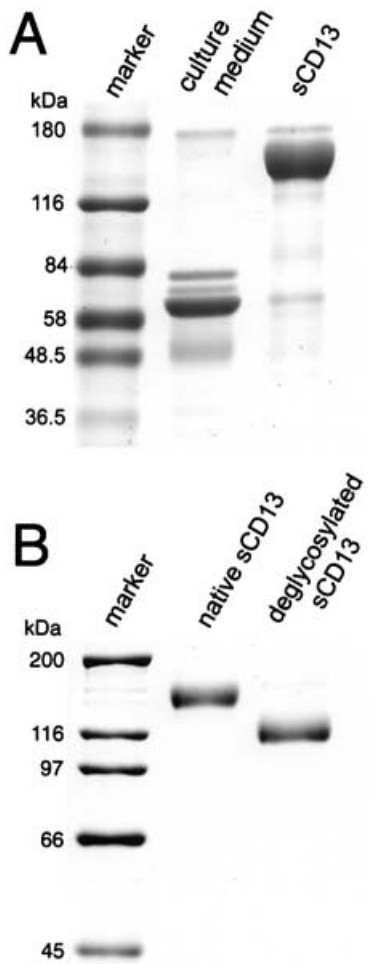

Figure 2 Characterization of recombinant CD13

(**A**) SDS/PAGE of the original culture medium and 10 μg of sCD13, purified as described in the Materials and methods section. (**B**) SDS/PAGE of 5 μg of native sCD13 or deglycosylated sCD13.

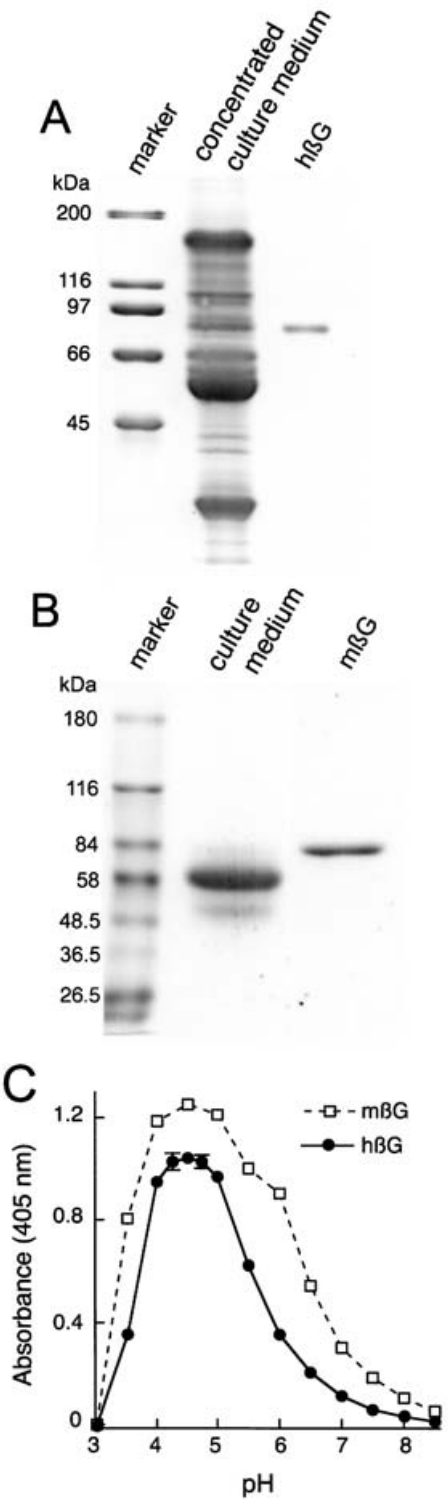

Figure 3 Characterization of purified β-glucuronidase

(**A**) SDS/PAGE of proteins in the binding buffer before purification on Ni-NTA (concentrated culture medium) and purified hβG. (**B**) SDS/PAGE of the original culture medium and purified mβG. (**C**) Activity of purified hβG and mβG (0.3 μg each) at different pH values.

not bind to CL1-5 cells (Figure 5B, squares), and free mAb L6 completely competed the binding of L6–hβG to CL1-5 cells (Figure 5B, triangles), showing that L6–hβG binding was through the L6 scFv. L6–hβG also retained complete hβG enzymic activity (Table 1).

We also tested whether stable producer cells could be isolated after LIPOFECTAMINE™ transduction and antibiotic selection of BALB/3T3 fibroblasts. Screening of single clones grown in the absence of antibiotic selection allowed the isolation of stable producer cells. As seen from Figure 5(C), CTLA4-γ1 and CD28-γ1 could be purified from the culture medium in a single step by Protein A affinity chromatography. Both proteins migrate as disulphide-linked dimers under non-reducing conditions and as monomers under reducing conditions. CTLA4-γ1 and CD28-γ1 were produced with final yields of 6 and 12 mg/l respectively.

Table 1 Comparison of the enzymic activities of recombinant proteins

Protein	Specific activity of recombinant His-tagged protein (μmol · h^{-1} · mg^{-1})		Published protein source	Reference
	Present study	Previous work		
hβG	1830 ± 25	1200	Seminal plasma	[20]
		1880	Recombinant	[21]
mβG	1210 ± 4	890	A/Jax mouse kidney	[22]
		780	C57BL/6J mouse urine	[23]
		2410	DMA mouse urine	[23]
CD13	1290 ± 10	446	Seminal plasma	[24]
		117	Prostate	[25]
L6–hβG	1990 ± 17[a]	NA[b]	NA	NA

[a] Corrected for the hβG content in L6–hβG.
[b] NA, not applicable.

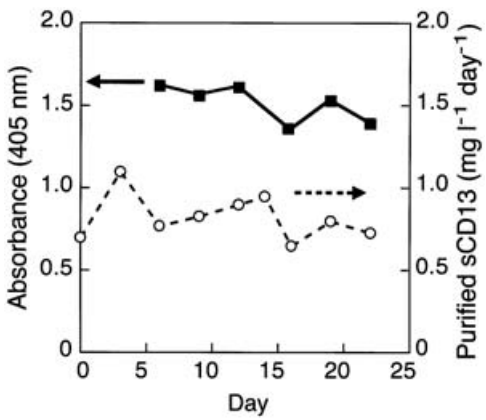

Figure 4 sCD13 production

Production of sCD13 was monitored by measuring the activity of CD13 in the culture medium (■) and the amount of purified sCD13 (○) obtained from each batch.

Discussion

Production of recombinant proteins with 'normal' post-translational modifications is important for a wide variety of laboratory and preclinical studies. Although baculovirus–insect cell systems are commonly employed to produce recombinant proteins that require post-translational modifications, the production of hβG in insect cells was low in the present study. hβG has been reported to be successfully produced in insect cells [10]. However, published yields of hβG were approx. 1 μg of hβG per mg of cell lysate with very low levels of enzyme detected in the culture medium [11]. N-glycosylation in insect cells also differs in many important respects from mammalian cells [12]. In contrast, we found that a wide variety of recombinant proteins could be secreted by murine fibroblasts at mg/l concentrations.

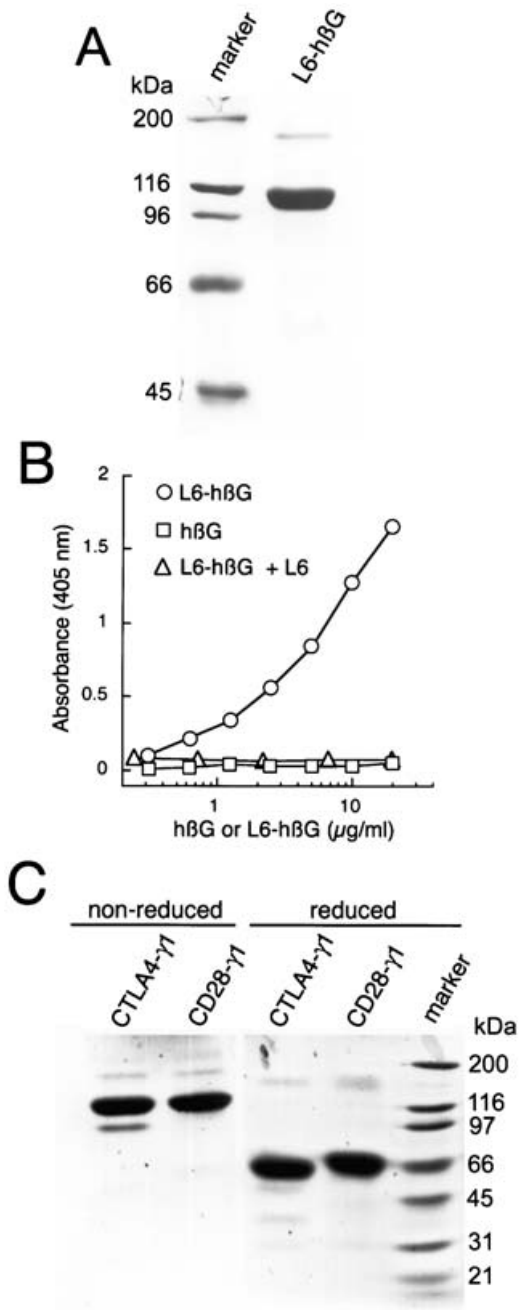

Figure 5 Production and characterization of recombinant fusion proteins

(**A**) SDS/PAGE of purified L6–hβG. (**B**) Graded concentrations of L6–hβG (○) or hβG (□) were allowed to bind to CL1-5 lung cancer cells, and βG activity was then measured. Excess free L6 antibody completely blocked L6–hβG binding to CL1-5 cells (△). (**C**) Purified CTLA4-γ1 and CD28-γ1 were subjected to SDS/PAGE under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions.

We also found that ammonium sulphate precipitation of the culture medium facilitated the recovery of polyhistidine-tagged proteins by Ni-NTA affinity chromatography.

Among the cell lines tested, BALB/3T3 fibroblasts displayed a good transduction efficiency (routinely 80–90 % with LIPOFECTAMINE™) and allowed the secretion of the highest amounts of hβG. A wide range of soluble proteins were produced and purified from BALB/3T3 cells, including recombinant hβG, mβG, sCD13 (aminopeptidase N), L6–hβG, CD28-γ1 and CTLA4-γ1. β-Glucuronidase is a homotetramer that catalyses the hydrolysis of β-D-glucuronic acid residues from the non-reducing termini of glycosaminoglycans. This lysosomal enzyme is of considerable biological and medical interest, since defects in βG activity or transportation can lead to lysosomal storage disease [13]. Both β-glucuronidase as well as the L6–hβG immunoenzyme are also potentially useful for antibody-directed enzyme prodrug therapy [14]. Aminopeptidase N (CD13) is a homodimer, transmembrane glycoprotein that is highly expressed in endothelial cells present in tumours [15] and appears to be involved in tumour angiogenesis [16]. Soluble CD28 and CTLA4 fusion proteins may be used for studying and regulating T-cell function. All the recombinant proteins were active on the basis of their catalytic or binding activities. The overall yields of the purified products were in the range 3–12 mg/l. The amount of L6–hβG produced (3–5 mg/l) was higher when compared with a similar antibody–hβG immunoenzyme produced in transiently transfected COS-7 cells (10 μg/l) [8] or in stable, cloned COS-7 cells (100 μg/l) [9], indicating that the BALB/3T3 expression system described here may be useful for the production of immunoenzymes and other recombinant proteins.

BALB/3T3 cells were transfected with the appropriate expression vector (or recombinant retrovirus) and selected in the antibiotic-containing medium. The cells were then cloned by limiting dilution without drug selection to isolate stable producer cells. The cells were expanded in DMEM containing 5 % serum until confluence; the serum concentration was decreased to 0.5 % and the culture medium was then harvested every 2–3 days. Polyhistidine-tagged proteins were precipitated with ammonium sulphate and dissolved in binding buffer for purification on Ni-NTA, whereas Ig fusion proteins could be directly purified from culture medium on Protein A–Sepharose. One litre of medium could be collected every 3 days from 6–7 large dishes, allowing the recovery of 30–50 mg of pure recombinant protein in a month.

The mechanism by which ammonium sulphate precipitation facilitates binding to Ni-NTA has not been identified so far. However, it does not appear to be simply due to the removal of low-molecular-mass interfering compounds, since dialysis against binding buffer was ineffective. Furthermore, re-addition of material that remained soluble during ammonium sulphate fractionation back to the crude protein did not affect binding or recovery of recombinant proteins from Ni-NTA (results not shown). The combination of high salt concentrations, which enhance polyhistidine binding to Ni-NTA [17], and increased protein concentration probably account for the good recovery after ammonium sulphate precipitation.

BALB/3T3 fibroblasts display some characteristics that are useful for laboratory-scale production of recombinant proteins. First, the cells grew rapidly, facilitating cloning and expansion of producer cultures. Secondly, BALB/3T3 cells can be maintained in serum at as low a concentration as 0.1 % under confluent conditions and still secrete large amounts of protein. Low serum concentrations markedly simplify the purification. Thirdly, the tight adherence of 3T3 cells allows rapid harvesting of the medium from standard culture dishes. Finally, transfection efficiencies of 80–90 % with LIPOFECTAMINE™ 2000 and retroviral titres of 10^5–10^6 cfu (colony forming units)/ml can be routinely achieved in BALB/3T3 cells.

In summary, expression of recombinant proteins in BALB/3T3 cells, combined with ammonium sulphate precipitation and affinity chromatography, provides a general, simple and relatively inexpensive method to obtain milligram levels of pure, active, glycosylated proteins for a variety of studies and applications, including the rapid production of standard proteins for quantitative proteomics and protein microarrays [18,19].

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