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High level expression of the large subunit of mouse ribonucleotide reductase in a baculovirus system

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The large subunit of ribonucleotide reductase from mouse has been overexpressed in *Spodoptera frugiperda* cells infected with recombinant baculovirus. The expressed protein was purified by affinity chromatography to apparent homogeneity as determined by SDS-PAGE. The homogeneous protein is recognized in Western blot analysis by a monoclonal antibody raised to the large subunit of ribonucleotide reductase from calf thymus, has the correct N-terminal sequence, and, in the presence of the small subunit of mouse ribonucleotide reductase and nucleoside triphosphate effectors, catalyzes the reduction of both purine and pyrimidine nucleoside diphosphates.

Ribonucleotide reductase; Expression; Baculovirus

1. INTRODUCTION

Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleoside diphosphate substrates to generate the deoxyribonucleotide precursors necessary for DNA biosynthesis. Mammalian RR is an allosteric enzyme comprised of two homodimeric subunits denoted R1 and R2, the expression of which are differentially regulated during the cell cycle [1]. Mouse and human R2 have been cloned [2,3] and an active form of recombinant R2 subunit has been purified from an *E. coli* expression system [4]. The R1 subunit, which contains the binding sites for the allosteric effectors, has also been cloned [2,5]. Up to now, cloned R1 has not been expressed in a soluble form capable of binding R2 and catalyzing the RR reaction. Furthermore, only limited amounts of R1 can be prepared conveniently from calf thymus. As a result, detailed mechanistic and structural studies of purified eukaryotic enzyme [6–10] have been narrow in scope. Here we report the expression and purification of active mouse R1 in baculovirus infected *Spodoptera frugiperda* (Sf9) cells. Recombinant R1 was approximately 8% of the total soluble protein in infected Sf9 cells and was purified in one step using an FTLDADF-Sepharose affinity column [9].

2. MATERIALS AND METHODS

2.1. *Construction of the vector*

The cDNA encoding the mouse ribonucleotide reductase (R1) was a gift from Dr. Ingrid Caras (Genentech). The R1 gene was amplified using PCR primers (mR1-Nter 5′-GGGGGCTAGCATGCATGTGATCAAG-3′ and mR1-Cter 5′-GGGGGCTAGCTCAGGATCCACACATCAG-3′) that add *Nhe*I restriction sites (underlined) to the ends of the DNA fragment. The amplification product was digested with *Nhe*I and inserted into the *Nhe*I site of pBlueBac2 (InVitrogen). Sf9 cells were transformed with the recombinant plasmid using the cationic liposome method [11]. Positive clones were selected by β-galactosidase blue/white screening and subsequently grown in 3 ml volumes in a 24-well dish. Plaques were subjected to PCR analysis with primers complementary to the polyhedron loci, BlueF 5′-TTTACTG TTTTCGTAACAGTTTTG-3′ and BlueR 5′-CAACAACGCACAG AATCTAG-3′ (InVitrogen), to select those free of non-recombinant baculovirus.

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2.2 *Growth and maintenance of Sf9 cells*

Spodoptera frugiperda (Sf9) cells are cultured at 27°C in Excell 400 medium (JRH Scientific) supplemented with 10% heat inactivated FCS (BRL), 50 μg/ml gentamycin and 2.5 μg/ml Fungizone that has been sterile filtered Cells grown in spinner flasks are also supplemented with 0.1% Pluronic F-68 (JRH Scientific) to reduce shear damage.

2.3. *Expression of recombinant R1*

Original stocks of recombinant virus were used to infect virgin Sf9 cells to screen for protein production and generate the second generation of virus. Approximately 2×10^6 cells were adhered to each well of a 24-well dish in a 3 ml volume and infected with 20, 100 and 500 μl of recombinant virus. Aliquots were removed and assayed for expression of R1 every 24 h for 4 days. Plaque number C3 was selected for further study. For large scale expression of R1 protein, 50 ml of cells were grown to a density of 1.3×10^6 cells/ml and infected with 10^8 virus particles. Cells were grown for 60–72 h then harvested by centrifugation at $1,000 \times g$ for 10 min.

2.4. *Purification of recombinant R1*

The cell pellet collected from a 50 ml culture was resuspended in 5 ml of lysis buffer (50 mM Tris-Cl, pH 7.6, 0.1 mM DTT, 2 mM PMSF) and subjected to two cycles of freeze-thaw. The lysate was centrifuged at $105,000 \times g$ for 30 min. The resulting supernatant was loaded directly onto an FTLDADF-Sepharose affinity column as previously described [9]. The column was washed with 50 mM Tris-Cl, 0.1 mM

DTT (buffer A) and then with buffer A + 100 mM KCl. R1 was eluted with 10 ml buffer A + 500 mM KCl. The column was regenerated by treatment with 6 M guanidine-HCl. Column fractions were monitored by the Bradford assay [12].

2.5. *RR assays*

Ribonucleotide reductase activity was assayed at 37°C using the method of Moore and Peterson [13] with minor modifications. Briefly, assay mixtures contained 60 mM HEPES, pH 7.6, 26 mM DTT, 7 mM NaF, 5 mM $Mg(OAc)_2$, 3 mM ATP, 0.05 mM $FeCl_3$, and either 10 μg of recombinant R2 prepared in our laboratories by the method of Mann et al. [4] or an equivalent volume of buffer A. For CDP reductase assays, 0.05 mM [5-^{3}H]CDP (20 Ci/mol) was added to assay mix, while for GDP reductase assays 1.5 mM dTTP and 0.02 mM [2,8-^{3}H]GDP (45 Ci/mol) were added to a final volume of 100 μl. Reactions were initiated by the addition of protein and incubated at 37°C for 5–15 min for initial rate determinations. Reactions were quenched by immersion in a boiling water bath for 4 min. Samples were frozen and lyophilized to dryness. Lyophilized samples were reconstituted in 1 ml of 50 mM Tris, pH 8.45 containing 100 mM $Mg(OAc)_2$ (buffer B). Samples were centrifuged at 10,000 × *g* for 10 min to precipitate denatured protein. The supernatant was then loaded onto 0.5 × 5 cm columns of aminophenylboronate (Amicon) which had been pre-equilibrated with 5 ml of buffer B. Deoxynucleoside diphosphates elute in 5 ml (for dCDP) to 12 ml (for dGDP) of buffer B. Unreacted ribonucleoside diphosphate substrates can be quantitatively recovered and columns can be regenerated by treatment with 10 ml of 50 mM sodium citrate, pH 5.9. Radioactivity in aliquots of both buffer B and citrate fractions were determined by liquid scintillation counting.

2.6. *Other methods*

A partial N-terminal sequence of the purified protein was determined by the Edman method [14] using an Applied Biosystems model 473A sequencer. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5% acrylamide as described by Laemmli [15] Western blots were performed as described [16] using monoclonal antibody AC1 (In Ro BioMedTek, Umeå, Sweden) raised against R1 from calf thymus. Protein concentration was determined according to Bradford [12].

3. RESULTS AND DISCUSSION

Initial attempts to produce active recombinant R1 in two *E. coli* expression systems, pZM [16] and pET [17], resulted in large quantities of insoluble protein in cytoplasmic inclusion bodies. The gene was subsequently inserted into the *NheI* site of pBlueBac2 for expression in Sf9 cells. After transfection, nine recombinant plaques were selected for further analysis. Putative positive clones were subjected to PCR analysis using primers that anneal to the polyhedron region and span the recombinant R1 gene. Of the selected plaques, only one contained an insert which was free of non-recombinant viral contamination. This sample, designated C3, was isolated for further study.

Sf9 cells were infected with recombinant virus stock, C3, at a multiplicity of infection of 1. Crude extracts, prepared by two cycles of freezing and thawing, were screened for R1 production by SDS-PAGE, Western blot and CDP reductase activity assays. Sf9 cells infected with a β-galactosidase producing baculovirus (In-Vitrogen) as well as uninfected Sf9 cells served as controls. No endogenous RR activity was detected in any soluble Sf9 cell lysate in the absence of recombinant R2 and no RR activity was detected in the crude lysates of either of the control cells in the presence of added recombinant R2. The level of R1 production in cells infected with recombinant virus was determined by assaying the crude lysate in the presence of recombinant R2. In 50 ml spinner flasks, R1 production was found to be independent of multiplicity of infection ranging from 1

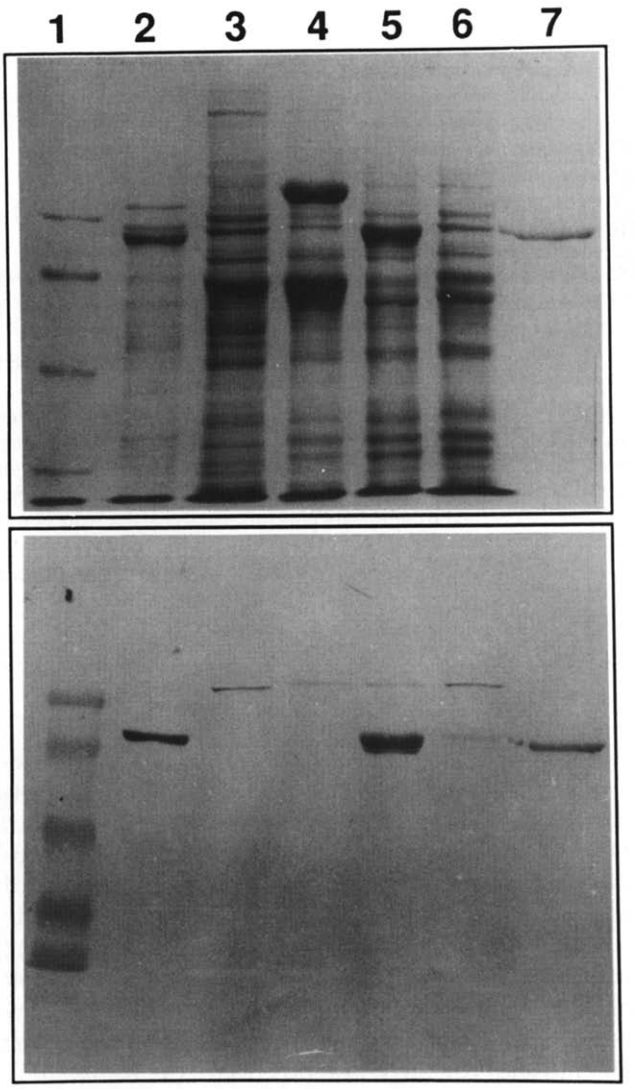

Fig. 1. SDS-PAGE (top) and Western blot (bottom) analysis of recombinant R1. Low m.wt. markers/prestained low m.wt. markers. phosphorylase *b* 97.4/106 kDa, BSA 66.2/80 kDa, ovalbumin 45/49.5 kDa, carbonic anhydrase 31/32.5 kDa, trypsin inhibitor 21.5/27.5, lysozyme 14.4/18.5 (lane 1 top/lane 1 bottom), authentic calf thymus R1 (lane 2), soluble cell lysate from uninfected Sf9 cells (lane 3), soluble cell lysate from Sf9 cells infected with baculovirus expressing β-galactosidase (lane 4), soluble cell lysate from Sf9 cells infected with recombinant virus stock C3 (lane 5), buffer A wash of C3-infected Sf9 cell lysate loaded FTLDADF-Sepharose column (lane 6), buffer A + 500 mM KCl wash of C3-infected Sf9 cell lysate loaded FTLDADF-Sepharose column (lane 7)

Table I

Purification of R1 on FTLDADF-Sepharose

Fraction	Protein (mg)	Activity (units)	Specific activity	% Crude activity
Crude lysate	18	145	8	100
[KCl] = 0	14.4	20	1.4	13
[KCl] = 100 mM	0.1	<4	-	<3
[KCl] = 500 mM	0.9	90	100	62
6 M guanidine-HCl	0.4	ND	ND	ND

One unit of activity is defined as the amount of protein required to produce 1 nmol of dCDP from CDP in 1 min. Specific activity is defined as nmol dCDP/min/mg protein. Protein concentrations are by Bradford and represent the average of three measurements. All protein activities were determined in duplicate with a variance of < 10%.

to 100. An incubation of 2–3 days was found to be optimal for the expression of this protein.

Western blot analysis shows the presence of a high molecular weight cross-reacting protein in the crude lysates of both C3-infected and control cells (Fig. 1, bottom). This protein may correspond to *Spodoptera* R1. If it does, then the lack of reductase activity upon addition of recombinant mouse R2 to control cell extracts would imply that mouse R2 is not able to form a productive complex with insect R1. The C-terminus of insect R2 may thus differ significantly from that of mouse R2 [9,18].

The purification of R1 on FTLDADF-Sepharose is described in Table I. The R1 protein was produced as 8% of the soluble protein in C3-infected Sf9 cells. 62% of R1 activity was collected as a homogeneous protein of molecular weight 85 kDa in the 500 mM KCl fraction (Fig. 1, column 7). More than 85% of the total protein loaded onto the column was recovered, and 75% of the reductase activity was accounted for. One preparation of purified R1 had a specific activity of 201 ± 11 U/mg for CDP reductase and 142 ± 13 U/mg for GDP reductase. Under the same conditions, R1 purified from calf thymus [9] gave specific activities for CDP and GDP reductase of 46 ± 5 U/mg and 32 ± 3 U/mg, respectively. Purification of a second lot of recombinant R1 which was contaminated with a lower molecular weight species, which appears to be a cleavage product, gave a specific activity of 100 ± 7 for CDP reductase. N-terminal analysis of the protein in the 500 mM KCl fraction gave a result that is consistent with that derived from the R1 gene sequence – MHVIKRDGR.

SDS-PAGE and Western blot analysis of the protein eluted by guanidine-HCl showed it to be predominantly R1. This indicates that future enhancements to this preparation may afford an even greater yield. Presently, purification of recombinant R1 from these cells takes less than a day, which compares favorably with the four to five days required for preparation of R1 from calf thymus.

In conclusion, we have succeeded in expressing R1 in baculovirus-infected Sf9 cells and purifying large quantities in good yield by a rapid one step procedure. This represents the first published expression of an active eukaryotic recombinant R1. It will now be possible to carry out detailed biochemical and structural experiments on the fully reconstituted mouse RR enzyme using recombinant R1 and recombinant R2. In addition, the baculovirus expression system is convenient for screening and production of mutant R1 proteins without an interfering background of Sf9 R1.

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