Characterization of human DNA ligase I expressed in a baculovirus–insect cell system

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The baculovirus expression system was used to overexpress human DNA ligase I (hLig I). Approx. 2 mg of recombinant hLig I was produced per 10^8 *Spodoptera frugiperda* Sf9 insect cells infected with the recombinant baculovirus. The optimum time point for the production of biologically active recombinant

hLig I was 48 h post-infection. Lig I activity was demonstrated by auto-adenylating, polynucleotide joining and DNA relaxation assays. The baculovirus system has the advantage over previously described methods for producing hLig I of generating large amounts of a full-length active protein.

INTRODUCTION

Normal mammalian cells harbour up to three distinct DNA-joining enzymes [1]. DNA ligase type I (Lig I) represents the major DNA ligase activity in nuclei of dividing cells, where it acts by joining the Okazaki fragments during lagging strand replication; a distinct, DNA unwinding (topoisomerase) activity associated with Lig I might serve to relieve the torsional forces accumulating during lagging strand growth [2]. In addition, Lig I has been implicated in the DNA excision–repair system in both resting and dividing cells. Accordingly, the expression of the Lig I gene correlates with the state of cell proliferation, and is stimulated by treatments generating DNA damage [3]. In contrast, the long half-lives of both mRNA and protein suggest that short-term control of Lig I activity takes place post-translationally. A model has been proposed according to which phosphorylation events directed onto the N-terminal ~ 24 kDa regulatory domain would activate the enzyme by unmasking the C-terminal ~ 78 kDa catalytic domain [4]. The N-terminal domain also harbours a putative nuclear localization signal(s) (NLS) [5,5a] whose functional regulation by phosphorylation could play a role in modulating the availability of Lig I in the nucleus.

Human Lig I (hLig I) cDNA has been isolated by complementation cloning in *Saccharomyces cerevisiae* [6]. Full-length hLig I overexpressed in *Escherichia coli* is inactive [7], whereas a mutant with a supernumerary Thr at the N-terminus was shown to be constitutively active, possibly owing to an altered N-terminal domain folding [8].

In the present report we describe the production and characterization of hLig I in the baculovirus–insect cells host system. Reportedly, this system permits recombinant protein synthesis at levels approaching those of bacteria, while providing the compartmentalization and the post-translational modifications characteristic of higher eukaryotes [9]. Indeed, baculovirus-overexpressed recombinant hLig I (r-hLig I) was shown to be active, largely targeted to the nucleus and partly phosphorylated. The system is thus well suited for further hLig I structure–function analysis.

MATERIALS AND METHODS

Cell culturing

Spodoptera frugiperda Sf9 cells were propagated in SF900 medium (Gibco), supplemented with 10 % (v/v) fetal bovine serum (FBS), either in monolayer or in spinner flasks. *Trichoplusia ni* Hi-5 cells were cultured in monolayer in the same medium.

Recombinant baculovirus production

A 3 kb fragment of hLig I cDNA (ATCC #65857, ref. [6]) spanning the complete coding region [from the *EcoRI* site 21 bp upstream from the start codon to the poly(A) tail] was inserted in the sense orientation into the expression cassette of the commercial baculovirus transfer vector pVL1392 (Invitrogen). The insert was positioned so as to be out-of-frame relative to the mutated polyhedrin start codon, to prevent spurious co-expression of polyhedrin–hLig I fusion polypeptides. The construct (pVLhLig) was lipofected together with a linearized, open reading frame 1621-deleted *Autographa californica* nuclear polyhedrosis virus genomic DNA into Sf9 cells. The first progeny of recombinant virus (rBac–hLig) was plaque-purified and further amplified and titrated according to published procedures [10].

Expression of hLig I in recombinant-virus-infected cells

For a preliminary analysis of r-hLig synthesis, Sf9 monolayers (3×10^8 cells/25 cm² flask) were infected at a multiplicity of infection (MOI) ≈ 5 and incubated at 27 °C for variable times (30–60 h). Cells were then washed with PBS and directly lysed in SDS/PAGE cracking buffer [25 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 1 % (v/v) β -mercaptoethanol, 10 % (v/v) glycerol]. For quantitative production, suspension-cultured Sf9 cells (1.5×10^6 per ml) were infected at MOI ≈ 5 and incubated at 27 °C for 48 h in SF900 medium supplemented with 10 % (w/v) FBS. SDS/PAGE and Western blot (WB) analysis were carried out as described [11]. As a specific anti-ligase I reagent, monoclonal antibody (mAb) 1A9 was used, kindly provided by Dr. Tomas Lindahl (ICRF, Clare Hall Laboratory, South Mimms, U.K.).

Abbreviations used: Ab, antibody; CBB, Coomassie Brilliant Blue; CIP, calf intestinal phosphatase; DTT, dithiothreitol; FBS, fetal bovine serum; hLig I, human DNA ligase I; Lig I, DNA ligase I; r-hLig I, recombinant hLig I; mAb, monoclonal Ab; MOI, multiplicity of infection; NLS, nuclear localization signal; rBac–hLig, recombinant baculovirus harbouring the r-hLig I cDNA; WB, Western blot.

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Preparation of cell-free extracts and purification of r-hLig I

All of the following procedures were carried out at 4 °C or on ice. Cells were collected by centrifugation, lysed by sonicating 3 times, for 1 min each, with a Branson sonifier in 4 volumes of extraction buffer [20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol (DTT), 5 mM EDTA, 1 µg/ml Pepstatin, 2 mM phenylmethylsulphonyl fluoride, 4 mM Na bisulphite, 1% (v/v) Nonidet P-40]. The sonicate was then centrifuged at 13000 *g* for 10 min. The supernatant was applied to a DEAE-cellulose column (12 ml of column per ml of cell pellet) equilibrated with buffer A (50 mM potassium phosphate, pH 7.5, 1 mM DTT, 0.1 mM EDTA) and after washing the column with 3 volumes of equilibration buffer, proteins were eluted with a linear KCl gradient (0–0.6 M) in buffer A. Fractions containing the r-hLig I, as determined by both SDS/PAGE and activity assays, were pooled and diluted 3 times with buffer A and immediately applied to a phosphocellulose column (5 ml of column per ml of cell pellet) previously equilibrated with buffer A. After washing the column with 3 volumes of equilibration buffer, r-hLig I was eluted with a linear gradient of KCl (0–0.6 M) in buffer A. Fractions containing the r-hLig I were pooled, diluted 4 times with buffer B (20 mM potassium phosphate, pH 7.5, 0.4 mM DTT, 0.04 mM EDTA) and applied to a heparin–Sephacrose HiTrap column (Pharmacia) previously equilibrated with buffer B. After washing the column with 5 volumes of equilibration buffer and then with 3 volumes of 0.14 M KCl in buffer B, r-hLig I was eluted with 1.5 volumes of 0.3 M KCl in buffer B. The fraction containing r-hLig I was dialysed against 200 mM Tris/HCl, pH 7.5, 0.05 mM EDTA, 0.5 mM DTT, 50% (v/v) ethylene glycol. The final r-Lig I concentration was 0.7 mg/ml. This fraction was used for further characterization of r-hLig I.

We exclude the possibility of interference by endogenous insect ligase in the purified r-hLig I fraction because, in both control labelling experiments done *in vivo* with ³²P and *in vitro* with [³²P]ATP on cells uninfected or infected with an unrelated recombinant baculovirus, we did not detect any autoradiographic signal within the SDS/PAGE migration region occupied by the r-hLig I.

Production of antiserum to r-hLig I

Antibodies (Abs) against r-hLig I were produced in 8-week-old BALB/c female mice. The first intraperitoneal injection consisted of 50 µg of purified protein in 100 µl of sterile PBS/complete Freund's adjuvant (1:1, v/v). Two boosting injections, containing the same amount of antigen but no adjuvant, were administered at 2-week intervals. In WB analysis, the serum was used at 1:500 dilution.

Immunofluorescence

To study the subcellular localization of r-hLig I, Hi-5 cells were seeded at 1.2×10^5 per cm² on glass coverslips. This cell line was preferred in these experiments, as its fibroblast-like morphology makes it ideally suited for cytological analysis. Cells were infected (MOI ≈ 1) either with rBac–hLig or with a baculovirus inducing the synthesis of a deleted version of human cytomegalovirus pp65 bearing an epitope tag (pp65–Etag), as a control. At 24 h post-infection, cells were washed twice with PHEM buffer (60 mM Pipes, 25 mM Hepes, pH 6.8, 10 mM EGTA, 3 mM MgCl₂) and fixed in 4% (v/v) paraformaldehyde in PHEM (on ice for 20 min). Cells were washed in PHEM and unreacted aldehyde was quenched with 150 mM NH₂Cl dissolved in the same buffer. PHEM was included in all subsequent treatments. Cells were exposed to 0.5% saponin for 30 min, post-coated with

0.2% gelatin for 30 min, and incubated with either mAb 1A9 or with mAb anti-Etag (1:500) in 1% FBS for 30 min at 37 °C in a humid chamber. After three washes in 1% FBS, the coverslips were incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody for 30 min at 37 °C in a humid chamber and washed again. Cells were finally counterstained with Evans Blue (1 µg/ml), mounted in glycerol and observed under an epifluorescence Leitz Orthoplan microscope, in the fluorescein channel.

Assays for DNA ligase activity

DNA ligase activity was determined by the method of Modrich and Lehman [12] according to which 1 unit is the amount of enzyme that converts 100 nmol poly[d(A-T)] to an exonuclease III-resistant form within 30 min at 30 °C. Proteins were quantified by the method of Bradford [13]. Ligation of pUC19, linearized either with *Eco*RI to produce cohesive ends, or *Pvu*II to produce blunt ends, was performed as follows: reaction mixtures (20 µl) containing 50 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 1 mM ATP, 250 µg of BSA were incubated at 37 °C for either 30 or 120 min, respectively. DNA relaxation activity was determined as previously described [14]. Recombinant hLig I was incubated in the following reaction buffer (20 µl): 20 mM Tris/HCl, pH 7.6, 3 mM MgCl₂, 1 mM dithioerythritol, 1 mM EDTA, 1 mM AMP, 100 µg/ml BSA, 100 ng of naturally supercoiled pUC19. After incubation at 37 °C for 30 min, the reaction mixture was made 0.04% in Bromophenol Blue, 0.01% in SDS and 12% (v/v) in glycerol. Bacteriophage T4 DNA ligase (Boehringer) was used as a positive control. Samples were then analysed on a 1% (w/v) agarose gel in 40 mM Tris acetate, 2 mM EDTA, pH 8, in the presence of 1 µg/ml ethidium bromide. Ligase-[³²P]AMP adducts were prepared as previously described [3]. The deadenylation reaction was performed by incubating 40 ng of purified protein in 20 mM Tris/HCl, pH 7.6, 3 mM MgCl₂, 1 mM DTT, 1 mM EDTA and 1 mM PP_i. The reaction product was analysed on 10% (w/v) SDS/PAGE and stained with silver.

Dephosphorylation of r-hLig I *in vitro*

Dephosphorylation of r-hLig I (40 ng/reaction mixture) was performed with calf intestinal phosphatase (CIP) (0.1 unit, Boehringer) in 20 µl of 50 mM Tris/HCl, pH 8.5, containing 0.1 mM EDTA, at 45 °C for 30 min. Samples were analysed on SDS/PAGE (8%). In this case, a 20 × 20 cm Bio-Rad Protean II apparatus was used to obtain a better resolution of the bands. The gel was stained with the Silver Stain Kit (Sigma).

RESULTS AND DISCUSSION

Time course of expression and cellular localization of r-hLig I

The baculovirus expression system is a useful method for producing large quantities of biologically active eukaryotic proteins [9]. In this study we report for the first time the production of full-length, functional hLig I in the baculovirus–insect cell system. Human Lig I cDNA [6] was ligated in the baculovirus transfer vector, pVL1392, and then cotransfected with the linearized baculovirus DNA into Sf9 cells. Recombinant baculovirus harbouring the r-hLig I cDNA (rBac–hLig) was isolated and checked for the presence of a correct insert by dot blot and Southern blot analysis of its genome (results not shown). Sf9 cells were infected with plaque-purified rBac–hLig, and examined at various times for the production of r-hLig I. A peak in r-hLig I expression was observed 48 h after infection, by

Figure 1 Electrophoretic characterization of baculovirus-induced hLig I

(a) SDS/PAGE analysis (10% resolving gel) of a crude lysate from Sf9 cells infected with r-Bac-hLig I (lane 1) or with an unrelated recombinant baculovirus (lane 2). The protein pattern was revealed by Ponceau staining after electrophoretic transfer of proteins onto nitrocellulose filter. (b) The lysates were separated on 8% SDS/PAGE and transferred to nitrocellulose for WB analysis. The position of molecular mass markers is shown on the left of each panel.

both total-protein staining and WB analysis with mAb to hLig I, whereas the protein was absent in cells infected under similar conditions with unrelated recombinants (Figure 1). The r-hLig I polypeptide exhibited a molecular mass of 125 kDa. DNA ligase I purified from mammalian cells shows an apparent molecular mass of 120–130 kDa by SDS/PAGE [15,16]. In a similar manner to observations in mammalian cells [17], r-hLig I was found to accumulate mainly within the cell nucleus by immunofluorescence analysis (Figure 2). In contrast, the control recombinant HCMV pp65-E tag protein was shown to be localized mainly in the cytoplasm (Figure 2).

Figure 3 Electrophoretic migration of r-hLig I during SDS/PAGE

(a) Silver staining of 40 ng of purified r-hLig I after migration in 8% polyacrylamide. Purified r-hLig I before (lane 1) and after (lane 2) incubation with CIP (see Materials and methods). (b) Immunoblotting with mAb of 480 ng of purified r-hLig I after migration in 8% polyacrylamide.

Biochemical characterization of r-hLig I expressed in Sf9 infected cells

Recombinant hLig I was purified from the extract of Sf9 infected cells by column chromatography on DEAE-cellulose and phosphocellulose, followed by a heparin–Sepharose affinity column, as described in Materials and methods. After these purification steps, approx. 2.8 mg of r-hLig I pure to near-homogeneity (Figure 3a) was obtained from each 80 ml batch of cell culture. However, the specific activity of r-hLig I was only 47 units per mg, about the same order as the hLig I from HeLa cells,

Cell host ... Hi-5 (*T. ni*)
Virus ... rBac hLig
Time after infection ... 24 h
Primary Ab ... MAb 1A9

Hi-5 (*T. ni*)
rBac pp65 Etag
24 h
Anti-Etag MAb

Figure 2 Immunofluorescence study on *T. ni* cells

Insect cells were infected with either rBac-hLig I or with a control virus (rBac–pp65–Etag) inducing the synthesis of an unrelated protein (a deleted version of HCMV pp65, bearing an epitope tag pp65–Etag). Specific recognition by either mAb is revealed by the white stain (corresponding to the yellow-green fluorescence of the original sample). Recombinant hLig I is demonstrated to be mainly nuclear, whereas the control protein is mostly cytoplasmically located.

Figure 4 Characterization of the enzymic activity of purified r-hLig I

(a) Autoradiographic pattern after migration on SDS/PAGE (10%) of the products of the reaction between r-hLig I and [α - 32 P]ATP. The reaction was stopped with EDTA (lane 1) or further incubated with 1 mM PP_i (lane 2) as described in Materials and methods. (b) Analysis on agarose gel (1%) of the sticky-ends ligation products of pUC19 DNA linearized with *Eco*RI. The reactions were stopped with EDTA to compare the initial products of reaction, DNA circles and multiple linear forms, still in the presence of the initial substrates. Lane 1, control incubated in the absence of ATP, to which markers of relaxed circular and supercoiled forms were added before loading onto the gel; lane 2, ligation with bacteriophage T4 DNA ligase (0.2 unit); lane 3, ligation with purified r-hLig I (0.1 unit). (c) Analysis on agarose gel (1%) of the blunt-ends ligation products of pUC19 DNA linearized with *Pvu*II. The reactions were stopped with EDTA as for (b). Lane 1, control linear DNA; lane 2, ligation with bacteriophage T4 DNA ligase (0.2 unit); lanes 3–5, ligation with purified r-hLig I (0.033, 0.1 and 0.15 unit, respectively). (d) Analysis on agarose gel (1%) of the AMP-dependent relaxation products of supercoiled pUC19 DNA. Lane 1, control supercoiled and relaxed DNAs; lane 2, relaxation with bacteriophage T4 DNA ligase (0.2 unit); lanes 3–5, relaxation with purified r-hLig I (0.066, 0.033 and 0.01 unit, respectively).

which is routinely purified in our laboratory (usually 16 units per mg; ref. [18]).

The purified protein is well resolved on SDS/PAGE gels as a triplet (Figure 3a). All three bands are recognized by monoclonal antibody to hLig I (Figure 3b). Human Lig I is a phosphoprotein and its phosphorylation seems to be necessary for the catalytic activity of the full-length protein. To determine whether the observed electrophoretic heterogeneity could derive from different levels of phosphorylation of the enzyme, r-hLig I was treated with CIP as described in Materials and methods. As shown in Figure 3(a), after exposure to CIP the r-hLig I triplet is reduced to a doublet and shows a slight increase in electrophoretic mobility. An increase in electrophoretic mobility has also been obtained by treatment of purified calf thymus DNA ligase I with bacterial alkaline phosphatase and acid phosphatase from potato [4]. It is consistent with the interpretation that r-hLig I is phosphorylated in insect cells that the protein was also found to be heavily labelled in rBac-hLig infected cells incubated with [32 P]orthophosphate (not shown).

To investigate whether a product adenylylated *in vivo* could contribute to the observed r-hLig I electrophoretic heterogeneity, we incubated the purified enzyme with PP_i (5 mM) to reverse the adenylylation process. None of the three bands was affected by such a treatment (not shown).

The catalytic properties of the purified protein were then investigated. Figure 4(a) shows that purified r-hLig I hydrolyses [α - 32 P]ATP to form the AMP-Lig I adduct with an apparent

Figure 5 Immunoblotting of crude cellular extracts with mouse antiserum against r-hLig I

(a) Immunostaining of human HeLa cell extract with preimmune (lane 1) and immunized (lane 2) mouse sera. (b) Immunostaining of mouse NIH 3T3 cell extract with preimmune (lane 1) and immunized (lane 2) mouse sera. Extracts were prepared from exponentially growing cells pelleted after washing with PBS. Cell pellets were resuspended 1:1 in 2 × cracking buffer (see Materials and methods), boiled for 10 min and sonicated 5 times for 1 min. After centrifugation at 13 000 *g* for 10 min, 2 μ l samples of supernatants were loaded in each well of a 7.5% polyacrylamide gel.

molecular mass of 125 kDa. This reaction can be reversed by addition of PP_i, as expected from an active DNA ligase molecule [19]. The purified enzyme is also able to catalyse (Figures 4b–4d) both sticky- and blunt-end ligation along with the AMP-dependent DNA relaxation that differentiates DNA ligase I from other mammalian DNA ligases [14]. Therefore the recombinant protein appears indistinguishable from the DNA ligase I purified from human cells, at least as far as enzymic activity is concerned.

Taken together, these results suggest that in insect cells the recombinant protein is active and phosphorylated.

Production of polyclonal Ab against hLig I

We have used the purified protein as an antigen for the production of polyclonal antibodies in mice, as described in Materials and methods. The antiserum obtained showed a titre of 1.7×10^6 (as measured in a direct ELISA assay) against the purified antigen (0.4 μ g per well) and could recognize a single polypeptide of the expected apparent molecular mass in both human (HeLa) and mouse (NIH 3T3) crude cell extracts (Figure 5).

CONCLUSIONS

This study demonstrates the feasibility of overexpressing functional full-length hLig I in a baculovirus system. hLig I was previously expressed in yeast (*S. cerevisiae*) and *E. coli*. The yeast system is capable of generating functional protein [6], but the yield is very low. In the *E. coli* system the protein cannot be phosphorylated and the catalytic activity is observed only associated either with fragments lacking the N-terminal domain [7] or with a mutated protein containing an extra Thr [8]. Therefore the baculovirus system shows the major advantage of producing large amounts of functional hLig I. This expression system is also well suited for the generation of mutated forms of enzyme that could help increase our understanding of the role of hLig I in the different cellular processes involving this enzymic activity.

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