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Targeting to the endoplasmic reticulum improves the folding of recombinant human telomerase reverse transcriptase

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Abstract

Telomerase is a specialized reverse transcriptase that catalyzes the addition of telomeric repeats, TTAGGG in all vertebrates, to the ends of chromosomes. The lack of recombinant purified human telomerase reverse transcriptase (hTERT) has hampered biochemical and structural studies. The primary problem in generating active recombinant hTERT appears to be protein folding, which may be due to the fact that telomerase is a multi-component ribonucleoprotein complex. When expressed in most heterologous systems, recombinant hTERT is largely insoluble. Here we describe a protein expression system using a baculovirus vector that can be used to prepare properly folded, enzymatically active, hTERT. In this system, the recombinant hTERT is directed to the endoplasmic reticulum (ER), which is rich in chaperones. This increases the expression of soluble recombinant hTERT, promoting proper folding using intrinsic ER chaperone proteins.

Introduction

Telomerase is a ribonucleoprotein complex that adds telomeric DNA repeats to the ends of chromosomes [1,2], which, in the absence of telomerase, progressively shorten in the successive rounds of DNA synthesis that accompanies cell division [3,4]. The progressive shortening eventually causes the loss of essential genetic information and death of the cell [5]. Telomerase is able to extend telomeres and counterbalance the natural shortening that occurs during DNA replication [6,7]. Telomerase activity has been detected in about 90% of cancer cells [8]. The activation of telomerase is involved in tumorigenesis and the level of telomerase activity correlates with clinical outcomes [9]. Telomerase is also active in stem cells [7], which have an unlimited ability to proliferate. Because telomerase is expressed in both cancer cells and stem cells, a better understanding of telomerase has considerable biomedical importance.

Not only is human telomerase a biomedically important enzyme, it also uses an unusual and poorly understood mechanism to synthesize DNA. Catalytic activity of human telomerase requires both hTERT and an intrinsic RNA template (hTR) [10,11]. Telomerase uses a specialized reverse transcription mechanism that processively adds multiple TTAGGG repeats to the 3' end of the G-rich strand of telomeres, by reiteratively copying an RNA template (hTR) that remains bound to the enzyme [12]. This repeated use of an intrinsic RNA template that is not degraded distinguishes telomerase from viral reverse transcriptases. In addition to the

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catalytic RT domain, hTERT contains a telomerase-specific N-terminal domain that has been implicated in template and primer binding, and C-terminal extension that presumably has a role in promoting enzyme processivity and in localization [13]. In cells, hTERT and the template RNA are part of a larger telomerase complex that includes a number of other proteins, including TLP1 [14], hsp90, hsp23 [15], and dyskerin [16].

The ability to generate active recombinant hTERT protein is crucial for a better understanding of the structure and function of this enzyme, and for drug development. Expressing and purifying properly folded, enzymatically active recombinant hTERT has been an exceptionally difficult problem. Despite a number of attempts using heterologous expression systems [17–19], it has not been possible to produce a large quantity of active, soluble human telomerase.

Baculovirus-insect cell expression systems are important tools for the production of correctly folded recombinant proteins. Baculovirus vectors can be used to express large proteins and to co-express multiple proteins [20]. This is an important consideration if the long-term goal is to express a protein complex, which is the goal for telomerase.

Others have used the baculovirus system to express the hTERT protein, however, most of the recombinant hTERT was insoluble [18], presumably due to the fact that the telomerase was not properly folded, and to the fact that key components of the telomerase complex were missing. Attempts to co-express the RNA template (hTR), an integral part of hTERT, did not significantly improve protein solubility [19]. We obtained similar results using a standard baculovirus expression system. The recombinant hTERT had a very low solubility (1–5%), and the solubility was not improved if hTERT was expressed as a maltose binding protein (MBP)-hTERT fusion, which increased the level of protein expression. We attempted to solubilize the MBP-hTERT in the pellet using various detergents and high pressure (5000psi) but were unsuccessful. In this study, we were able to express soluble, enzymatically active recombinant hTERT using the chaperones in the endoplasmic reticulum (ER).

Materials and Methods

Plasmid construction

The plasmid *pCI-Neo-hTERT*, which encodes hTERT (genebank locus AF018167), was kindly provided by Dr. Robert Weinberg. A series of hTERT protein expression plasmids were constructed; they are listed in Fig. 1. These constructs were built using the plasmid *pFastBac*, a donor vector in the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). The plasmids were constructed as follows: The plasmid *GFP-hTERT*, which was used to express the GFP-TERT fusion protein, was generated from GFP and hTERT coding sequences as described below. All the sequences of primers that were used in this study are given in Table 1. The GFP fragment was PCR amplified from *peGFP-N1* (Clontech, Mountain View, CA) with the GFPfor primer which contains an NcoI site (lowercase), and the GFPrev primer which contains an EcoRI site (lowercase) and nucleotide sequences encoding the TEV protease recognition sequence ENLYFQg (underlined). The hTERT cDNA was PCR amplified from the *pCI-Neo-hTERT* plasmid using the hTERTfor primer containing an EcoRI site (lowercase), and the hTERTrev primer containing a SalI site (lowercase), termination codon (bold) and sequences encoding the FLAG (DYKDDDDK) epitope (underlined). The NcoI-EcoRI-digested GFP fragment and EcoRI-SalI-digested hTERT fragment were then inserted by a three-way ligation into the NcoI-SalI-digested *pFastBac* vector yielding the plasmid *GFP-hTERT*. To facilitate protein purification, a *GFP-hTERT-biotin* plasmid was made as follows. First, a new GFP-hTERT fusion was made without the termination codon using the primers as described above except that, in the hTERTrev primer, the termination codon TTA was omitted, generating the plasmid *GFP-hTERTin*. Next, two complementary oligonucleotides (BIOTINfor and BIOTINrev), encoding a 23-aminoacid biotin tag (underlined),

MASSLRQILDSQKMEWRSNAGGS, which can be biotinylated by the BirA enzyme in *E. coli*, and a stop codon (bold) were annealed and inserted into Sall/XbaI sites of *GFP-hTERTin*, resulting in *GFP-hTERT-biotin* plasmid. The plasmid *GFP-hTERT-hTR*, which was designed to co-express hTR RNA component with GFP-hTERT fusion protein, was constructed as described below. The hTR gene was PCR amplified with the hTRfor primer and the hTRrev primer containing Sall and KpnI sites (lowercase) respectively. The Sall-KpnI-digested hTR fragment was inserted into the *GFP-hTERT* plasmid that had been digested with Sall/KpnI, resulting in *GFP-hTERT-hTR*. Plasmids *MBP-hTERT*, *MBP-hTERT-biotin*, and *MBP-hTERT-hTR* were constructed in the same way as *GFP-hTERT*, *GFP-hTERT-biotin* and *GFP-hTERT-hTR* described above, except replacing GFP with MBP. MBP was PCR amplified from parental vector C2 (NEB) with the MBPfor primer and the MBPrev primer containing NcoI/EcoRI sites (lowercase) and a TEV protease recognition sequence (underlined). Plasmids for targeting to the ER including *erGFP-hTERT*, *er-GFP-hTERT-biotin*, *erGFP-hTERT-hTR*, *erMBP-hTERT*, *er-MBP-hTERT-biotin*, and *erMBP-hTERT-hTR*, were made by adding the signal sequence upstream of the GFP and MBP coding regions in the expression plasmids. The ER signal peptide was based on the human BiP (AAF42836) N-terminal 18 amino acids (MKLSLVAAAMLLLLSAARA). First, complementary DNA oligonucleotides (BiP-BamH and BiP-NcoI), encoding the BiP signal peptide (underlined), were annealed and inserted into the BamHI/NcoI sites of the *pFastBac* vector. Next, DNA fragments including GFP-hTERT, GFP-hTERT-biotin, GFP-hTERT-hTR, MBP-hTERT, MBP-hTERT-biotin, and MBP-hTERT-hTR described above were inserted immediately downstream of the NcoI site of the BiP signal sequence, resulting in *erGFP-hTERT*, *erGFP-hTERT-biotin*, *erGFP-hTERT-hTR*, *erMBP-hTERT*, *erMBP-hTERT-biotin*, and *erMBP-hTERT-hTR* plasmids respectively. The plasmid *erGFP-hTERT-KDEL*, in which the retention signal was added at the C-terminal end, was made by inserting annealed complementary oligonucleotides (KDEL-Sall and KDEL-XbaI), encoding KDEL (underlined) and the termination codon (bold), with Sall/XbaI sites of *erGFP-hTERT* and yielding the plasmid *erGFP-hTERT-KDEL*. Plasmids *mtGFP-hTERT*, and *mtMBP-hTERT* were made for targeting the fusion proteins to the mitochondria. The N-terminal 39 a.a. signal peptide (MASVWQRLGF YASLLKRQLN GGPDPVIKWER RVIPGCTRS) [21] of human mitochondrial tRNA^{Leu} synthetase (mt-tRNA^{Leu}) were used. The complimentary oligonucleotides (mtRNA-BamH and mtRNA-NcoI), encoding the mt-tRNA^{Leu} signal peptide (underlined), were inserted using the same sites as ER signal sequence to generate *mtGFP-hTERT* and *mtMBP-hTERT* plasmids. The plasmid *BirA*, which expresses the BirA protein, was made as follows. BirA1 (NC_000913) gene from *E. coli* K-12 strain was PCR amplified from DH5 α cell lysates with the BirAfor primer containing an EcoRI site (lowercase), and the BirArev primer containing an XbaI site (lowercase). The EcoRI-XbaI-digested PCR fragment was inserted into the *pFastBac* vector. Baculoviruses expressing human hsp70 and hsp90 are kind gifts from Dr. David Toft at the Mayo Clinic. In vitro site-directed mutagenesis was used to introduce the D868A mutation into *erMBP-hTERT-hTR* with primers D868For and D868Rev, using Quick-Change II (Stratagene, La Jolla, CA) kit, following the manufacturer's recommendation. The resulting plasmid was sequenced to ensure that the desired mutation was introduced.

Virus production

The plasmids described above were used to generate recombinant baculoviruses carrying the desired inserts. Baculoviruses vectors were created following the procedures described for the Bac-to-Bac system. Briefly, each donor plasmid was transformed into DH10Bac cells containing the baculovirus genome bacmid. After recombination, white colonies containing recombinant bacmids with the insert were isolated. The recombinant bacmids were amplified and analyzed by PCR for the presence of the hTERT sequence. 1 μ g of each of the purified bacmids were used to transfect 10⁶ Sf9 cells using cellfectin (Invitrogen), according to the manufacturer's protocol. The viruses were harvested 3 days post-transfection and were used

to amplify the viral stocks. The final amplified baculoviruses were titered before being used for protein expression.

Protein expression and purification

Spodoptera frugiperda cell lines Sf9 and Sf21 were maintained in suspension culture at 120 rpm using serum-free media HyQ SFX-insect (HyClone, Logan, UT) at 27°C. Cells were grown to a density of 3×10^6 /ml and infected by recombinant baculoviruses at a multiplicity of infection (MOI) of 2 to 10. Fetal bovine serum of 3 to 5 % (v/v) was added at the time of infection. GFP fluorescence was measured using a million infected cells plated on a 60-mm dish two-day post-infection. The cells were visualized by fluorescence microscope Zeiss Axiovert 200 (Zeiss, Germany) and the images were taken with SPOT imaging system (Diagnostic Instrument Inc., Sterling Height, MI). For protein purification, cells expressing the MBP fusions were harvested by centrifugation 48 to 72 hours post infection. The pellet was washed once with PBS, collected by centrifugation and frozen at -80°C until use. The cell pellets of the ER transported MBP and hTERT fusions were resuspended with 20 volumes of ice-cold lysis buffer containing 50 mM $\text{NH}_3\text{CH}_2\text{O}$, pH 7.8, 10% v/v glycerol, 50 mM NaCl, 1 mM MgCl_2 , 1% triton, 5 mM DTT, and protease inhibitor cocktail (Roche, Switzerland). The cell lysate was placed on ice for 20 minutes and sonicated briefly. Lysates were cleared by centrifugation and were subjected to affinity chromatography. Two types of affinity columns were used for purification: amylose resin (NEB, Ipswich, MA) for MBP affinity and anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO). For MBP affinity purification, the amylose resin was equilibrated with solubilization buffer and then incubated with cleared lysate with gentle mixing at 4°C for 2 hours. Resin was collected in an empty column and washed twice with 20 resin volumes of TMG (50 mM Tris, pH 7.5, 1 mM MgCl_2 , 50 mM NaCl, 1 mM DTT, protease inhibitors cocktail) buffer. The protein was eluted from the amylose column by adding 4 resin volumes of TMG buffer containing a final concentration of 10 mM maltose. For FLAG epitope affinity purification, the anti-FLAG affinity gel was equilibrated, incubated with cleared lysate, and washed as described above for MBP affinity purification. The protein was eluted from the anti-FLAG affinity gel by adding 4 gel volumes of TMG buffer containing 1 mg/ml of the FLAG peptide. The eluants were subjected to SDS-PAGE and Western blotting analysis. The MBP and hTERT were linked by a TEV protease recognition sequence, which can be cleaved by TEV protease. 50 µg of partially purified MBP-hTERT was incubated with 1 µg of TEV in a reaction buffer containing 50 mM Tris, pH 8.0, 5 mM EDTA, for 2 to 6 hours at 30°C. The cleaved products were analyzed by SDS-PAGE and Western blot.

Western blotting

Samples are fractionated on a NuPage 4–12% Bis-Tris (Invitrogen) gradient gel. After electrophoresis, samples were either stained by Simple Blue (Invitrogen), according to the manufacturer's recommendations, or transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NY). The blots were blocked by incubating in 100 mM Tris-HCl pH 7.5, 125 mM NaCl, 0.1% Tween-20 (TBS-T) containing 5% dry milk for 4–18 hours. The primary antibody anti-hTERT H-231 (Santa Cruz, Santa Cruz, CA) was diluted 1:1000 in TBS-T solution and incubated with the blots for 2 hours. Blots were washed three times in TBS-T for 15 minutes and then incubated with peroxidase-labeled anti-rabbit IgG at 1:2000 dilution in TBS-T for 1 hour. Blots were washed three times and developed with WestPico chemiluminescence substrate (Pierce, Rockford, IL). For detection of biotinylation, blots were reacted with streptavidin-horseradish peroxidase conjugated antibody (GE Healthcare) at 1:1000 dilution in TBS-T for 1 h, washed three times with TBS-T, and detected with the WestPico substrate.

Telomerase activity assay

Telomerase activity was detected using the telomeric repeat amplification protocol (TRAP). The assay was performed with the TRAPeze kit (Chemicon, Temecula, CA). Briefly, the TS primer was radio-labeled with [γ - 32 P]ATP (3000 Ci/mmol; GE Healthcare) using T4 DNA kinase. About 5000 uninfected or infected Sf9 cells were lysed with 100 μ l of CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM Benzamidine, 5mM β -mercaptoethanol, 0.5% CHAPS, and 10% Glycerol). Fifty microliter reactions contained 1x TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, and 1 mM EGTA), 25 μ M dNTP, 0.1 μ g of TS primer and reverse primers, 1 μ l of cell lysate or partially purified hTERT (~0.1 μ g), and 2 U of Taq DNA polymerase (Invitrogen). The reaction mixture was placed in the thermocycler and incubated at 30°C for 30 minutes. A 2-step PCR was performed (94°C/30s, and 59°C/30s for 30 cycles). 0.1 atto-mole of the control template TSR8, a synthetic oligonucleotide with eight TTAGGG repeats, was used as a positive control. PCR products were separated in 12.5% non-denaturing PAGE gel. Dried gels were analyzed quantitatively using phosphorimager or recorded using film.

Deglycosylation

Endoglycosidase H (Endo H) and N-glycosidase F (PNGase F) (NEB) were used to treat erMBP-hTERT. In a 10 μ l reaction, 0.5 μ g of purified erMBP-hTERT was denatured in buffer containing 0.5% SDS and 40mM DTT at 100° for 10 minutes. 1/10 volume of 10x reaction buffer (0.5M sodium citrate, pH 5.5) was added to the tube followed by 0.25 μ l of Endo H (500U/ μ l) and the reaction was incubated at 37°C for 1 hour. The products were analyzed by SDS-PAGE. The PNGase F reactions were similar except the 10x reaction buffer containing 0.5 M sodium phosphate pH 7.5, and 10% NP-40. 10 μ g of RNase B was used as a positive control to show that the deglycosylation reactions worked.

Confocal microscopy

Infected Sf9 cells were plated onto poly-d-Lysine coated glass bottom dishes (MatTek, Ashland, MA) for 2 days. The cells were either fixed prior to antibody labeling or labeled with ER-tracker red and DAPI (Invitrogen). For ER-tracker red and DAPI staining, the live cells were labeled according to the manufacturer's protocols. Briefly, infected cells were washed 2 times with PBS, incubated at room temperature for 20 min with 1 μ M red ER-tracker, washed with PBS and incubated with 300 nM DAPI in PBS for 5 min at room temperature, washed and imaged. For ER labeling using the anti-KDEL antibody (Stressgen, San Diego, CA), infected cells were fixed with 3.7% formaldehyde at room temperature for 1 hour, and 0.1 M glycine was added to quench free aldehyde groups. The fixed cells were blocked with 3% BSA/PBS for 1 hour. The cells were then incubated for 1 hr with 5 μ g/ml of the anti-KDEL antibody, washed, and incubated with Alexa-594-conjugated anti-mouse IgG (Invitrogen) for 30 min at room temperature. The cells were imaged using an Olympus IX-71 inverted microscope and analyzed with Delta Vision Software (Applied Precision Inc., Seattle, WA).

Results

Using GFP fluorescence to monitor the folding of hTERT expressed in insect cells

In the baculovirus expression experiments, we used the Bac-to-Bac system to generate recombinant baculoviruses that use the viral polyhedron promoter to express hTERT and hTERT fusion proteins. Sf9 or Sf21 cells were infected by recombinant baculoviruses at an m.o.i. of 1 to 5 and the cells were harvested 48 to 72 hours post-infection. There was no obvious difference in the expression level in Sf9 and Sf21 cells; all the results reported here were obtained with Sf9 cells.

Because the problem of the recombinant hTERT appeared to be solubility/folding, we decided to use a GFP fusion protein to monitor the folding of hTERT. A C-terminal GFP fusion has been commonly used as a folding reporter to distinguish proteins that are properly folded from those that are misfolded [22]. We generated an hTERT-GFP fusion to use as a folding reporter for hTERT. The level of expression of the hTERT-GFP fusion was very low, and it was not used in the folding experiments. However an N-terminal GFP-hTERT fusion (Fig. 1A) was expressed at a higher level and we used GFP fluorescence from the GFP-hTERT fusion to monitor the folding of hTERT. All of the hTERT fusion proteins contained a FLAG epitope, which made it simple to monitor the level of expression and could be used in the purification of the protein. We also added a C-terminal 23 amino-acid tag that can be biotinylated by the BirA [23,24] protein. This additional tag was included so that it could be used for tandem affinity purification of the hTERT fusion proteins. All of the fusions we generated had a TEV protease cleavage site inserted between hTERT and its fusion partner (GFP or MBP, see Fig. 1). When the GFP-hTERT fusion was expressed using the standard baculovirus expression system, little or no green fluorescence (Fig. 2) was seen by microscopy, suggesting that the fusion protein was not properly folded.

Co-expression of hTR and the chaperones hsp70, 90 with the GFP-hTERT fusion

Telomerase is a multiprotein complex that includes an integral RNA component, hTR, and chaperone proteins including hsp90 [15] and possibly hsp70. The proper folding of hTERT may require either hTR, or the cellular chaperone proteins, or both. We co-expressed hTR or the heat shock proteins hsp70 and hsp90 with the GFP-hTERT fusion in various combinations. Co-expression of these components did not enhance the GFP fluorescence, and by implication, folding and solubility (data not shown).

Expression of the GFP-hTERT fusion in specific subcellular locations enhanced GFP fluorescence

Because the expression of the hsp70 or hsp90 chaperone proteins did not increase the solubility of the GFP-hTERT fusion, we asked whether the endogenous chaperone proteins in insect cells could be used to improve the folding of GFP-hTERT. We directed the newly synthesized GFP-hTERT protein to the mitochondria and the ER, which are chaperone-rich. This approach was used to improve the solubility of the polyglutamine (74Q) aggregate present in the Huntington's disease [25]. We modified the baculovirus expression vector to include N-terminal leader sequences that would direct the GFP-hTERT fusion protein to the mitochondria or the ER (see *Materials and methods*). A signal peptide containing the N-terminal 39 amino acids from human mitochondria tRNA^{Leu} synthetase [21] was used to direct the fusion proteins to the mitochondria (mt). For the ER (er) targeting, we used the N-terminal 18 amino acids from the human BiP signal peptide and added an ER retention signal at the C-terminus (KDEL). To perform these experiments, the plasmids listed in the Fig. 1B and 1C were prepared. As a control, we expressed an unfused GFP protein that contained these peptides to ask whether the peptides were able to direct GFP to the proper cellular compartments and to see if the peptides affected protein expression and/or GFP folding. The control experiments showed that the signal peptides directed GFP to the proper compartments and that the addition of the peptides had no significant effect on the level of protein expression and/or the folding of GFP (Fig. 2).

We then expressed GFP-hTERT fusions that include the signal peptides. When the GFP-hTERT fusion was directed to the mitochondria (mtGFP-hTERT), there was little or no fluorescence. However, when we directed GFP-hTERT fusion to the ER (erGFP-hTERT-KDEL), there was a significant increase in the fluorescence (Fig. 2). The KDEL sequence, which is an ER retention signal, was added to increase the time that the fusion protein was retained in the ER. To determine whether the fluorescence came from the erGFP-hTERT-KDEL that was transported to the ER, colocalization studies were carried out with a marker

specific for the ER (ER-tracker) and the nucleus (DAPI). Staining of live and fixed Sf9 cells is shown in Fig. 3. Live Sf9 cells infected by baculovirus expressing erGFP-hTERT-KDEL were stained with the ER-tracker (red). The cells showed a staining pattern similar to that obtained with fixed cells expressing erGFP-hTERT and were reacted with anti-KDEL antibody followed by FITC-labeled IgG (red). As expected, GFP fluorescence in cells expressing erGFP-hTERT-KDEL or erGFP-hTERT colocalized with the red fluorescence of the ER markers (Fig. 3), indicating that the fusion protein was localized in the ER.

To verify that this increase in fluorescence was due to an increase in the solubility of the erGFP-hTERT-KDEL protein, the infected cells were lysed and the lysates were analyzed by SDS-PAGE and Western blotting with hTERT antibody. Both the whole cell lysates (Fig. 4A) and soluble fractions (Fig. 4B), contained a novel protein that migrated with a molecular weight of approximately 150 kDa which is consistent with the expected size of a fusion of GFP and hTERT (27 and 127 kDa). The Western blot with anti-hTERT antibody confirmed that this protein was the hTERT fusion. The data show that directing the fusion protein to the ER increased the amount of the GFP and hTERT fusion protein, presumably because proper folding stabilized the protein.

We expected that erGFP-hTERT-KDEL, which had the ER retention signal, might behave differently from an erGFP-hTERT fusion without this signal (Fig. 1C), because the KDEL version should remain in the ER while the fusion lacking KDEL could enter the secretion pathway. We compared the GFP fluorescence and solubility of erGFP-hTERT-KDEL and erGFP-hTERT fusion proteins. Interestingly the erGFP-hTERT without the ER retention signal had better biochemical properties with respect to GFP fluorescence and protein solubility (Fig. 4C). The fluorescence intensity of erGFP-hTERT-KDEL is less than that of erGFP-hTERT (data not shown). However, a similar proportion (~70%–80%) of both erGFP-hTERT-KDEL and erGFP-hTERT remained in the ER, as determined by fluorescence microscopy (data not shown) whether or not the fusion proteins contained the retention signal. Because the retention signal did not increase the production of soluble protein, we used versions without the retention signal in the subsequent experiments.

We also tested an MBP-hTERT fusion, which accumulates to high levels in insect cells whether it was expressed in the cytoplasm (MBP-hTERT) or was directed to the ER (erMBP-hTERT) (Fig. 1). Because both proteins were expressed at a similar level, we used a semi-quantitative Western blotting to measure the fraction of the two fusion proteins that was soluble (Fig. 5A). We estimate that about 2% of total expressed MBP-hTERT was soluble using the standard expression system. The soluble fraction increased to about 25% of the total expressed erMBP-hTERT when the fusion protein was directed to the ER, showing that directing the MBP fusion protein to the ER increased its solubility. ER transport in insect cells is co-translational, which suggests that it might be important to fold the protein as it is synthesized.

Enzymatic activity of the ER-transported hTERT

Having produced soluble erMBP-hTERT, we wanted to ask whether the fusion protein produced using the ER localization method had proper enzymatic activity. We measured the telomerase activity in insect cell lysates expressing the unmodified and ER transported MBP-hTERT fusions using a TRAP assay [26] in which the telomere repeats that were added to the primer by hTERT were amplified by PCR. Lysates of uninfected Sf9 cells, and Sf9 cells expressing MBP-hTERT and erMBP-hTERT without hTR showed no detectable telomerase activity (Fig. 6A). Because hTR is essential for the activity of telomerase, we inserted hTR RNA component *in cis* into the expression vector *erMBP-hTERT-hTR* [27] (Fig. 1C). Lysates of Sf9 cells expressing erMBP-hTERT-hTR showed strong and processive telomerase activity in the TRAP assay. As a control, we prepared a version of the fusion protein with a mutation at the polymerase active site (erMBP-hTERT-hTR D868A), which showed little or no activity

in the TRAP assay. This demonstrated that the telomerase activity came from the recombinant erMBP-hTERT-hTR we expressed.

Having demonstrated that ER transported erMBP-hTERT had a proper enzymatic activity if it is co-expressed with hTR, we scaled up the infections and partially purified the fusion protein. We first screened a number of buffer conditions to improve the extraction of erMBP-hTERT-hTR (Fig. 5B). We then partially purified the erMBP-hTERT-hTR fusion from lysates of insect cells using the optimized solubilization conditions. The erMBP-hTERT-hTR was constructed such that it contained an N-terminal MBP, and a C-terminal FLAG epitope that is a hydrophilic octapeptide with the sequence DYKDDDDK, to facilitate affinity purification. We partially purified the soluble erMBP-hTERT-hTR fusion protein either using amylose affinity column chromatography (NEB) or an anti-FLAG M2 affinity gel (Sigma). After a partial purification, we were able to obtain several hundred micrograms of erMBP-hTERT-hTR from a one liter Sf9 culture (Fig. 7A). Both the amylose and anti-FLAG M2 affinity columns gave a similar yield. We used a TRAP assay to test the activity of this partially purified MBP-hTERT-hTR; the enzyme was active and processive (Fig. 6B). We also purified the hTERT D868A active site mutant in parallel, and as expected, it had little or no activity, confirming that the activity we measured is from erMBP-hTERT-hTR fusion. As has already been mentioned, the erMBP-hTERT-hTR contains a TEV protease recognition site between MBP and hTERT. TEV protease was used to digest the fusion protein to separate MBP and hTERT. The digested protein was analyzed by SDS-PAGE and Western blotting to show that the digestion was nearly complete (Fig. 7B). The activity was not affected, indicating that the MBP fusion partner does not affect the enzymatic activity: The properly folded hTERT protein remained soluble after cleavage from MBP.

We also generated an erMBP-hTERT-luciferase fusion protein to ask whether a significant amount of the fusion protein was exported from the cells. Based on the luciferase assay, approximately 95% of the soluble erMBP-hTERT-luciferase fusion protein remained within the cells (data not shown).

We were concerned, because some cellular proteins transported to the ER are modified by glycosylation, that the erMBP-hTERT might be glycosylated. We treated the erMBP-hTERT with glycosidases, including endoglycosidase H (Endo H) and N-glycosidase F (PNGase F), and saw no change in the migration of treated erMBP-hTERT protein on the SDS-PAGE (Fig. 8). In control experiments, the RNase B protein was properly deglycosylated by both EndoH and PNGase F.

Discussion

Purified proteins are required for biochemical and structural analysis. The required proteins are often generated by expression as recombinant proteins; however there can be folding problems when proteins were expressed in heterologous systems. The difficulties are often greater for proteins that normally are components of protein complexes. Because the TERT protein is normally part of a ribonucleoprotein complex, expressing this protein has been difficult, and others have expressed individual domains from TERTs from various species. Screening for fragments of *T. thermophila* TERT that could be expressed in *E. coli* led to the expression [28] and eventual crystal structure of a soluble N-terminal domain of this protein, consisting of residues 2-191 [29]. However expressing individual TERT domains does not allow reconstitution of a functional telomerase.

We have expressed and partially purified full-length, enzymatically active hTERT fusion proteins by taking advantage of the fact that the ER is enriched in chaperones and provides a good environment for protein folding. This system provides an effective way to produce

recombinant hTERT protein. Because the method utilizes a signal peptide to direct proteins to the ER, it can be applied to many proteins of interest, and may be a generally useful method that can be used to assist the folding of other recombinant proteins that are difficult to express in a properly folded state.

Purified recombinant hTERT can be used to understand the regulation of telomerase activity. Because germ cells, stem cells, and the vast majority of cancer cells express high levels of telomerase, it is important to understand how telomerase activity is regulated in normal and pathological conditions. The level of hTERT mRNA expression seems to be an important rate-limiting step for telomerase activity [30]. Post-translational modifications of TERTs also play an important role in regulating telomerase activity. hTERT is phosphorylated by signaling kinases which regulate the telomerase activity [31–33]. The recombinant hTERT protein we reported here can be used to study interactions between hTERT and other telomerase components and monitor the effects of post-translational modifications. Telomerase activity is also regulated by interactions between the telomere complex and the telomerase complex. Telomerase-mediated telomere maintenance and elongation is modulated by the structure of telomere, and involves telomere binding proteins [34,35]. Purified hTERT will make it possible to use biophysical and biochemical tools to study hTERT and its interactions with the other components of the telomerase complexes and, ultimately with the telomere. The enzymatically active recombinant hTERT will also facilitate the screening and development of compounds that would modulate (or block) telomerase activity.

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Fig. 1.

The hTERT protein fusions. (A) Plasmids were constructed to express GFP-hTERT and MBP-hTERT fusion proteins, including *GFP-hTERT*, *GFP-hTERT-hTR*, *MBP-hTERT*, and *MBP-hTERT-hTR*, using the *pFastBac* donor vector. Each plasmid contained either a GFP or MBP at the N-terminus, followed by the TEV protease recognition sequence, hTERT, and a FLAG epitope. The TEV protease recognition site was inserted between GFP/MBP and hTERT to allow these components to be separated by TEV cleavage. Parallel constructs were prepared that did and did not express the RNA component hTR. (B) Plasmids were made that expressed the GFP/MBP and hTERT fusions and directed them to the mitochondria (mt), including *mtGFP-hTERT*, and *mtMBP-hTERT*, using the human mitochondria tRNA^{Leu} synthetase 39 a.a. signal peptide. (C) Plasmids were made that directed the fusions to the endoplasmic

reticulum (er), including *erGFP-hTERT*, *erGFP-hTERT-hTR*, *erMBP-hTERT*, *erMBP-hTERT-hTR*, *erGFP-hTERT-biotin*, *erMBP-hTERT-biotin*, and *erGFP-hTERT-KDEL*. All had the N-terminal signal peptides from human BiP. Plasmids *erGFP-hTERT-biotin* and *erMBP-hTERT-biotin* contained a C-terminal biotin tag that can be biotinylated by the BirA protein. The plasmid *erGFP-hTERT-KDEL* contained a C-terminal ER retention signal. Constructs that expressed an unfused GFP were used for control experiments, including *GFP*, *mtGFP*, and *erGFP* (not shown).

Fig. 2.

GFP fluorescence from the GFP-hTERT fusion was enhanced when the fusion protein was transported to the ER. GFP fluorescence of live insect cells was monitored after the cells were infected by recombinant baculoviruses expressing GFP or GFP-hTERT fusion proteins. Cells were visualized with a Zeiss Axiovert 200 microscope using appropriate filters. The left column shows proteins that contain signal peptides that direct either GFP alone or GFP-hTERT to the mitochondria or ER. The GFP controls (middle column) show that directing GFP to the mitochondria or ER does not affect fluorescence. The GFP-hTERT fusion protein (right column) showed very weak fluorescence when it was expressed in the cytosol, in the presence/absence of hsp70/hsp90/hTR, or when it was expressed in the mitochondria, indicating that GFP-hTERT folding was inefficient compared to the GFP control. When GFP-hTERT was directed to ER, the level of GFP fluorescence is comparable to the GFP control, indicating the folding of the fusion protein was greatly improved.

Fig. 3.

Localization of the ER-transported GFP and hTERT fusions in Sf9 cells by fluorescence microscopy. (A1) Live Sf9 infected by baculovirus expressing erGFP-hTERT-KDEL, stained with the (A2) ER-tracker (red), an ER-specific marker, and (A3) DAPI (blue). (B1) Fixed Sf9 cells that expressed erGFP-hTERT were reacted with (B2) anti-KDEL antibody followed by FITC-labeled IgG (red), and (B3) the merged image. (C1) A control experiment performed with fixed Sf9 cells expressing GFP, with (C2) anti-KDEL labeling, and (C3) the merged image. Results showed most ER-transported GFP and hTERT fusions were localized in the ER.

Fig. 4.

Expression of hTERT fusion proteins in insect cells. Proteins were separated on 4–12% SDS-PAGE gradient gels. The gels were stained with Coomassie blue or used for Western blotting with anti-hTERT H231 antibody unless specified. (A) The whole cell lysates of uninfected and infected Sf9 were fractionated: Lane M, molecular weight marker (in kilodaltons); lane 1, uninfected Sf9; lane 2, Sf9 expressing GFP-hTERT; lane 3, mtGFP-hTERT; lane 4, erGFP-hTERT; and lane 5, erMBP-hTERT. A band in lane 4 that migrates with molecular weight (m.w.) of 150 kDa corresponds to the fusion erGFP-hTERT (left arrow). A band in lane 5 that migrates with m.w. of 160 kDa corresponds to erMBP-hTERT (right arrow). Proteins in lanes 1–5 were subjected to Western blotting (shown in lanes 6 to 10). (B) Soluble fractions of cell lysates expressing GFP and hTERT fusions were monitored by Western blotting: Lane 1, uninfected Sf9 lysate; lane 2, GFP-hTERT; lane 3, mtGFP-hTERT; lane 4, erGFP-hTERT-KDEL; and lane 5 erGFP-hTERT without the KDEL retention signal. (C) Whole cell lysates of infected Sf9 cells: Lane 1, Sf9 expressing erGFP-hTERT-biotin; lane 2, erGFP-hTERT-biotin and BirA (red arrow); lane 3, erMBP-hTERT-biotin; and lane 4, erMBP-hTERT-biotin and BirA. Samples in lanes 1–4 were subjected to Western blotting using either anti-hTERT H231, shown in lanes 5–9, or using streptavidin-HRP, shown in lanes 9–12. Results indicated

erGFP-hTERT-biotin and erMBP-hTERT-biotin were biotinylated by BirA. The lower band in lanes 9–12 was an endogenous biotin-containing protein present in Sf9 cells.

Fig. 5.

The solubility of MBP-hTERT and erMBP-hTERT. (A) Cell lysates: Lane 1, total lysate of Sf9 cells expressing MBP-hTERT and lane 2, erMBP-hTERT. Both lanes showed a similar level of expression. Western blotting: Lane 3, total lysate of Sf9 cells expressing MBP-hTERT; lane 4, soluble MBP-hTERT; and lane 5, soluble erMBP-hTERT. Soluble fractions were extracted using buffer containing 0.2% Triton, Tris 50 mM pH 7.5, NaCl 50 mM and 1 mM MgCl₂. By semi-quantitative measurement, soluble MBP-hTERT is about 2% of the total expressed MBP-hTERT, whereas soluble erMBP-hTERT is about 25%. (B) Extraction of erMBP-hTERT was measured by Western blotting using solubilization buffers that contain different detergents and salt concentrations. The following conditions were used: Lane 1, 0.5% MEGA-9, 500 mM NaCl; lane 2, 0.5% CHAPS, 500 mM NaCl; lane 3, 0.5% NP-40, 350 mM NaCl; land 4, 0.5% NP-40, 0.5% Cholate; and lane 5, 1% Triton, 500 mM NaCl. All of these buffers used contained 50 mM Tris, pH 7.5. Lane 6, 0.2 M ammonium bicarbonate (AB), pH 7.8; lane 7, 0.2 M AB, Triton 1%, and lane 8, 0.2 M AB, 0.5% Triton, 50 mM KCl. Solutions containing ammonia bicarbonate extracted more soluble erMBP-hTERT. The solution used in lane 7 was used for erMBP-hTERT solubilization and purification.

Fig. 6.

Telomerase activity of recombinant hTERT. (A) TRAP assays with cell lysates of uninfected and infected Sf9 cells: Lane 1, uninfected, lane 2, MBP-hTERT; lane 3, erMBP-hTERT; lane 4, erMBP-hTERT-hTR; and lane 5, erMBP-hTERT-hTR D868A active site mutant. Lane 6, 293 cell lysate as a positive control, and lane 7, erMBP-hTERT-hTR D712A. (B) TRAP assays with purified proteins: Lane 8, purified erMBP-hTERT-hTR; lane 9, TSR8 control template; lane 10, purified D868A mutant erMBP-hTERT-hTR; and lane 11, purified erMBP-hTERT-hTR that was digested by TEV protease.

Fig. 7.

Affinity purification of erMBP-hTERT-hTR. (A) Lane *M*: the marker; lane *1*, partially purified erMBP-hTERT-hTR using amylose resin that was eluted with 10 mg/ml of maltose. (B) Lane *1*: partially purified erMBP-hTERT-hTR using an anti-FLAG affinity gel eluted with excess FLAG peptide (1 mg/ml). The eluted erMBP-hTERT-hTR in lane *1* was subjected to Western blotting (lane 2–4). Western blotting showed partially purified erMBP-hTERT-hTR (arrow 1) cleaved by TEV protease for 2 hours (lane 3), and for 6 hours (lane 4), indicating most of the fusion protein was cleaved (arrow 2). The heavy chain of mouse IgG from M2 antibody-FLAG affinity was also eluted by the elution buffer and reacted (arrow 3) in the Western blot.

Fig. 8. Recombinant hTERT is not glycosylated. (A) Western blotting of the partially purified erMBP-hTERT reacted with deglycosidases: Lane 1, undigested; lane 2, digested by Endo H; and lane 3, digested by PNGase F. There was no detectable change in the apparent molecular weight of erMBP-hTERT before and after the digest. (B) SDS-PAGE of the RNase B reacted with deglycosidases as a control: Lane 1, undigested; lane 2, digested by Endo H; and lane 3, digested by PNGase F.

The sequences of primers used in this study. The DNA oligonucleotides were used to generate the expression constructs shown in Fig. 1. Each primer may contain a restriction site (lowercase), termination codon (bold), or specific coding sequence (underlined) which encodes the TEV protease recognition sequence in GFPrev² and MBPprev¹⁰ primers; the FLAG epitope in hTERTrev⁴; the biotin tag in BIOTINfor⁵ and BIOTINrev⁶; the BiP signal peptide in BiP-BamH¹¹ and BiP-NcoI¹²; the ER-retention signal KDEL in KDEL-SalI¹³ and KDEL-XbaI¹⁴; the signal peptide of human mitochondrial tRNA^{Leu} synthetase in mtRNA-BamH¹⁵ and mtRNA-NcoI¹⁶ primers. Please see *Materials and Methods* for a detailed description of the primers.

Table 1

DNA oligonucleotides	Sequence
GFPfor ¹	CATGccatggTGAGCAAGGGGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGT
GFPrev ²	GcaattcGCCCTGAAAAATACAGGTTTTCCTTGTAACAGCTCGTCCATGCCGAGAGTGA TC
hTERTfor ³	GgaattcATGCGCGCGGCTCCCGCTGCCGAGCCGTTGGCTCCCTGCTGGCGCAGCCACTA
hTERTrev ⁴	ACGcgcga cTTT ACTTATCTGTCGTCATCCTTGTAATCGTCCAGGATGGTCTTGAAGTCTGAGG
BIOTINfor ⁵	TGACATGGCTTCTTCCCTGAGGCAAAATCCTGGACTCTCAAAAGATGGATGGAGATCTAACGGTGGCGGATCCT TAAT
BIOTINrev ⁶	CTAGATTAGGATCCGCCAGCGTTAGATCTCCACTCACTCTTCTGAGAGTCCAGGATTTCCTCAGGGAAGAAAGCCATG
hTRfor ⁷	CCGctcragTGAGGGTTGGGAGGGTGGGCC
hTRrev ⁸	CGGggtaccTTTTTATTTTTCATGTGTGAGCC
MBPfor ⁹	CATGccatggAAA TCGAAGAAGGTAAACTGGTAACTCTGGATTAAACGG
MBPrev ¹⁰	GcaattcGCCCTGAAAAATACAGGTTTTCAGTCTGCGCGTCTTC
BiP-BamH ¹¹	GATCCATGAAGCTGAGCGCTGGTGGCCGCCATGCTGCTCTGCTCAGCGCGCCAGGGCCC
BiP-NcoI ¹²	CATGGGGCCCTGGCGGCGCTGAGCAGGAGCAGCATGGCGGCCACCAGGCTCAGCTTCATG
KDEL-SalI ¹³	TGACAAAGGACGAGCTGTAA T
KDEL-XbaI ¹⁴	CTAGATTACAGCTCGTCTCTG
mtRNA-BamH ¹⁵	GATCCATGGCCAGCGTGTGGCAGAGGCTGGGCTTCTACGCCAGCTGTCTGAAGAGGCAGCTGAACGGCGGCCCGACGTGATCAAGTGGGAGAGGAGGGTGAATCCCCGGCTGACCCAGGAGCC
mtRNA-NcoI ¹⁶	CATGGGCTCCTGTGTGCAGCGGGGATCACCTCTCTCTCCCACTTGATCAGTCTGGGGCCCGCTTCAGCTGCCCTTCAGAGGCTGGCTAGAAAGCCAGCCCTCTGCCACACGCTGGCCATG
BirAfor ¹⁷	GgaattcATGAAGGATAAACACCGTGGCCACTGA AAATTG
BirArev ¹⁸	GGctagaTTATTATTCTTCTGCACACGCAGGGA
D868For ¹⁹	GGGCTGCTCTCGCTTGGTGGCTGATTCTTGTTGGTG
D868Rev ²⁰	CACCAACAAGAAATCAGCCACCAACCAACGAGGAGCAGCCC