Telomerase Activity Reconstituted in Vitro with Purified Human Telomerase Reverse Transcriptase and Human Telomerase RNA Component*

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Telomerase is a specialized reverse transcriptase that catalyzes elongation of the telomeric tandem repeat, TTAGGG, by addition of this sequence to the ends of existing telomeres. Human telomerase reverse transcriptase (hTERT) has been identified as a catalytic enzyme involved in telomere elongation that requires telomerase RNA, human telomerase RNA component (hTR), as an RNA template. We established a new method to express and purify soluble insect-expressed recombinant hTERT. The partially purified FLAG-hTERT retained the catalytic activity of telomerase in a complementation assay in vitro to exhibit telomerase activity in telomerase-negative TIG3 cell extract and in a reconstitution assay with FLAG-hTERT and purified hTR in vitro. FLAG-hTERT (D712A) with a mutation in the VDV motif exhibited no telomerase activity, confirming the authentic catalytic activity of FLAG-hTERT. The reconstituted complex of FLAG-hTERT and hTR in vitro was detected by electrophoretic mobility shift assay, and its activity was stimulated by more than 30-fold by TIG3 cell extract. This suggested that some cellular component(s) in the extract facilitated the reconstituted telomerase activity in vitro. Geldanamycin had no effect on the reconstituted activity but partially reduced the stimulated activity of the reconstituted telomerase by the TIG3 cell extract, suggesting that Hsp90 may contribute to the stimulatory effect of the cellular components.

The telomere is a specialized structure at the ends of linear eukaryotic chromosomes that provides a mechanism for maintaining chromosome length and has critical functions in maintaining chromosome stability (1, 2). The telomere structure consists of long tandem repeats (TTAGGG), telomere repeats, and specific DNA-binding proteins (2). Telomerase, a ribonucleoprotein complex, is composed of template RNA, and several proteins elongate telomeres (2, 3). Human telomerase reverse transcriptase, hTERT,1 has been identified as the catalytic enzyme required for telomere elongation (4–7). TERT contains motifs found in many reverse transcriptase, and these motifs are highly conserved among species from budding yeast to human (2, 4, 5). Human TERT is the rate-limiting factor for telomerase activity both biologically and enzymatically (6, 8–12). Introduction of hTERT into normal human primary cells overcomes senescence and extends their lifespan and has been reported to cause cellular immortalization without crisis in the transformed cells (8–11). Transient expression of hTERT results in telomerase activity in telomerase-negative normal human cells (6). Telomerase is highly active in most cancer cells and immortalized cells, whereas telomerase activity is suppressed in somatic cells (13–15). Thus, hTERT may play an important role in cellular senescence and carcinogenesis (16). Recently, some groups reported in vitro reconstitution of telomerase and demonstrated the essential role of hTERT and human telomere RNA, hTR, as an RNA template (17–20). One group demonstrated that Hsp90 and p23 were essential for telomerase activity with recombinant hTERT synthesized de novo in rabbit reticulocyte extract in vitro (20). Another group reported that the production of active recombinant telomerase of Tetrahymena requires a factor in rabbit reticulocyte extract that promotes ribonucleoprotein assembly (21, 22). In these systems, certain factor(s) carried over with rabbit reticulocytes may influence the native telomerase activity. Therefore, it remains unclear whether these two components, hTERT and hTR, are sufficient for in vitro telomerase reconstitution. The production of purified hTERT and hTR is necessary to answer this question and to provide an experimental system in which to identify factors that are essential for or that stimulate telomerase activity in vitro.

Here, we report a method of expression and purification that allows recovery of a large amount of the soluble form of FLAG-tagged hTERT from insect cells infected with hTERT expression recombinant baculovirus. We demonstrated that the partially purified FLAG-hTERT and hTR retained the authentic catalytic activity of telomerase that was further stimulated by some factors in telomerase-negative cell extracts.

EXPERIMENTAL PROCEDURES

Cells and Viruses—SF9 cells and the BaculoGold Starter Package including BaculoGold linear baculovirus DNA were purchased from PharMingen Co. Ltd. High5 cells were purchased from Invitrogen Co. Ltd. SF9 cells were grown in suspension in TNM-FH insect medium (Sigma) supplemented with 10% fetal calf serum. High5 cells were also

repeat amplification protocol; ELISA, enzyme-linked immunosorbent assay; GA, geldanamycin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CBB, Coomassie Brilliant Blue; EMSA, electrophoretic mobility shift assay.

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¶ The abbreviations used are: hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA component; m.o.i., multiplicity of infection; DTT, dithiothreitol; MEGA-9, n-nonanoyl-N-methylglucamide; PAGE, polyacrylamide gel electrophoresis; TRAP, telomere

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grown in suspension in High5 serum-free medium (Invitrogen Co. Ltd.).

Plasmid Construction—The EcoRI-SalI fragment containing the hTERT cDNA was subcloned from pc1-Neo-hTERT, which was kindly provided by Dr. Robert A. Weinberg (Whitehead Institute, MIT). This fragment was inserted into the EcoRI-SalI sites of the plasmid pNZFLAG (23, 24). The FLAG-hTERT baculovirus DNA was constructed by inserting the NotI-BglII fragment of the FLAG-hTERT cDNA derived from pNZX-FLAG-hTERT into the NotI-BglII sites of the pVL1393 Baculovirus Transfer Vector (PharMingen Co. Ltd.). An aspartic acid to alanine mutation at position 712 (D712A) was introduced via polymerase chain reaction site-directed mutagenesis (25). pGRN164 contains the hTERT cDNA, which was kindly provided by Dr. Gregg B. Morin (Geron Corporation) (18, 25).

Generation of Recombinant Baculoviruses—Aliquots of 10^7 of SF9 cells were seeded in 9-cm^2 dishes 30 min before transfection with 2.5 μg of pBKM-FLAG-hTERT mixed with 0.25 μg of Baculogold-linearized baculovirus DNA (PharMingen Co. Ltd.). Other components for co-transfection were as recommended by the manufacturer (PharMingen Co. Ltd.). The cells were incubated for 5 days at 27 °C and the supernatant was used for amplification of the FLAG-hTERT expression recombinant virus, BVKM-FLAG-hTERT, on SF9 cells. The recombinant viruses amplified on SF9 cells were titrated by plaque assay as according to the manufacturer’s protocol. High titer suspensions of BVKM-FLAG-hTERT (>1.0 × 10^7 plaque-forming units/ml) were used for infection of High5 cells.

Purification of FLAG-tagged hTERT Protein from Infected Cells—For expression of FLAG-hTERT protein, aliquots of 1.0 × 10^7 High5 cells were seeded onto 5 × 25-cm^2 dishes before infection. The cells were infected with BVKM-FLAG-hTERT at a multiplicity of infection (m.o.i.) of about 0.2. These infected High5 cells were incubated for 5 days at 27 °C and then scraped off the plates and centrifuged at 4000 rpm for 10 min. All subsequent steps were performed at 4 °C, and all buffers contained 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A (Sigma), 10 μg/ml leupeptin (Sigma), 10 μg/ml aprotinin (Roche Molecular Biochemicals), 10 μg/ml phenanthroline (Sigma), 16 μg/ml benzamidine (Sigma), and 1 mM DTT (Nakalai Tesque Co. Ltd.). The collected cells were resuspended in 5 ml of buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1% Nonidet P-40, 150 mM NaCl, 10 mM MgCl2, 3 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 units/μl RNasin), and aliquots of the cell extracts were stored at −80 °C (6, 27). The TiG3 cell extract and FLAG-hTERT were mixed with or without hTERT in a total volume of 20 μl and incubated at 33 °C for 1 h for telomerase reaction. Telomerase activity was detected using the two methods described above.

Results of Geldanamycin (GA) on Telomerase Activity—Complementation assay of telomerase activity with TIG3 extract and in vitro reconstitution of human telomerase were performed with the benzoquinone ansamycin, GA (Calbiochem). Increasing amounts of GA were added after the assembly step (preincubation), and telomerase reactions were carried out.

Expression of FLAG-tagged hTERT—We were unable to express and purify hTERT by several methods using bacterial expression systems (data not shown). A baculovirus system was therefore applied to express hTERT in insect cells. We compared the expression level of FLAG-hTERT in SF9 cells with that in High5 cells infected with the recombinant virus BVKM-FLAG-hTERT. The expression level of FLAG-hTERT was much higher in High5 than in SF9 cells, and therefore we chose the High5 cells to express FLAG-hTERT. The amount of expressed FLAG-hTERT in High5 cells infected with BVKM-FLAG-hTERT at a m.o.i. of 0.2 was similar to or slightly higher than those in cultures infected with the virus at m.o.i. of 5–10. The expression level of the protein was almost similar during day 4–6 after infection and were higher than those at day 2 and 3 (Fig. 1C). Therefore, the conditions used for efficient expression of FLAG-hTERT using High5 cells had an m.o.i. of around 0.2, which was determined to be 0.2. The time course of changes in expression level of FLAG-hTERT in High5 cells infected with BVKM-FLAG-hTERT at a m.o.i. of 0.2 was determined. The expression levels of the protein were almost similar during day 4–6 after infection and were higher than those at day 2 and 3 (Fig. 1C). Therefore, the conditions used for efficient expression of FLAG-hTERT using High5 cells had an m.o.i. of around 0.2, which was determined to be 0.2. The time course of changes in expression level of FLAG-hTERT in High5 cells infected with BVKM-FLAG-hTERT at a m.o.i. of 0.2 was determined.

Purification of Catalytically Active hTERT—Recombinant FLAG-hTERT expressed by insect cells was poorly solubilized in buffers containing several kinds of detergents including Triton X-100, Nonidet P-40, and CHAPS, and low or moderate concentrations of salt. However, we found that FLAG-hTERT
could be solubilized in buffer containing a high salt concentration and the nonionic detergent MEGA-9 (29, 30). We exploited this property to partially purify the FLAG-hTERT proteins first using buffers in which most cellular protein could be solubilized (Fig. 2A, lanes 1, 3, and 5). Under these conditions, the majority of FLAG-hTERT protein remained in the pellet but could be eluted by a subsequent step using high stringency buffer (Fig. 2A, lanes 4 and 6). As shown in Fig. 2A, most of the cellular proteins were eluted from the cell pellet with buffers containing low concentrations of Nonidet P-40, glycerol, and NaCl (Fig. 2A, S1, S2, and see “Experimental Procedures”). In contrast, FLAG-hTERT was eluted efficiently only with the buffer containing MEGA-9 and high salt (Fig. 2A, S2 and S3). Therefore, FLAG-hTERT was enriched in S2 and S3.

We attempted to purify the protein from these supernatants (S4 also see “Experimental Procedures”) with anti-FLAG monoclonal antibody M2-bound Sepharose (Kodak Bioscientific Imaging Systems Co. Ltd.), but the recombinant FLAG-hTERT protein could not be immunoprecipitated by this procedure (data not shown). The FLAG-hTERT fractionated by SDS-PAGE was successfully detected by Western blotting using M2 antibody (Figs. 1B and 2C). These results strongly suggested that the FLAG tag at the N terminus is not exposed in native FLAG-hTERT but is exposed under denaturing conditions. Therefore we attempted to partially purify the FLAG-hTERT (see “Experimental Procedures”) by heparin column chromatography based on the properties of hTERT (3, 18, 19, 21, 31, 32). FLAG-hTERT was found to bind heparin-Sepharose and was eluted from the resin with high concentrations of NaCl (Figs. 2B, lane 10, and C). Similarly, FLAG-hTERT can bind poly(U)-Sepharose with a similar efficiency to heparin-Sepharose CL-6B (data not shown).

Telomerase Activity of FLAG-hTERT and hTR Reconstituted in Vitro—We attempted to detect telomerase activity of the purified FLAG-hTERT (heparin-Sepharose fraction) and in vitro transcribed hTR (see “Experimental Procedures”) reconstituted in vitro. Telomerase activity was detected by TRAP assay only when FLAG-hTERT and hTR were present although FLAG-hTERT alone exhibited no activity (Fig. 3A). This result indicates that the reconstituted components in vitro exhibited telomerase activity. The telomere synthesis was observed after a lag time of 10 min and continued for at least 1 h in an apparently linear fashion at 33 °C as determined by TRAP assay (Fig. 3C). Maximum catalytic activity of FLAG-hTERT occurs at a reaction temperature between 30 °C and 37 °C, and the activity requires the presence of magnesium ions at 4 mM (data not shown). The telomerase activity of the reconstituted components in vitro was examined by another in vitro assay, TRAP ELISA (see “Experimental Procedures”), to measure telomerase activity quantitatively in the presence of varying molar ratios of the recombinant FLAG-hTERT and hTR (Fig. 3B). Maximum telomerase activity was observed when the two components, FLAG-hTERT and hTR, were present at approximately equimolar ratio in the reaction. Judging from the results obtained by these two methods, reconstitution of equimolar amounts of FLAG-hTERT and hTR seemed to occur resulting in the optimal telomerase activity, suggesting efficient complex formation of the two components in vitro.

The Catalytic Activity of FLAG-hTERT Complements Telomerase-negative Cell Extracts, which Has Stimulating Effects on in Vitro Reconstituted Telomerase—TERT has been reported to be a rate-limiting factor for telomerase activity in cultured cell
We examined whether the catalytic activity of FLAG-hTERT could complement that missing in telomerase-negative cell extracts. We constructed a mutant FLAG-hTERT baculovirus expression plasmid (see “Experimental Procedures”), in which the VDV motif was replaced by VAV. FLAG-hTERT and FLAG-hTERT(D712A) were expressed in insect cells and purified (Fig. 4A), and total cell extracts were prepared from the telomerase-negative normal human fibroblast cell line (TIG3). Neither TIG3 cell extract nor the purified FLAG-hTERT alone showed any telomerase activity (Figs. 4B, lane 3, and 3A, lane 2). The wild-type FLAG-hTERT exhibited telomerase activity only when the TIG3 cell extract was added, whereas the mutant protein, FLAG-hTERT(D712A), showed no telomerase activity even in the presence of the cell extract (Fig. 4B, lanes 4 and 5). The catalytic activity of FLAG-hTERT was necessary to complement the telomerase-negative cell extracts because the wild-type FLAG-hTERT and not the mutant FLAG-hTERT (D712A), complemented the telomerase-negative TIG3 cell extract. FLAG-hTERT(D712A) showed no catalytic activity in the reconstitution assay (data not shown).

If hTR is the only factor supplied by TIG3 extract for telomerase activity, telomerase activity reconstituted in vitro may not be affected in the presence of TIG3 extract. Interestingly, TIG3 extract strongly augmented telomerase activity reconstituted with FLAG-hTERT and hTR in vitro as determined by two as-

![Fig. 3. Telomerase activity with recombinant hTERT and hTR reconstituted in vitro.](image)

![Fig. 4. FLAG-hTERT complemented telomerase activity of the telomerase-negative TIG3 extract, and the TIG3 extract augmented the reconstituted telomerase activity reconstituted with FLAG-hTERT and hTR.](image)
Telomerase is present in germ cells but is repressed in most human somatic cells during development (1, 33). In contrast, this enzyme is highly active in most cancer cells (13, 15, 34–36). hTERT was identified as the catalytic component of telomerase and is a limiting factor for its activity (4–7). Induction of hTERT overcame senescence and extended the lifespan of normal human primary cells, and ectopic expression of hTERT in combination with two oncogenes results in direct tumorigenic conversion of normal human epithelial and fibroblast cells (8, 16). These results suggested an important role of hTERT in preventing cell senescence and in carcinogenesis. Thus, hTERT is therefore a likely target for methods to prevent cell senescence or carcinogenesis.

Here, we established a method to express and purify a large amount of soluble recombinant hTERT. Attempts to express and purify bacterial recombinant hTERT were not successful, and therefore the baculovirus expression system was applied. The expression level of FLAG-hTERT was very low in Sf9 cells, and therefore the baculovirus expression system was applied. hTERT overcame senescence and extended the lifespan of normal human primary cells, and ectopic expression of hTERT in combination with two oncogenes results in direct tumorigenic conversion of normal human epithelial and fibroblast cells (8, 16). These results suggested an important role of hTERT in preventing cell senescence and in carcinogenesis. Thus, hTERT is therefore a likely target for methods to prevent cell senescence or carcinogenesis.

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better harvesting time that were different from those recommended in the standard protocol (28). This efficiency of expression may have been because of the slow growth rate of the cells infected with BVKM-FLAG-hTERT. The recombinant FLAG-hTERT protein was recovered at 2.0 mg/ml^10^ infected cells, a level not much different from those of other recombinant proteins. hTERT could be solubilized only with the nonionic detergent MEGA-9 in the presence of high salt concentrations (29, 30).

We constructed hTERT tagged at the N terminus with FLAG because C-terminal tagging with the hemagglutinin epitope tag prevents the biological activity of telomerase (9). The FLAG epitope seemed to not be exposed under native conditions because the soluble recombinant FLAG-hTERT was not recovered by immunoprecipitation with anti-FLAG antibody M2, but FLAG-hTERT fractionated by SDS-PAGE was successfully recognized by Western blotting using the same antibody. Although the other FLAG-tagged hTERT was successfully immunoprecipitated with the anti-FLAG antibody in a previous study, the latter recombinant hTERT was tagged at the C terminus (7).

Here, we clearly showed that the purified recombinant FLAG-hTERT retained the catalytic activity of human telomerase detected in different assays. Our results are the first indicating that hTERT and hTR are the minimal essential components for telomerase activity in vitro. Efficient complex formation was detected by EMSA assay when equimolar amounts of hTR and FLAG-hTERT were mixed, and the maximum telomerase activity was observed with the reconstituted components. However, for detection by TRAP assay, higher amounts of the reconstituted telomerase were required. The active form of telomerase may require proper folding or multimerization of the complex, which can be facilitated by auxiliary factors or complexes. The telomerase activity of the reconstituted components was augmented by more than 30-fold in the presence of telomerase-negative TIG3 cell extract. A similar stimulatory effect of cell extract was also observed with another telomerase-negative cell line, IMR-90, and in the complementation assay in vitro (data not shown). The telomerase-negative extract might contain some component(s) capable of stimulating telomerase activity reconstituted in vitro. Holt et al. (20) proposed that Hsp90 and p23 are essential components for telomerase activity reconstituted in vitro using hTR and hTERT synthesized with rabbit reticulocyte extract. Our results showed that GA partially inhibited the stimulated telomerase activity in the presence of the cell extract but did not affect the telomerase activity of the two components reconstituted in vitro. The partial inhibition of GA on the stimulated telomerase activity was consistent with their results, although we demonstrated that purified hTERT and hTR exhibited telomerase activity. Active telomerase may be detected only when hTERT and hTR are present at higher concentrations. The GA resistant stimulatory effect of the TIG3 extract implied the presence of some component(s) in addition to Hsp90 and p23 required for the reconstituted telomerase. TERT and TR have been reported to interact with other components to form a ribonucleoprotein complex. The component(s) in ribonucleoprotein are different in different organisms, e.g. p43 in Euploites (38, 39), p80 and p95 in Tetrahymena (21, 40–42), and Ext1, Ext3, and Cdc13 in Saccharomyces cerevisiae (43–45). These components may have roles in active complex formation or catalytic activity of telomerase. The assay using reconstituted telomerase may serve as an experimental tool to identify components with stimulatory effects on telomerase.

The baculovirus expression system and the purification method reported here will be useful not only for studies to determine the molecular functions and structure of telomerase but also for development of strategies of drug design targeting hTERT, which is a good strong candidate to prevent cell senescence and carcinogenesis.