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メタデータ	言語: eng 出版者: 公開日: 2017-10-05 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/14551

Human Telomerase Exists in Two Distinct Active Complexes *In Vivo*

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Received November 30, 2006; accepted February 13, 2007; published online March 23, 2007

Telomerase, a stable complex of telomerase reverse transcriptase (TERT) and template RNA (TERC), is responsible for telomere maintenance. During purification trials of recombinant human telomerase of the two components reconstituted in insect cells, we identified two complexes of human telomerase of molecular masses 680 and 380 kDa, both of which retain telomerase activity *in vitro*. We show here that the former complex does not include Hsp90 (heat shock protein 90) and its telomerase activity is resistant to Hsp90 inhibitors, whereas the latter contains Hsp90 and its telomerase activity is sensitive to Hsp90 inhibitors. N-terminal of FLAG-hTERT in the former is exposed, as this complex was efficiently purified with anti-FLAG M2 affinity resin. We also identified two different telomerase complexes in HeLa cells, in addition to ectopically expressed hTERT. Most of endogenous hTERT and FLAG-hTERT was detected around 680 kDa. These two complexes in HeLa cells have the same properties as their respective reconstituted telomerases. The unstable property of the telomerase complex with Hsp90, especially in the presence of Hsp90 inhibitors, was due to proteasome-mediated degradation of hTERT, since proteasome inhibitors prevented hTERT degradation *in vivo*. To our knowledge, this is the first demonstration of two distinct active complexes of human telomerase ectopically expressed in insect and mammalian cells.

Key words: GA, Hsp90, hTERC, hTERT.

Abbreviations: GA, geldanamycin; Hsp90, heat shock protein 90; hTERC, human telomerase RNA component; hTERT, human telomerase reverse transcriptase subunit; TRAP, telomere repeat amplification protocol.

Telomeres are physical ends of eukaryotic chromosomes, which consist of long tandem repeats (TTAGGG), and are responsible for maintaining chromosomal stability and integrity (1, 2). Telomerase is a ribonucleoprotein (RNP) composed of human telomerase reverse transcriptase (hTERT), the catalytic subunit, and its template RNA (hTERC) (2–5). As direct evidence that telomere erosion plays a major role in cellular senescence, ectopic expression of hTERT in normal human cells with endogenous hTERC resulted in activation of telomerase, stabilization of telomere lengths and extension of cellular life span (6, 7).

The homeostasis of mammalian telomeres is regulated by a number of telomere-binding proteins. These proteins form a physical complex of molecular mass 550–1000 kDa (8–10). Among these proteins, TRF1 and TRF2 directly bind double-stranded telomere DNA and interact with a number of proteins to maintain telomere length and structure (11, 12).

hTERT is the rate-limiting factor for telomerase activity both biologically and biochemically (6, 13–16). Although telomerase activity is regulated by hTERT gene expression (17), it is also regulated post-translationally.

Post-translational modification of telomerase may also involve the interaction of hTERT with telomerase-associated proteins, chaperones and polypeptide modifiers (18, 19). Heat shock protein 90 (Hsp90), a molecular chaperone, has been reported to be functionally associated with hTERT, and both Hsp90 and p23 are required for efficient telomerase assembly *in vitro* and *in vivo*, although both of these proteins, as well as other chaperones, are associated with hTERT in its unassembled or inactive form (20, 21). The Hsp90 inhibitor, geldanamycin (GA), has been shown to prevent the assembly of active telomerase, as well as to promote ubiquitination and the proteasome-mediated degradation of hTERT (20, 22). These reports clearly demonstrate the involvement of Hsp90 in telomerase assembly and function.

We previously identified GA-resistant telomerase activity using *in vitro* reconstituted telomerase with partially purified FLAG-hTERT expressed in insect cells and *in vitro* transcribed hTERC (13). We further purified the recombinant FLAG-hTERT and found that its *in vitro* telomerase activity was reduced as it was purified (data not shown). We therefore attempted to express recombinant telomerase by reconstituting hTERT and hTERC in insect cells.

Here we report the properties of reconstituted telomerase in the lysate of insect cells. We found that

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this activity could be separated into two different complexes, of molecular masses 680 (peak I) and 380 kDa (peak II), using biochemical fractionation methods. We also detected similar complexes, of approximate molecular masses 680 and 400 kDa, in the lysates of HeLa cells and HeLa cells stably expressing FLAG-hTERT. The telomerase activity of the former complex was resistant to Hsp90 inhibitors, whereas the activity of the latter complex was sensitive to Hsp90 inhibitors. To our knowledge, this is the first demonstration of two distinct active complexes of human telomerase ectopically expressed in insect and mammalian cells.

EXPERIMENTAL PROCEDURES

Baculovirus Expression Vectors—The FLAG-hTERT baculovirus expression vector, pBKM-FLAG-hTERT, has been described (13, 23). The hTERC cDNA was prepared by PCR using pGRN164 as a template with the primer set, hTERCFor, which contains artificial *NcoI* site, and hTERCRev, which contains artificial *BamHI* site. The 5' processing ribozyme cDNA was prepared by annealing of following oligos: RibFor (5'-GGATCCGTCGACGGATC TAGATCCGTCCTGATGAGTCCGTGAGGACGAAACGG ATCC-3'), and RibRev (5'-GGATCCGTTTCGTCCTCAG GACTCATCAGGACGGATCTAGATCCGTGACGGATC C-3'), each of which contains a *BamHI* site. SV40 polyA signalling sequence was prepared by PCR using pNKZ-FLAG-hTERT as a template with the primer set, SVFor, which contains artificial *BamHI* site, and SVRev, which contains artificial *BglII* site. The hTERC-PolyA baculovirus expression vector, pBKM-hTERC-PolyA, was constructed by inserting the *NcoI*-*BamHI* fragment of hTERC cDNA and the *BamHI*-*BglII* fragment of the SV40 polyA cDNA into the *EcoRV* and *BglII* sites of the pVL1393 baculovirus transfer vector. The hTERC-Rib baculovirus expression vector, pBKM-hTERC-Rib, was constructed by inserting the *NcoI*-*BamHI* fragment of hTERC cDNA, the *BamHI*-*BamHI* fragment of 5' processing ribozyme cDNA and the *BamHI*-*BglII* fragment of SV40 polyA cDNA into the *EcoRV* and *BglII* sites of the pVL1393 baculovirus transfer vector.

Insect Cell Lines and Generation of Recombinant Baculoviruses—Sf9 and High5 insect cell lines were cultured as described, and recombinant baculoviruses were prepared as described (13, 23). High titre suspensions of BVKM-FLAG-hTERT, BVKM-hTERC-PolyA and BVKM-hTERC-Rib ($>1.0 \times 10^7$ plaque-forming units/ml) were used for infection of High5 cells.

Plasmid Construction—The hTERC-Rib phagemid expression vector pGRN-hTERC-Rib was constructed by inserting the *BamHI*-*BamHI* fragment of 5' processing ribozyme cDNA into the *BamHI* site of the pGRN164 phagemid vector. FLAG-GST cDNA was prepared by PCR using pNKZ-GST as a template with the primer set, FLAGFor, which contains artificial *FbaI*-FLAG-*EcoRI* sites, and FLAGRev, which contains artificial *XhoI*-*Sall* sites. The FLAG-GST retrovirus expression vector pBabe-puro-FLAG-GST was constructed by inserting the *FbaI*-*XhoI* fragment of FLAG-GST cDNA into the *BamHI*-*Sall* sites of the pBabe-puro vector. The GST

cDNA of pBabe-puro-FLAG-GST was replaced by the *EcoRI*-*Sall* fragment containing hTERT cDNA resulting in retrovirus delivery vector, pBabe-puro-FLAG-hTERT.

Mammalian Cell Lines and Retrovirus Delivery—HeLa (human carcinoma of cervix) and 293T cells (human kidney cell lines) were cultured by standard methods in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; CELLECT R GOLD). Recombinant retrovirus packaging, infection and selection of FLAG-hTERT expressing stable transformations of HeLa cells were performed essentially as described (24).

Partial Purification of *in vivo* Reconstituted Telomerase Complex from Insect Cells by Anti-FLAG M2 Affinity Resin—For expression of *in vivo* reconstituted telomerase, High5 cells were co-infected with both BVKM-FLAG-hTERT and BVKM-hTERC-Rib (or BVKM-hTERC-PolyA) at a multiplicity of infection (MOI) ratio of 2.0:4.0. The cells were cultured for 5 days at 27°C, scraped off the plates and centrifuged at $2500 \times g$ for 10 min. A total of 5×10^7 High5 cells were suspended in 5 ml of buffer A [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM 2-mercaptoethanol, 20% glycerol, 0.1% Triton, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin and 10 mM aprotinin] and the suspension was sonicated three times for 10 s each. The lysate was centrifuged for 10 min at $10,000 \times g$. The supernatant (S1) was removed and detergent concentration of S1 was adjusted to 0.5% by 20% Triton-X100 (S2). S2 was mixed with 500 μ l of 50% slurry of anti-FLAG M2 affinity resin (Sigma), and rotated for 4 h at 4°C. After 1 min centrifugation at $4500 \times g$, the supernatant was removed, and the resin with bound proteins was washed three times with buffer A. 500 μ l of buffer B (buffer A containing, 100 μ g/ml FLAG peptide) was added to washed resin and the mixture was rotated for 30 min at 4°C. After 1 min centrifugation at $4500 \times g$, the supernatant (M2-bound fraction 1) was recovered. Moreover, 500 μ l of buffer B was added to washed resin and the mixture was rotated for 30 min at 4°C. After 1 min centrifugation at $4500 \times g$, the supernatant (M2-bound fraction 2) was recovered. M2-bound fractions 1 and 2 were mixed (M2-bound fractions). FLAG-tagged hTERT protein was quantified by SDS-PAGE on 8% polyacrylamide gels, and gels were stained with Coomassie Brilliant Blue (CBB).

Northern Blot Analysis—10 μ g of total RNA from each infected High5 cells were run for each lanes, electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N⁺ membrane (Amersham Biosciences), crosslinked by UV for 2 min and subjected for hybridization. Membrane was probed with ³²P-labelled *in vitro* transcribed hTERC (full-length). Hybridization was performed according to the manufacturer's recommendations (Amersham Biosciences), and the autoradiograms were scanned on a BAS2000 bio-imaging analyser (FUJIX).

Preparation of Cell Lysates, Immunoprecipitation—Cells were harvested, washed with PBS(−) and sonicated in buffer A. The lysate of 5×10^6 cells was diluted 10-fold in buffer A and incubated at 4°C for 3 h with 10 μ l of GammaBind G resin containing pre-bound anti-FLAG

M2 (Sigma), followed by three washes with buffer A. The bound proteins were separated by SDS-PAGE and visualized by western blotting.

Fractionation of Cell Lysates—M2-bound fractions or the lysate of 5×10^7 cells were fractionated on HiLoad 16/60 Superdex 200 pg gel filtration columns (Amersham Biosciences) in running buffer A without protein inhibitors. The resulting fractions were resolved by SDS-PAGE and probed with various antibodies. For the calibration of the column, the high molecular weight calibration kit from Amersham Biosciences was used.

Antibodies and Western Blot Analysis—For western blot analysis, the total cell lysate and their fractions from gel filtration column were separated by SDS-PAGE and transferred to nitrocellulose membrane and probed with anti-FLAG M2, anti-Hsp 90 α / β (Santa Cruz Biotechnology) or anti-TERT 2C4 (Novus Biologicals, Inc.) primary antibodies, followed by incubation with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (Amersham Biosciences) for anti-FLAG M2 and anti-Hsp 90 α / β antibodies, or horseradish peroxidase conjugated goat anti-mouse IgM secondary antibody (Pierce) for anti-TERT 2C4 antibody.

Telomerase Activity Assays—Telomerase activity was measured by two different methods. (i) TRAP assay, a polymerase chain reaction-based telomere repeat amplification protocol assay (TRAP assay), was carried out with TRAPEZE kit (Intergen Co. Ltd) according to the manufacturer's protocol. Each reaction product was amplified in the presence of a 36-bp internal telomerase assay standard (ITAS). The polymerase chain reaction products were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR Green I (Molecular Probes Co. Ltd). (ii) TRAP enzymelinked immunosorbent assay (ELISA), telomerase activity was quantitatively measured using a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd) according to the manufacturer's protocol.

Real-time Quantitative RT-PCR—Real-time quantitative RT-PCR was performed for hTERT using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primers and the TaqMan probe for hTERT were designed using the primer design software Primer ExpressTM (Applied Biosystems). The forward primer for hTERT was 5'-GGTGGTGGCCATTTTTC GTC-3'. The reverse primer for hTERT was 5'-CTAGAATGAACGGTGAAGGC-3'. The TaqMan probe for hTERT was 5'-CGCGCTGTTTTTC TCGCTGACTTTC-3' (25). The probe was labelled with a reporter fluorescent dye (6-carboxy-fluorescein) at the 5' end and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine) at the 3' end. The PCR conditions were 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

RESULTS

Partial Purification Trials of *in vivo* Reconstituted Telomerase Complex from Insect Cells by M2 Affinity Resin—We previously reported that telomerase activity

reconstituted *in vitro* using partially purified FLAG-hTERT expressed in insect cells and *in vitro* transcribed hTERC was resistant to GA (13). During further purification of the recombinant FLAG-hTERT, we found that the highly purified FLAG-hTERT preparations reconstituted lower telomerase activity *in vitro* (data not shown). The peak fractions of FLAG-hTERT from heparin cellulose column chromatography, in which FLAG-hTERT was estimated to be ~70%, failed to reconstitute any telomerase activity *in vitro* in the presence of hTERC. We therefore attempted to co-express FLAG-hTERT and hTERC in insect cells and to purify *in vivo* reconstituted telomerase. For this purpose, we constructed baculovirus delivery plasmids of hTERC-expression baculovirus. To mimic as close as possible the intrinsic transcription pathway for TERC, we chose Polyhedrin promoter and a polyadenylation (polyA) tailing to transcribe hTERC in insect cells (BVKM-hTERC-PolyA). Once hTERC transcription is successfully initiated and terminated, we prefer to eliminate the polyA tail, since it was possible that the factors complexed with the polyA tail may interfere with high-grade purification and further biochemical studies. Additionally, there is no report showing that polyA-tailed hTERC exists in human cells. We attempted to remove the polyA tail by splicing *in vivo* using a self-cleaving hammer-head ribozyme, or by cleavage *in vitro* using targeting ribozyme. The polyA tail could not be cleaved *in vitro* using targeting ribozyme. To ensure function of self-cleaving hammerhead ribozyme, 5'-processing ribozyme was inserted into the pGRN164 phagemid vector after the hTERC coding sequence and 100 bp upstream to the digestion site that was used to linearize the vector to prepare a template for *in vitro* transcription. In this case, two bands instead of one appear on a gel electrophoresis as a product of *in vitro* transcription due to ribozyme self-splicing (Fig. 1A, compare lanes 2 and 3). The hTERC origin of the resulting bands was identified by northern blot with hTERC probe (Fig. 1A, lanes 4–6). Having confirmed the effectiveness of 5'-processing ribozyme, we constructed a recombinant baculovirus producing hTERC with a self-cleaving ribozyme at the 5'-end. The schematic presentation of baculovirus transfer vector expressing hTERC under the control of polyhedrin promoter is shown in Fig. 1B. SV40 polyA signal was inserted downstream of hTERC coding sequence. The 5'-processing ribozyme was inserted into the baculovirus transfer vector after the hTERC coding sequence and before SV40 polyA signal (pBKM-hTERC-Rib). High5 cells were infected with BVKM-FLAG-hTERT and BVKM-hTERC-PolyA or BVKM-hTERC-Rib. Expression of the intact hTERC was detected by northern blot only when the hTERC- and FLAG-hTERT expression viruses were co-infected and the size of hTERC produced by BVKM-hTERC-Rib was similar to that of *in vitro* transcribed hTERC (Fig. 1C, lanes 1 and 3). However, hTERC produced by BVKM-hTERC-PolyA had larger size due to the polyA incorporation (Fig. 1C, lane 2). The hTERC RNA could not be detected by northern blotting, when hTERC-expression virus alone, either BVKM-hTERC-PolyA or BVKM-hTERC-Rib, was infected, suggesting that hTERC RNA might be degraded in

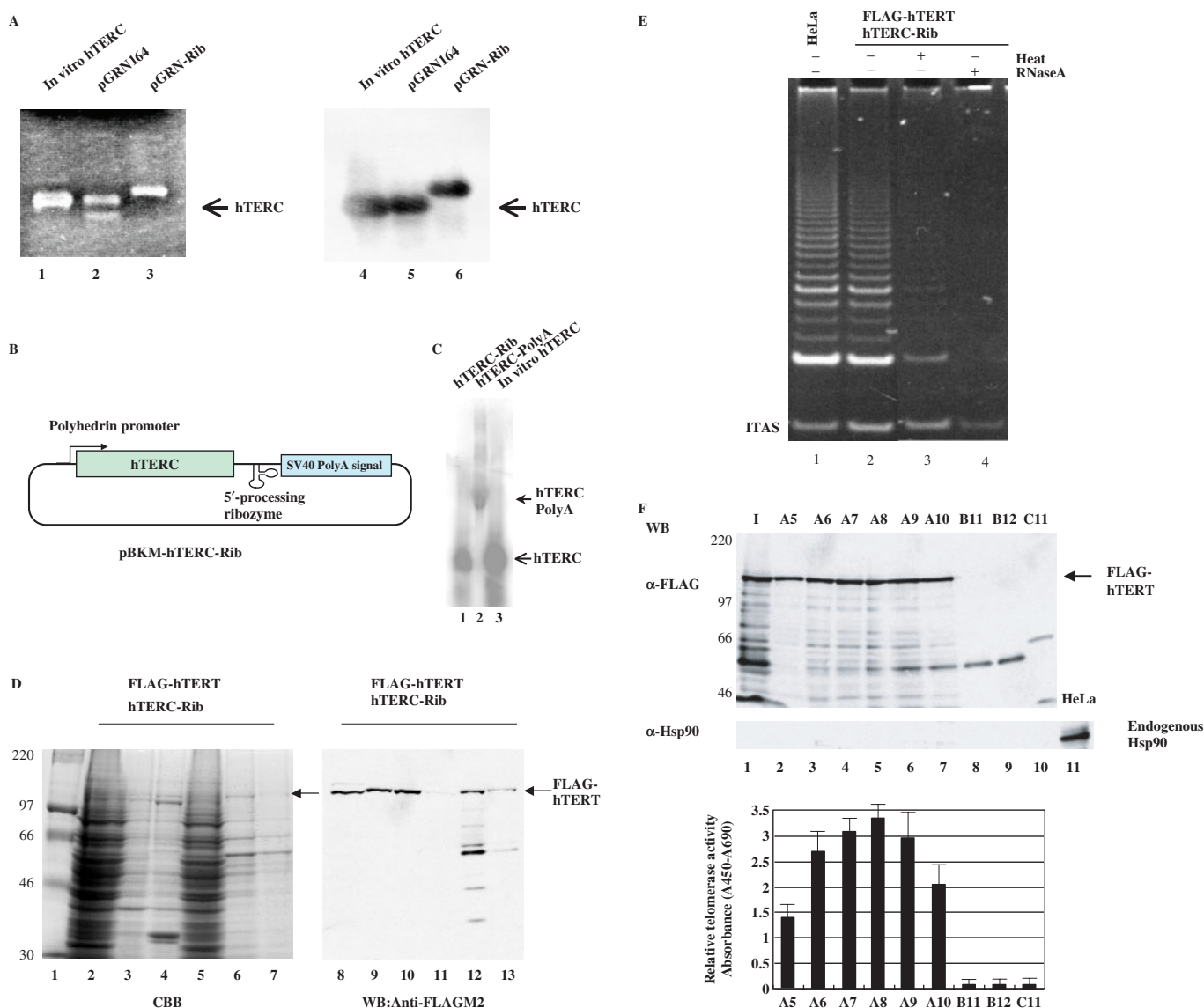


Fig. 1. Partial purification trials of *in vivo* reconstituted telomerase complex in insect cells by M2 affinity resin. (A) pGRN164 and pGRN-Rib were linearized by digestion with *Pvu*I and subjected to *in vitro* transcription (pGRN164 and pGRN-Rib, respectively). pGRN164 was linearized by digestion with *Fsp*I and subjected to *in vitro* transcription (*in vitro* hTERC). *In vitro* transcribed RNAs were fractionated on denaturing agarose-gel electrophoresis and detected by either EtBr (left panel) or northern blot with hTERC probe (right panel). Lanes 1 and 4, *in vitro* hTERC; lanes 2 and 5, pGRN164; lanes 3 and 6, pGRN-Rib. (B) Schematic presentation of baculovirus transfer vector expressing hTERC under the control of the polyhedrin promoter. The 5' processing ribozyme was inserted between hTERC coding sequence and the SV 40 polyadenylation (PolyA) signal. (C) Total RNAs isolated from High5 cells infected with both BVKM-FLAG-hTERT (FLAG-hTERT) and BVKM-hTERC-PolyA (hTERC-PolyA) or BVKM-hTERC-Rib (hTERC-Rib) were assayed by northern blotting with ³²P-labelled hTERC as a probe. Lane 1, hTERC-Rib; Lane 2, hTERC-PolyA; Lane 3, *in vitro* hTERC. (D) High5 cells were infected with recombinant baculoviruses BVKM-FLAG-hTERT (FLAG-hTERT) and BVKM-hTERC-Rib (hTERC-Rib) at MOI ratio of 2.0:4.0, and lysate of infected cells were prepared and subjected to affinity chromatography with anti-FLAG M2 resin (see 'EXPERIMENTAL PROCEDURES'). Each sample was separated by 8% SDS-PAGE, and gels were stained with CBB and subjected to

western blot analysis with anti-FLAG M2 antibody. Lanes 2 and 8, total lysate of the infected High5 cells; lanes 3 and 9, supernatant S1; lanes 4 and 10, M2 unbound fractions; lanes 5 and 11, anti-FLAG M2 resin; lanes 6 and 12, M2-bound fraction 1; lanes 7 and 13, M2-bound fraction 2. The arrows indicate FLAG-hTERT. (E) Two microlitres of M2-bound fractions and HeLa cell lysate were subjected to TRAP assay. Bound fractions were treated by incubating at 85°C for 10 min, or treated with 100 µg/ml RNase A at 30°C for 15 min. Lane 1, HeLa; lane 2, M2-bound fraction (no treatment); lane 3, M2-bound fraction treated by heat; lane 4, M2-bound fraction by RNase. ITAS represents the internal telomerase assay standard. (F) M2 bound fractions were fractionated on HiLoad 16/60 Superdex 200pg gel filtration columns in running buffer containing 200mM NaCl and 0.1% Triton-X100. The molecular sieving peak corresponded to approximate molecular masses of 680kDa. The fractions were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-FLAG M2 or anti-Hsp90 antibodies. Lane 1, as 'I' (= input) is M2-bound fractions; lane 2, fraction No. A5; lane 3, fraction No. A6; lane 4, fraction No. A7; lane 5, fraction No. A8; lane 6, fraction No. A9; lane 7, fraction No. A10; lane 8, fraction No. B11; lane 9, fraction No. B12; lane 10, fraction No. C11; and lane 11, as control, HeLa cell lysate. The arrows indicate FLAG-hTERT. Relative telomerase activity of fractions was measured by TRAP ELISA.

insect cells in the absence of hTERT (data not shown). Insect cell lysate expressing FLAG-hTERT and hTERC-Rib was fractionated using anti-FLAG M2 resin. Most of FLAG-hTERT could be eluted from anti-FLAG M2 resin with the buffer containing FLAG peptide (see 'EXPERIMENTAL PROCEDURES' and Fig. 1D). We found that the fractionation behaviour of two types of telomerase with hTERC-PolyA and hTERC-Rib expressed in insect cells was almost identical, except the telomerase consisting of FLAG-hTERT and hTERC-PolyA bound more efficiently to polyU resin than did the telomerase consisting of FLAG-hTERT and hTERC-Rib (data not shown). The partially purified *in vivo* reconstituted telomerase complex exhibited comparable telomerase activity to that of endogenous telomerase from HeLa cells detected by the TRAP assay (Fig. 1E, lanes 1 and 4). This activity was abolished by heat treatment or RNase treatment (Fig. 1E, lanes 2 and 3). Since the expression level, fractionation behaviour and activity of the two types of telomerase reconstituted *in vivo* were almost identical, we assayed the properties of recombinant telomerase consisting of FLAG-hTERT and hTERC-Rib. The expression level of FLAG-hTERT in High5 cells infected with BVKM-FLAG-hTERT and BVKM-hTERC-Rib at MOI of 2.0 and 4.0 (see 'EXPERIMENTAL PROCEDURES'), respectively, was higher than those obtained from other combinations of MOI (data not shown). For the purpose of additional steps towards higher purity, M2 bound fractions of insect cells expressing FLAG-hTERT and hTERC-Rib was subjected to gel filtration on Superdex 200 pg column. The fractions were assayed for telomerase activity and the elution profile was compared with those of the marker proteins of known molecular mass. Telomerase activity of M2 bound FLAG-hTERT peaks in fraction No. A8 (Fig. 1F, TRAP ELISA), which corresponds to an approximate molecular mass of 680 kDa (Fig. 1F, upper panel). We could not detect FLAG-hTERT in other peaks (fraction No. B11 and B12 were of molecular mass 250 kDa, No. C11 was of molecular mass 150 kDa). We previously reported that telomerase activity reconstituted *in vitro* using partially purified FLAG-hTERT expressed in insect cells and *in vitro* transcribed hTERC was resistant to GA, in which Hsp90 was not detected (13). Similarly, we could not detect Hsp90 in the M2-bound fraction and thus gel filtration fractions (Fig. 1F, lower panel).

Fractionation of Insect Cell Lysate Expressing Recombinant Telomerase—For initial purification trials, we applied gel filtration chromatography. When the soluble fraction of the lysate of insect cells expressing FLAG-hTERT and hTERC-Rib was subjected to gel filtration, FLAG-hTERT was distributed in two peaks, of around 680 kDa (peak I) and 380 kDa (peak II) in parallel with the distribution of telomerase activity (Fig. 2A, upper panel, and B). FLAG-hTERT was not distributed, and telomerase activity was not detected in the fractions between peak I and peak II (fraction No. B1, B2), confirming the specificity of distribution pattern. Relative amount of hTERT in peak II to peak I was ~0.44 (Fig. 2A, upper panel), and that of telomerase activity was ~0.84 which is higher than that of hTERT (Fig. 2B). Anti-FLAG M2 antibody recognized two bands

in peak I and peak II (Fig. 2A, upper panel arrows and asterisks indicated). The slower migrating band was likely a cross-reacting protein of the insect cells, as it was also detected in lysate of non-infected High5 cells (Fig. 2A, lower panel arrows and asterisks indicated). Furthermore, there was no telomerase activity in all fractions of non-infected High5 cells (data not shown).

Most of the telomerase activity and FLAG-hTERT were recovered when peak I fractions were subjected to anti-FLAG M2-resin affinity chromatography in the presence of 0.5% of Triton-X100, but telomerase activity and FLAG-hTERT in peak II fractions could not be trapped by affinity chromatography with anti-M2 resin in the presence of 0.5% of Triton-X100 (Fig. 1F, data not shown).

Distribution of hTERC in peak I and peak II was quantified by real time RT-PCR using the primer set for hTERC. The relative amount of hTERC in peak II to peak I was ~0.39 (Fig. 2C) that is similar to the relative amount of hTERT measured by western blot.

Next we analysed the distribution of endogenous Hsp90. Hsp90 was exclusively distributed in peak II but not in peak I (Fig. 2A, middle panel lanes 7–10). Endogenous Hsp90 was found to be complexed with FLAG-hTERT in peak II, as Hsp90 was recovered in immunoprecipitates of anti-FLAG antibody in the presence of 0.1% Triton-X100 (Fig. 2D, lane 4).

These results indicate that there are two different complexes of the reconstituted telomerase in insect cells, the complex without Hsp90 in peak I and the complex with Hsp90 in peak II.

Characterization of Two Complexes Separated by a Molecular Sieving in Insect Cells—To further characterize two different telomerase complexes, we tested whether GA affects two complexes *in vivo*. FLAG-hTERT expressing insect cells was treated for 4 h with varying concentration of GA, and soluble lysates were prepared and subjected to gel filtration. Again, two complexes of FLAG-hTERT and telomerase activity were observed at both peaks I and II. GA did not affect the telomerase activity and the amount of FLAG-hTERT in peak I treated with varying concentration of GA (Fig. 3A). In contrast, incubation with GA reduced the telomerase activity of peak II fractions in a dose-dependent manner, accompanied by a dose-dependent decrease of FLAG-hTERT in peak II (Fig. 3B). GA had little effect on the recovery of Hsp90, suggesting that the reduction in FLAG-hTERT was not due to changes in Hsp90 levels (Fig. 3B). Similar results were obtained by treatment with novobiocin (26), another Hsp90 inhibitor.

These results suggest that telomerase activity in insect cell extracts is found in two different fractions, one is resistant, and the other is sensitive to Hsp90 inhibitors.

Fractionation of HeLa Cell Lysate—To examine whether there were also two different telomerase complexes in mammalian cells, HeLa cell lysate was fractionated by molecular sieving. Two different hTERT complexes were detected at around 680 kDa (peak I) and 400 kDa (peak II) (Fig. 4A, upper panel) that was slightly larger than peak II of insect cells. Endogenous hTERT

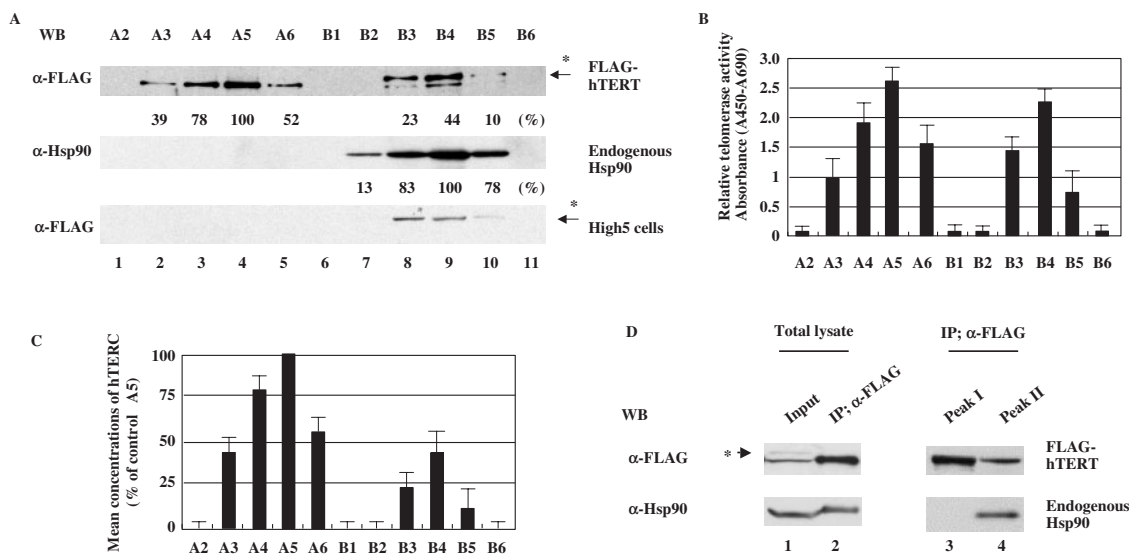


Fig. 2. Fractionation of the insect cell lysate expressing recombinant telomerase. (A) High5 cells were infected with recombinant baculoviruses BVKM-FLAG-hTERT and BVKM-hTERT-Rib at MOI ratio of 2.0:4.0, and cell lysate was fractionated on HiLoad 16/60 Superdex 200pg gel filtration columns in running buffer containing 200mM NaCl and 0.1% Triton-X100. The telomerase active peaks corresponded to approximate molecular masses of 680kDa (peak I) and 380kDa (peak II). The fractions in peaks I and II were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-FLAG M2 or anti-Hsp90 antibodies. Non-infected High5 cell lysate was fractionated on the same column in the same running buffer. Fraction samples were subjected to western blot analysis using anti-FLAG M2 antibody. Lane 1, fraction No. A2; lane 2, fraction No. A3; lane 3, fraction No. A4; lane 4, fraction No. A5; lane 5, fraction No. A6; lane 6, fraction No. B1; lane 7, fraction No. B2; lane 8, fraction No. B3; lane 9, fraction No. B4; lane 10, fraction No. B5; and lane 11, fraction No. B6. The arrows and

asterisks indicate the band in insect cell lysates cross-reactive with anti-FLAG M2 antibody. (B) Relative telomerase activity of the fractions in peaks I and II was measured by TRAP ELISA. (C) hTERT in peaks I and II was quantified by real time RT-PCR (see 'EXPERIMENTAL PROCEDURES'). Relative quantity of fractions was expressed as a percentage of max concentration in fraction No. A5. (D) Total lysate and peak I and peak II fractions from High5 cells infected with the recombinant baculoviruses BVKM-FLAG-hTERT and BVKM-hTERT-Rib were immunoprecipitated with anti-FLAG M2 resin. The bound proteins were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-FLAG M2 or anti-Hsp90 antibodies as indicated. Western blot analysis of total lysate is equal to 5% of the input shown in lane 1 (= input). Lane 2, total lysate, IP; anti-FLAG antibody; lane 3, peak I fractions, IP; anti-FLAG antibody; and lane 4, peak II fractions, IP; anti-FLAG antibody. The arrows+asterisks indicate the band in insect cell lysate cross-reactive to anti-FLAG M2 antibody.

was much distributed in peak I (Fig. 4A, lanes 2–5). Relative amount of hTERT in peak II to peak I was ~ 0.39 that is almost similar to the relative amount of hTERT measured by real time RT-PCR (0.32) (Fig. 4B, real time RT-PCR). Distribution of telomerase activity of the samples was quantified by TRAP ELISA, and relative activity of telomerase activity in peak II to peak I was ~ 0.85 (Fig. 4B, TRAP ELISA) that is almost similar to the relative amount of telomerase activity in insect cells (Fig. 2B). Similarly to insect cells, endogenous Hsp90 was distributed in peak II but not in peak I (Fig. 4, lower panel). These results indicate that HeLa cells contain two different hTERT complexes, one with and one without Hsp90.

Fractionation of HeLa Cell Lysate Stably Expressing FLAG-hTERT—Since most of the endogenous hTERT in HeLa cells was distributed in peak I, whereas most of the FLAG-hTERT in insect cells was also present in peak I (Figs 2 and 4), we examined the distribution of ectopically expressed hTERT in HeLa cells. HeLa cells were stably transfected with the FLAG-hTERT expression virus (vBabe-puro-FLAG-hTERT), and the cell lysate was fractionated by molecular sieving. We found that the distribution of endogenous hTERT was similar

to that in HeLa cell lysate (Figs 4 and 5 upper panel); furthermore, ectopically expressed FLAG-hTERT was mainly distributed in peak I (Fig. 5, middle panel), similar to finding in insect cells (Fig. 2). The distribution patterns of endogenous hTERT, FLAG-hTERT and hTERT were almost identical, and relative activity of telomerase was also similar to that from HeLa cells (Figs 4B and 5B). As in the case of HeLa cell extracts, Hsp90 protein was detected only in peak II fractions (Fig. 5A, lower panel lanes 7–10). Incubation of transfected total cell lysate with anti-FLAG antibody immunoprecipitated endogenous Hsp90 (Fig. 5C, lane 2). As observed for the peak II complex in insect cells, endogenous Hsp90 was similarly recovered in anti-FLAG immunoprecipitates from the peak II complex in HeLa cells stably expressing FLAG-hTERT (Fig. 5C, lane 4).

These results suggest that, similarly to the recombinant telomerase reconstituted in insect cells, there are also two different telomerase complexes in HeLa cells stably expressing FLAG-hTERT, the complex without Hsp90 in peak I and the complex with Hsp90 in peak II.

Characterization of the Complexes in HeLa Cells Stably Expressing FLAG-hTERT—To determine whether the hTERT complexes in HeLa cells stably expressing

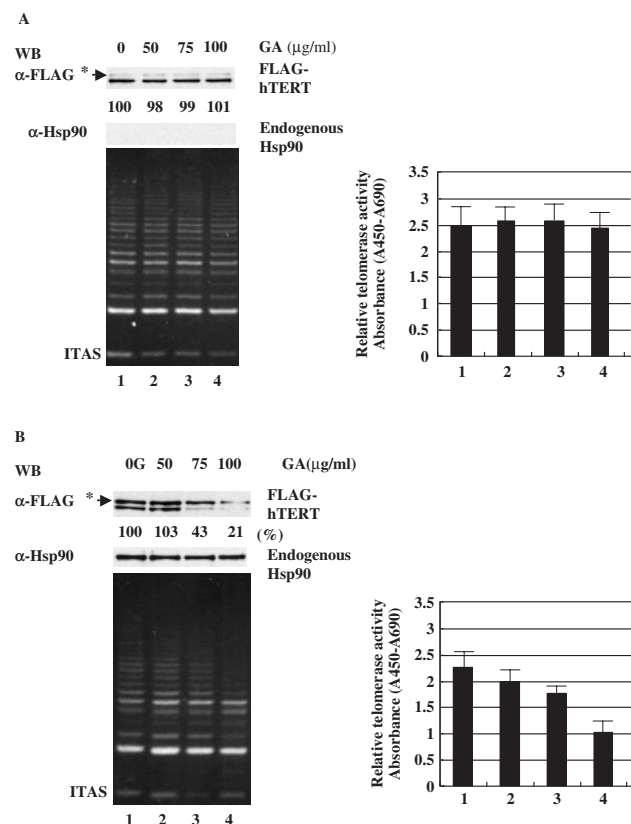


Fig. 3. Characterization of the two telomerase complexes in insect cells. (A), (B) High5 cells infected with the recombinant baculoviruses BVKM-FLAG-hTERT and BVKM-hTERT-Rib were treated for 4h with various GA concentration indicated, and each cell lysate was fractionated on HiLoad 16/60 Superdex 200pg gel filtration columns in running buffer containing 200mM NaCl and 0.1% Triton-X100. Peak I (A) and peak II (B) fractions were separated by 8% SDS-PAGE and, subjected to western blotting using anti-FLAG M2 or anti-Hsp90 antibodies as indicated. The arrows+asterisks indicate the band in insect cell lysate cross-reactive to anti-FLAG M2 antibody. The fractions of peak I and peak II (~20 ng protein/each fraction) with varying concentration of GA were subjected to TRAP and TRAP ELISA. (A), (B) Lane 1, GA at 0 μg/ml; lane 2, GA at 50 μg/ml; lane 3, GA at 75 μg/ml; lane 4, GA at 100 μg/ml.

FLAG-hTERT were sensitive to GA, HeLa cells stably expressing FLAG-hTERT were treated for 4h with varying concentrations of GA. GA treatment reduced the amounts of endogenous hTERT and FLAG-hTERT, as well as telomerase activity, in a dose-dependent manner (Fig. 6A). To determine whether GA-induced down-regulation of hTERT was due to proteasome-mediated degradation *in vivo* (22), we pre-treated HeLa cells stably expressing FLAG-hTERT with MG132, a proteasome inhibitor, for 2h before GA treatment. We found that the GA-induced reduction of both endogenous hTERT and FLAG-hTERT was mostly prevented by pre-treatment with MG132 (Fig. 6A, lane5), indicating that GA reduction of endogenous hTERT and FLAG-hTERT is due to proteasome-mediated degradation *in vivo*. Furthermore, the

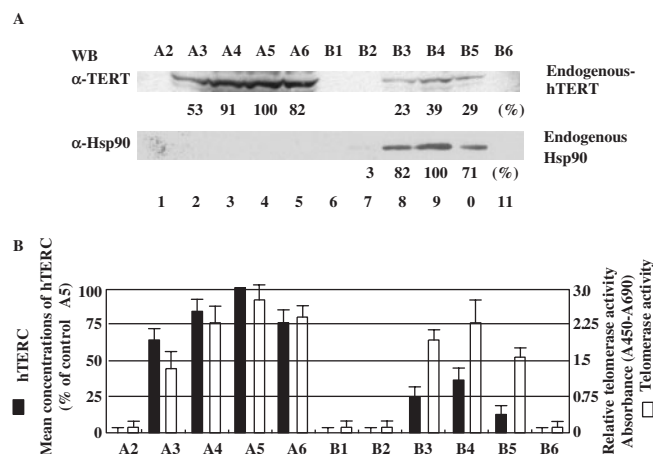


Fig. 4. Fractionation of HeLa cell lysate. (A) HeLa cell lysate was fractionated on HiLoad 16/60 Superdex 200pg gel filtration columns in running buffer containing 200mM NaCl and 0.1% Triton-X100. The telomerase activity peaks were at approximate molecular masses of 680 kDa (peak I) and 400 kDa (peak II). The fractions in peaks I and II were separated by 8% SDS-PAGE and subjected to Western blot analysis using anti-TERT (2C4) or anti-Hsp90 antibodies as indicated. Lane 1, fraction No. A2; lane 2, fraction No. A3; lane 3, fraction No. A4; lane 4, fraction No. A5; lane 5, fraction No. A6; lane 6, fraction No. B1; lane 7, fraction No. B2; lane 8, fraction No. B3; lane 9, fraction No. B4; lane 10, fraction No. B5; lane 11, fraction No. B6. (B) The quantification of hTERT in HeLa cells was measured by real time RT-PCR as shown in Fig. 2C. Relative telomerase activity of the fractions in peaks I and II (fraction No. A2–B6) was measured by TRAP ELISA.

GA-induced reduction pattern between endogenous hTERT and FLAG-hTERT was almost identical. Under these conditions, there was no change in the levels of Hsp90 (Fig. 5A, lower panel), suggesting that the reduction of hTERT is due to its degradation and not due to changes in the Hsp90 level. In the absence of GA treatment, incubation of HeLa cells with MG132 alone did not cause a significant change in the levels of hTERT expression relative to untreated controls (data not shown). To confirm whether GA-induced degradation of endogenous hTERT and FLAG-hTERT targeted the hTERT complexed with Hsp90 in peak II, and whether MG132 prevents the degradation of telomerase in peak II, lysates of HeLa cells stably expressing FLAG-hTERT cultured in medium with various concentrations of GA, before or not before pre-treatment with MG132, were fractionated by molecular sieving. GA and GA plus MG132 had little effect on endogenous hTERT, FLAG-hTERT and telomerase activity in peak I (Fig. 6B). Otherwise GA decreased the amounts of endogenous hTERT and FLAG-hTERT, and reduced telomerase activity in the peak II fractions, and MG132 rescued the GA-induced down-regulation of endogenous hTERT and FLAG-hTERT (Fig. 6C). The effect of GA or GA plus MG132 on endogenous hTERT in HeLa cells was the same as that of the HeLa cells stably expressing FLAG-hTERT (data not shown). The result indicates that telomerase complexed with Hsp90 in peak II is sensitive to Hsp90 inhibitors, which induce degradation

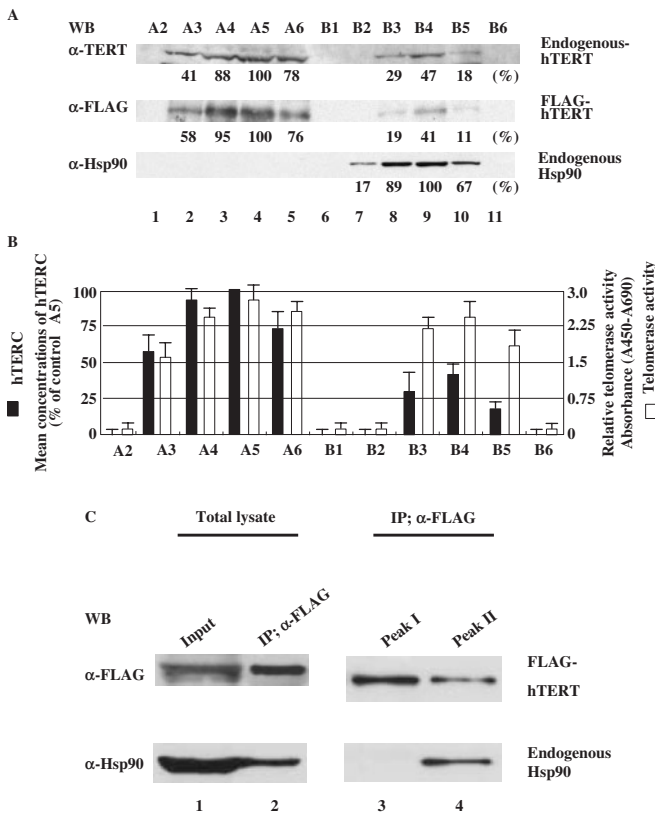


Fig. 5. Fractionation of HeLa cell lysate stably expressing FLAG-hTERT. (A) FLAG-hTERT was stably expressed in HeLa cells, and the lysate was fractionated on HiLoad 16/60 Superdex 200 pg gel filtration columns in running buffer containing 200 mM NaCl and 0.1% Triton-X100. The telomerase activity peaks were at approximate molecular masses of 680 kDa (peak I) and 400 kDa (peak II). The fractions in peaks I and II were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-TERT (2C4), anti-FLAG M2 or anti-Hsp90 antibodies as indicated. Lane 1, fraction No. A2; lane 2, fraction No. A3; lane 3, fraction No. A4; lane 4, fraction No. A5; lane 5, fraction No. A6; lane 6, fraction No. B1; lane 7, fraction No. B2; lane 8, fraction No. B3; lane 9, fraction No. B4; lane 10, fraction No. B5; lane 11, fraction No. B6. (B) The quantification of hTERT in HeLa cell stably expressing FLAG-hTERT was measured by real time RT-PCR. Relative telomerase activity of the fractions in peaks I and II (fraction No. A2–B6) was measured by TRAP ELISA. (C) Total lysate, peak I and peak II fractions from HeLa cells stably expressing FLAG-hTERT were immunoprecipitated with anti-FLAG M2 resin. The bound proteins were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-FLAG M2 or anti-Hsp90 antibodies as indicated. Western blot analysis of total lysate is equal to 5% of the input shown in lane 1 (= input). Lane 2, total lysate, IP; anti-FLAG antibody, lane 3, peak I fractions, IP; anti-FLAG antibody; and lane 4, peak II fractions, IP; anti-FLAG antibody.

of endogenous hTERT and FLAG-hTERT through a proteasome-mediated pathway. Telomerase complex in peak I was neither affected by GA nor by GA plus MG132. These results with the lysate of HeLa cells stably expressing FLAG-hTERT demonstrate that there are two different complexes of telomerase with Hsp90 and without Hsp90, both of which retain telomerase activity.

DISCUSSION

Telomerase activity is tightly regulated by several control mechanisms (27). The limiting component of telomerase, TERT, is under transcriptional and post-transcriptional controls (28), and recruitment of telomerase to the telomere is regulated in a cell-cycle-dependent manner (29, 30). Modification and degradation of hTERT are also involved in the assembly of telomerase, subcellular trafficking and telomerase activity itself (30). Since endogenous telomerase is a limiting component in cells, it is difficult to use biochemical approaches to gain insight into the mechanisms of telomerase regulation without using recombinant forms of telomerase. We therefore prepared recombinant human telomerase reconstituted in insect cells, as well as *in vitro*, from hTERT and hTERC.

Here we report two different complexes of recombinant human telomerase in insect cells, as well as two different complexes of endogenous and stably expressed FLAG-tagged human telomerase in HeLa cells. Distribution pattern of hTERT and hTERC in the complex of 680 kDa and the complex of 380–400 kDa from insect cells was almost similar to that from HeLa cells. Moreover, the relative amount of telomerase activity in the complex of 380–400 kDa to the complex of 680 kDa between insect cells and HeLa cells was almost identical (Figs 2, 4 and 5). Telomerase activity of the complex of 380–400 kDa was sensitive to Hsp90 inhibitors and was rather unstable in proteasome-dependent pathway(s) due to the tight complex of telomerase and Hsp90. In contrast, telomerase activity of the complex of 680 kDa was free from Hsp90, resistant to Hsp90 inhibitors, and rather stable. The distribution and biochemical properties of these complexes from HeLa cells are similar to those from the insect cells. The mechanism to rescue the telomere shortening by reverse transcription of telomere repeats in eukaryotes is completely different from that in insect cells in which non-long terminal repeat (non-LTR) retrotransposons transpose on the chromosomal ends (31–33). In fact, telomerase activity has been not or barely detected in insect cells (31–33). Therefore, the biochemical properties of the human telomerase complexes reconstituted *in vivo* in insect cells in these reports do not reflect or mimic the rescue mechanism of chromosome ends in insect cells, but give an insight of the possible complex formation of human telomerase observed in insect cells that contains some ubiquitous factors such as Hsp90. Molecular weight of Hsp90 is 90 kDa in mammalian cells, and 82 kDa in insect cells. Since the complex of 380–400 kDa contains Hsp90 both in mammalian and insect cells, the slight difference in the molecular masses in HeLa cells (400 kDa) from that in insect cells (380 kDa) may be due to the difference in molecular weight of Hsp90 in these cells.

Several different telomerase complexes of molecular masses 550–1000 kDa have been reported. Wenz *et al.* (8) showed that both *in vitro* reconstituted telomerase and endogenous telomerase from HeLa cells formed a complex of an approximate molecular mass of 600 kDa (8). They demonstrated that this complex purified by an affinity purification method, using a biotinylated

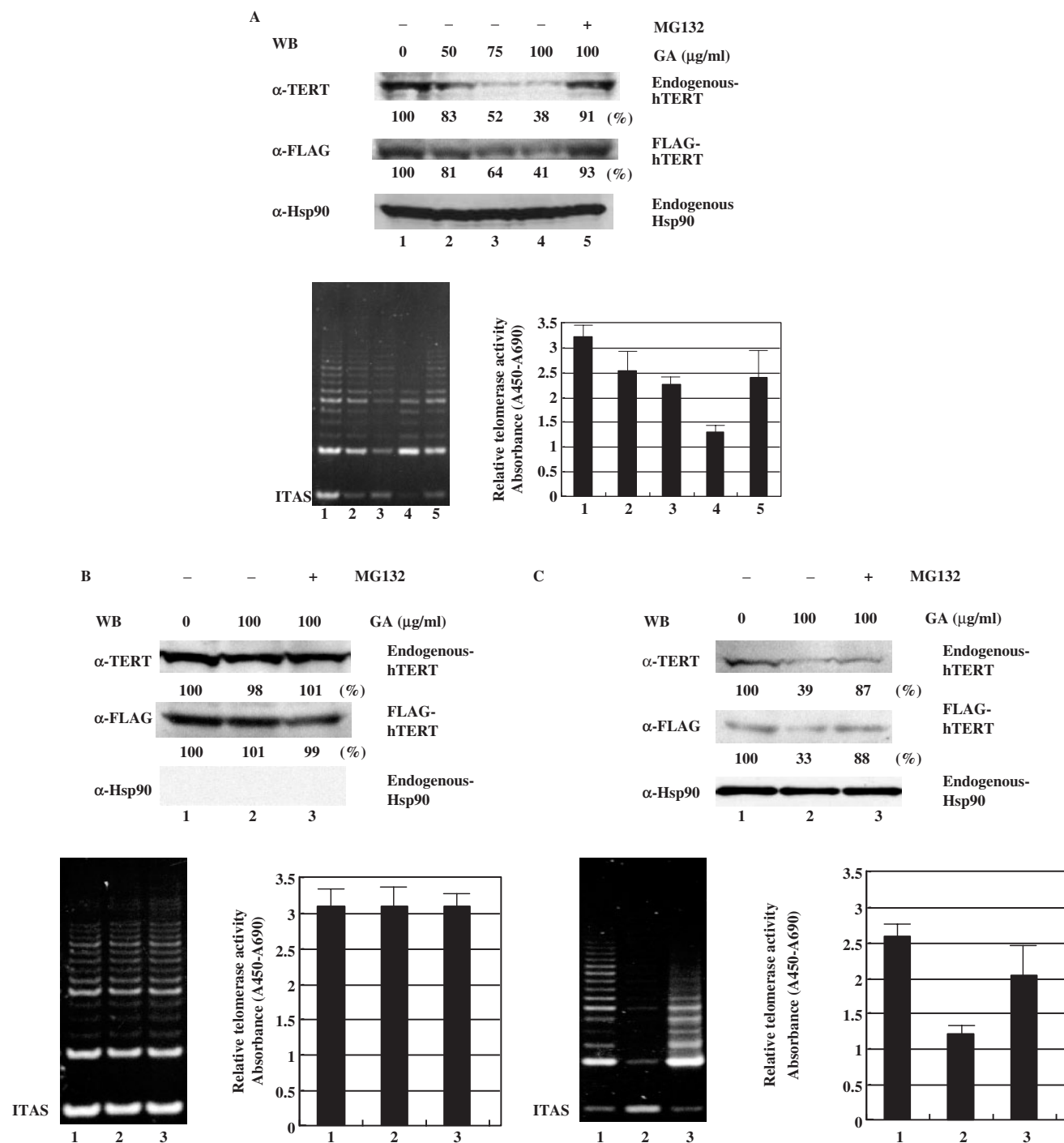


Fig. 6. Characterization of the complexes in HeLa cells stably expressing FLAG-hTERT. (A) HeLa cells stably expressing FLAG-hTERT were treated for 4h with the GA concentration indicated; where indicated, cells were pre-treated with MG132 at 10mM for 2h prior to treatment with GA. Each lysate were separated by 8% SDS-PAGE and then subjected to western blot analysis using anti-TERT (2C4), anti-FLAG M2 or anti-Hsp90 antibodies as indicated. The activity of each lysate was determined using the TRAP assay and TRAP ELISA. Lane 1, GA at 0 $\mu\text{g/ml}$; lane 2, GA at 50 $\mu\text{g/ml}$; lane 3, GA at 75 $\mu\text{g/ml}$; lane 4, GA at 100 $\mu\text{g/ml}$; lane 5, GA at 100 $\mu\text{g/ml}$ plus MG132 at 10mM. (B), (C) HeLa

cells stably expressing FLAG-hTERT were treated with 100 $\mu\text{g/ml}$ GA for 4h; where indicated, cells were pre-treated with MG132 at 10mM for 2h before treatment with GA. Each lysate were fractionated on HiLoad 16/60 Superdex 200 pg gel filtration columns in running buffer containing 200mM NaCl and 0.1% Triton-X100. Peak I (B) and peak II (C) fractions were separated by 8% SDS-PAGE and subjected to western blot analysis using anti-TERT (2C4), anti-FLAG M2 or anti-Hsp90 antibodies as indicated, and analysed for telomerase activity by the TRAP assay and TRAP ELISA. (B), (C) Lane 1, GA at 0 $\mu\text{g/ml}$; lane 2, GA at 100 $\mu\text{g/ml}$; lane 3, GA at 100 $\mu\text{g/ml}$ plus MG132 at 10mM.

oligonucleotide complementary to hTERC, was telomerase dimer consisting of two hTERT (127 kDa) and two hTERC (125 kDa) (8). Schnapp *et al.* (9) reported that the affinity-purified telomerase complex had a molecular mass of 550 kDa, although the telomerase RNP in crude extracts had a molecular mass of 1000 kDa. The telomerase complex of 680 kDa is similar in molecular mass to the dimer form of telomerase reported (8), although we do not have the direct evidence at present. The telomerase complex of 680 kDa cannot be converted to smaller molecular masses or monomer form even in the presence of 2 M NaCl or 1% Triton-X100 (data not shown). Consistent to the previous report (9), telomerase complex in crude lysate of HeLa cells apparently had a molecular mass of ~1000 kDa (data not shown). However, two separated complexes (680 and 380–400 kDa) were observed instead of ~1000 kDa in our conditions in contrast to a single complex of ~550 kDa (9). The discrepancy of telomerase complexes among the previous and our reports may be due to the different conditions including buffer conditions and affinity purification methods. The telomerase complex of 380–400 kDa is smaller than a dimer form consisting of two hTERT and two hTERC molecules, but may be a trimeric complex of hTERT, hTERC and Hsp90.

As Holt *et al.* (20) firstly demonstrated, Hsp90 has been shown to be functionally a critical factor for telomerase activity *in vivo* and *in vitro* with transcription-coupled reconstituted telomerase (21), although GA-sensitive property of telomerase activity has not been reported with the purified reconstituted telomerase (8, 9, 13). Here we show that the telomerase complex with Hsp90 in the complex of 380–400 kDa is not only sensitive to GA, but also unstable during incubation. This property is evidently due to proteolytic degradation via proteasome pathway(s), since the proteasome inhibitors prevented the degradation of endogenous hTERT and FLAG-hTERT in the complex of 380–400 kDa. Hsp90 functions in a variety of aspects (34–36), and Hsp90 inhibitors prevent the degradation of some client proteins, and facilitate the degradation of other client proteins (22, 37). With telomerase, Hsp90 may function in a way distinct from its role as chaperone that serves premature state of client proteins into mature proteins. Keppler *et al.* (38) suggested two different roles for Hsp90: one in telomerase assembly and another in primer loading. They reported that a lower concentration of GA (IC₅₀ of 8.4 μ M) inhibited the formation of active telomerase if present during the assembly, but that a higher concentration (IC₅₀ of 53 μ M) was required to inhibit telomerase if added after the assembly. The different effects of GA on the two complexes in this report were observed at the GA concentration that could inhibit both roles of Hsp90 (38). Under these conditions, any telomere shortening of the GA-treated cells was not observed (data not shown).

It is noteworthy that anti-FLAG M2-resin affinity chromatography can purify FLAG-tagged hTERT in the complex of 680 kDa but not that in the complex of 380–400 kDa in the presence of 0.5% Triton-X100

(‘EXPERIMENTAL PROCEDURES’), although the latter can be recovered in the immunoprecipitates with anti-FLAG M2 antibody in 0.1% of Triton-X100. The availability of the N-terminal-tagged FLAG in the complex of 380–400 kDa was actually affected by the different concentrations of Triton-X100, since ectopically expressed N-terminal-tagged hTERT functions have been reported to be biologically active in telomerase (39, 40). The anti-FLAG M2 antibody could immunoprecipitate FLAG-hTERT and telomerase activity of the complex of 380–400 kDa only in the presence of 0.1% Triton-X100, whereas it could recover FLAG-hTERT and telomerase activity of the complex of 680 kDa at both concentrations. The result suggests that N-terminal of FLAG-hTERT in the complex of 380–400 kDa is not available to the antibody due to some conformation change or dissociation of its interacting partner(s) induced in the high concentration of the detergent.

As Hsp90 is a critical factor for telomerase activity *in vivo* (20, 21, 41), the complex of 380–400 kDa seems to be biologically functional for telomerase activity *in vivo*. There are several possibilities on biological roles of the complex of 680 kDa. First, the telomerase complex of 680 kDa may be a precursor form of the complex of 380–400 kDa. This seems unlikely since cycloheximide treatment, which blocks *de novo* synthesis of hTERT and thus of telomerase, did not affect the distribution pattern of hTERT in the two complexes (data not shown). Second, excess telomerase may be reserved in a different state (the complex of 680 kDa) from the working complex in the complex of 380–400 kDa. This possibility may be consistent with the result that more than half of telomerase activity is sensitive to GA *in vivo*, although the complex of 680 kDa is the major one in the lysate. Third, the complex of 680 kDa may be a working enzyme not for telomere synthesis but for another function of telomerase that may be required for DNA damage responses (42). These possibilities remains to be examined in future studies. This is the first demonstration of two distinct active complexes of human telomerase that may contribute to further understanding of several aspects of function(s) and regulation of telomerase.

We are grateful to T. Shimakami, N. Oishi and T. Kusakawa for encouraging discussion. We thank K. Kuwabara, T. Ohashi and M. Yasukawa for their technical assistance. This work was supported in part by Researches Grants-in-Aid for scientific research (B), 17390091, and development and a Grant-in-Aid for scientific research on priority area (C) in oncogenesis, 18012018, from the Ministry of Education, Sports, Culture and Technology, Japan.

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