Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the human telomerase complex. Growing evidence suggests that hTERT also contributes to the cell physiology independently of telomere elongation. However, its role in bacterial infection is unknown. Here we show that hTERT is critical for Listeria monocytogenes infection, as the depletion of hTERT impaired bacterial intracellular replication. In addition, we observed that L. monocytogenes caused a decrease in hTERT levels at early time points of the infectious process. This effect was mediated by the pore-forming toxin listeriolysin O (LLO) and did not require bacterial entry into host cells. Calcium influx through the LLO pores contributed to a proteasome-independent decrease in hTERT protein levels. Together, our data provide evidence that these bacteria trigger hTERT degradation, an event that is detrimental to bacterial replication.

Received 11 June 2012 Returned for modification 10 July 2012 Accepted 16 September 2012

Published ahead of print 24 September 2012

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Supplemental material for this article may be found at http://iai.asm.org/

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doi:10.1128/IAI.00614-12
methyl ester (Calbiochem), 200 μM; KCl (Sigma), 135 mM; and EGTA (Sigma). The incubation times for each drug are indicated in the legends to Fig. 4 and 6 and to Fig. S2 and S3 in the supplemental material.

L. monocytogenes strains were grown in brain heart infusion (BHI) medium (BD Difco) at 37°C. The bacterial strains used in this study were Listeria innocua (BUG 499), L. innocua overexpressing InlB (BUG 1642), L. monocytogenes EGD-e (BUG 1600), L. monocytogenes EGD (BUG 600), L. monocytogenes EGD ΔinlA ΔinlB (BUG 949), L. monocytogenes EGD ΔInlY (BUG 2132), L. monocytogenes L028 (BOF 343), L. monocytogenes L028 Tn::llo (BOF 415), and L. monocytogenes L028 Tn::llo (BOF 415) overexpressing LLO (BUG 210).

To quantify intracellular bacteria by immunofluorescence, the infected cells were washed and intracellular bacteria were released by 0.1% Triton X-100. The cell lysates were plated on BHI agar plates to quantify the intracellular bacteria.

To evaluate the extent of L. monocytogenes infection or Listeria entry by counting the CFU, infected cells were washed and intracellular bacteria were released by 0.1% Triton X-100. The cell lysates were plated on BHI agar plates to quantify the intracellular bacteria.

To quantify intracellular bacteria by immunofluorescence, the infected cells were washed and fixed in 4% paraformaldehyde, and extracellular bacteria were stained with an anti-L. monocytogenes R11 antibody (18) and an Alexa Fluor 546 goat anti-rabbit antibody (Invitrogen) before permeabilization of the host cell plasma membrane. After permeabilization, intracellular bacteria were stained with an anti-L. monocytogenes R11 antibody and an Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen).

Transfection of HeLa cells. Control small interfering RNA (siRNA) (siGENOME nontargeting siRNA 1) and hTERT1 siRNA (described in reference 32) were purchased from Dharmacon, while hTERT2 siRNA was custom designed (5'-UCAGACAGCACUUGAAAG-3') and purchased from Eurofins MWG Operon. For siRNA transfection, we used Oligofectamine (Invitrogen) according to the manufacturer’s instructions. To infect transfected cells, we adjusted the multiplicity of infection (MOI) of 50 with bacteria that were washed three times in medium without serum prior to infection. For a longer infection process, infected cells were grown in growth medium supplemented with 20 μg/ml gentamicin to kill the extracellular bacteria.

To determine the role of hTERT in the infectious process, we treated HeLa cells with hTERT or control siRNAs and then infected them with L. monocytogenes EGD. (A) Thirty minutes after the beginning of infection, cell-associated bacteria were quantified and normalized on control siRNA-treated cells. (B) Thirty minutes after the beginning of infection, cells were treated with 100 μg/ml gentamicin for 30 min. The intracellular bacteria were counted and normalized on control siRNA-treated cells. (C) One hour after infection, the cells were treated with 20 μg/ml gentamicin for 4 h. The percentages of intracellular bacteria are relative to the control siRNA-treated cells. The experiments were performed three times, and the results are shown as means ± standard error of the means. The asterisks mark P values of <0.05, and ns marks nonsignificant differences.

For Western blot analysis, cells were lysed with 2× Laemmli loading buffer (124 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.02% bromophenol blue, 0.03% dithiothreitol [DTT]), sonicated for 2 s, and then boiled for 5 min. The samples were loaded on 6% gels or 4 to 15% Mini-Protein TGX gradient gel (Bio-Rad). The proteins were transferred on a nitrocellulose membrane (GE Healthcare) that was then blocked in 10% milk. The primary antibodies were anti-actin (catalog no. A5441; Sigma), anti-hTERT (manufacturer part no. 600-401-252; Rockland), anti-phospho-IκB (Cell Signaling Technology), and anti-hemagglutinin (HA) tag (clone 6E2; Cell Signaling Technology). Rabbit polyclonal antibodies against UBC9 (R201) and LLO (R176) were raised by immunizing rabbits with purified recombinant LLO and UBC9 proteins.

RESULTS

hTERT is important for intracellular Listeria replication. To determine the role of hTERT in the infectious process, we treated HeLa cells with hTERT or control siRNAs and then infected them with L. monocytogenes for either 30 min to assess bacterial adhesion and entry or for 5 h to measure intracellular replication. After 30 min of infection, we observed that the number of cell-associated bacteria in hTERT-depleted cells was not significantly different from that of control cells (Fig. 1A). In order to assess the effect on bacterial entry, we infected HeLa cells for 30 min and then treated them with 100 μg/ml gentamicin for another 30 min. No
significant differences in bacterial entry were observed when comparing control cells with hTERT1 or hTERT2 siRNA-treated cells (Fig. 1B). However, 5 h after infection, the number of intracellular bacteria was significantly reduced in the cells treated with hTERT siRNA compared to those in the control (Fig. 1C). We confirmed this effect by quantifying intracellular bacteria via an immunofluorescence approach (see Fig. S1 in the supplemental material). These results suggest that hTERT is important for the intracellular replication of Listeria.

**Listeria monocytogenes induces a decrease in hTERT levels.** Based on the observation discussed above that hTERT plays a role in *Listeria* infection, we examined whether infection with different wild-type strains of *L. monocytogenes* or incubation with *L. innocua*, a nonpathogenic *Listeria* species, would affect hTERT at the protein level. The level of hTERT was analyzed by Western blotting. We observed a decrease in hTERT levels upon infection with *L. monocytogenes* for 1 h (Fig. 2A). To confirm the results obtained with the antibody against hTERT (50), we transfected HeLa cells with a plasmid expressing hTERT-HA and infected these cells. LLO expression and the degradation of UBC9, a host enzyme, were employed as markers of *L. monocytogenes* infection (43). As observed with the endogenous protein, the level of hTERT-HA decreased in the cells infected with *L. monocytogenes* (Fig. 2B).

We next examined the levels of hTERT mRNA 1 h after infection of HeLa cells by *L. monocytogenes* or incubation with *L. innocua*. We did not observe a significant difference in hTERT mRNA levels between the infected and uninfected cells (Fig. 2C). Moreover, we performed a similar experiment 3 h after infection and found that *Listeria* infection did not modify hTERT mRNA levels in those host cells. Trichostatin A (TSA), which is known to inhibit histone deacetylases and to activate the hTERT promoter, was used as a positive control for hTERT transcription activation (9). As expected, and in contrast to *Listeria* infection, TSA induced an approximately 1.72-fold increase in hTERT mRNA levels (see Fig. S2 in the supplemental material). Taken together, our data show that *L. monocytogenes* decreases the levels of the hTERT mRNA without affecting its transcription.

**The decrease of hTERT occurs early after infection.** We next examined the kinetics of the decrease in hTERT protein levels. A time course analysis conducted 5 h after infection indicated that *L. monocytogenes* reduces the levels of hTERT as soon as 1 h after the addition of bacteria to HeLa cells (Fig. 3A). This effect was transient, as the hTERT levels in infected and uninfected cells were similar 5 h after infection. We also observed that the decrease in hTERT levels was dependent on the multiplicity of infection used to infect the cells (Fig. 3B). These results definitively establish that *L. monocytogenes* induces a decrease in hTERT levels during the initial stages of infection.

**L. monocytogenes induces a decrease in hTERT levels before bacterial entry.** We next examined whether a diminution in hTERT levels depends on bacterial invasion. We infected cells with wild-type *L. monocytogenes* or a ΔinlA ΔmlB mutant that is strongly impaired in the invasion of HeLa cells. Both bacterial strains induced a decrease in the hTERT levels, as assessed by Western blotting (Fig. 4A). In contrast, the cells exposed to wild-type *L. innocua* or *L. innocua* expressing InlB (which confers to *L. innocua* the capacity to invade cells [6]) did not exhibit a decrease in hTERT levels (Fig. 4A), suggesting that hTERT levels do not decrease upon uptake of the nonpathogenic bacterium *L. innocua*.

Similar results were observed with hTERT-HA-expressing cells (Fig. 4B). In addition, the pretreatment of cells with cytochalasin D, an inhibitor of actin polymerization that prevents *L. monocytogenes* entry (15), did not block hTERT decrease (Fig. 4A). These results demonstrate that the decrease in hTERT levels does not depend on bacterial invasion and is specific to *L. monocytogenes*.

Listeriolysin O is responsible for the reduction in hTERT levels. Because hTERT was degraded in the presence of extracellular bacteria, we next investigated whether a secreted factor could pro-
Calcium contributes to hTERT degradation induced by L. monocytogenes. To address the mechanism by which LLO induced the decrease in hTERT levels, we examined whether it could be prevented by inhibiting the activity of the proteasome with the inhibitors MG132 and lactacystin (LC). The inhibition of proteasome activity had no effect on the LLO-induced decrease of hTERT (Fig. 6A), although as expected, the proteasome inhibitors provoked accumulation of ubiquitin-protein conjugates in treated cells (see Fig. S3A in the supplemental material).

To further investigate the molecular basis of the decrease in hTERT levels, we pretreated cells with inhibitors of aspartyl proteases (pepsatin methyl ester), cysteine proteases (loxistatin and leupeptin), metalloproteases (bestatin methyl ester), and serine proteases (AEBSF and leupeptin). We monitored the inhibitory activity of pepstatin methyl ester through the partial resistance to degradation that it conferred to UBC9 in the presence of LLO (see Fig. S3B in the supplemental material) (43). The activities of leupeptin and loxistatin were tested through the inhibition of phospho-IkB activation of serine proteases, as shown by the decreased activation (phosphorylation) of IκB (see Fig. S3C and D in the supplemental material) (34), while AEBSF inhibited the activation of serine proteases, as shown by the decreased activation (phosphorylation) of IκB (see Fig. S3C in the supplemental material) (24, 34) and the inhibitory effect on the degradation of phospho-IkB (see Fig. S3D in the supplemental material). The addition of LLO decreased the levels of hTERT in the presence of all tested protease inhibitors (Fig. 6B). We conclude that, under...
the conditions that we examined, the proteolytic activity necessary for the reduction in hTERT levels in the presence of LLO could not be impaired.

LLO pore formation provokes the permeability of the plasma membrane to K\textsuperscript{+} and Ca\textsuperscript{2+} ions (4). K\textsuperscript{+} efflux leads to histone H3 dephosphorylation (20), while Ca\textsuperscript{2+} influx plays a role during bacterial entry and induces mitochondrial fragmentation (13, 47). To study the implication of the two ions in the decrease of hTERT levels, we prevented K\textsuperscript{+} efflux by incubating cells in high extracellular concentrations of KCl, while Ca\textsuperscript{2+} influx was blocked by incubating cells with EGTA, a calcium chelator. As shown in Fig. 6C and D, the pretreatment of cells with EGTA impaired the decrease in hTERT levels induced by LLO, while blocking K\textsuperscript{+} influx had no effect. These results suggest that Ca\textsuperscript{2+} influx, rather than K\textsuperscript{+} efflux, contributes to the decrease in hTERT levels in the presence of LLO.

**DISCUSSION**

In the present study, we provide the first evidence that hTERT is important for *Listeria* infection. In addition, we show that *L. monocytogenes* is able to induce a decrease in hTERT levels through the formation of LLO pores in the plasma membrane prior to host cell invasion. The pores formed by LLO induce a decrease of the hTERT level that is proteasome independent but requires Ca\textsuperscript{2+} influx.

*Listeria monocytogenes* induced an hTERT decrease without affecting transcription of the hTERT gene. Because the half-life of hTERT is around 6 h (28), the decrease of the hTERT level observed 20 min after LLO treatment probably does not result from...
a decrease in hTERT translation but, rather, from posttranslational regulation of the hTERT protein. Several studies have reported that hTERT is degraded through the ubiquitin-proteasome pathway (27, 29, 38). Here, we observed a decrease in hTERT levels that was proteasome independent. Similarly, a proteasome-independent pathway was suggested previously for the degradation of UBC9 upon treatment with LLO (43). However, the degradation of UBC9 involves an aspartyl protease and is calcium independent (43). In contrast, we observed a contribution of calcium to the pathway leading to the decrease of hTERT levels. Calcium is known to contribute to the activation of cysteine proteases, such as calpains (46). However, other proteins belonging to serine proteases, aspartyl proteases, or metalloproteases can also be activated by the presence of calcium (16, 23, 35). We blocked these classes of proteases using well-characterized inhibitors, but under the conditions tested, none of the protease inhibitors impaired the decrease in hTERT levels. Two possible explanations exist for the inability to prevent the reduction in hTERT levels induced by LLO: (i) the doses of protease inhibitors and the duration of the treatment were not sufficient to prevent the decrease in hTERT levels, and (ii) proteolytic cleavage can be very specific. For example, identification of the protease responsible for the cleavage of paxillin upon treatment with the pore-forming toxin α-hemolysin from uropathogenic Escherichia coli relied on a highly specific trypsin-like serine protease inhibitor (tosyl-l-lysine-chloromethyl ketone), while it was insensitive to the chymotrypsin-like serine protease inhibitor tosyl-l-phenylalanine-chloromethyl ketone (12). Our current work is focusing on identifying the molecular basis of the degradative pathways activated by LLO via proteomic analysis and genome-wide siRNA screening approaches.

We found that hTERT was important for Listeria infection. Our siRNA experiments show that reduced hTERT expression early during infection does not impair bacterial adhesion and entry, yet it results in a decrease of the intracellular bacterial load at later time points. The degradation of hTERT could therefore represent an event that protects host cells at a specific stage of the Listeria infectious cycle. In the absence of siRNA treatment, hTERT levels start recovering 5 h after infection, allowing for the full intracellular replication of L. monocytogenes, further suggesting that this antibacterial effect is time restricted. In addition, the recovery of hTERT levels may contribute to cell survival, given the proposed antiapoptotic role of hTERT.

A decrease in hTERT levels could have important consequences during an in vivo infection. Telomerase activity has been detected in human adult stem cells, including hematopoietic and nonhematopoietic stem cells (25). Secreted LLO could diffuse to such progenitor cells or stem hematopoietic cells in colonized organs. An LLO-induced decrease in hTERT levels in LLO-targeted cells would lower their self-renewal capacity and therefore impair the immune response and promote L. monocytogenes infection (26, 36). It remains to be tested whether hTERT levels are affected in vivo.

The first characterized role of hTERT concerns telomere elongation that contributes to the extension of cellular life span (5). To detect this effect, cells have to be followed through several generations. However, given that long-term infections (for more than 48 h) are toxic in HeLa cells and the observed L. monocytogenes-induced reduction of hTERT levels was followed by a recovery process, we did not expect to provoke detectable effects on telomere length in HeLa cells. In agreement with this, all of our attempts to detect a change in telomere length were unsuccessful. Most virus-induced tumor cells possess high telomerase activity but short telomeres (3). Indeed, hTERT can extend the cellular life span without inducing net telomere lengthening (37, 51). It is possible that L. monocytogenes affects the recently described noncanonical functions of hTERT (31). As mentioned, hTERT was shown to play roles in processes as diverse as DNA damage, Wnt signaling, and the decrease of the RNA component of a mitochondrial RNA-processing endoribonuclease (RMRP). These roles of hTERT seem to be at least partially independent of each other (37). Indeed, while hTERT induced cell proliferation independently of an increase in Wnt signaling, it was associated with a decrease in RMRP levels (37). The next challenge will thus be to determine whether noncanonical functions of hTERT are necessary for bacterial infection and which specific function of hTERT is targeted by bacterial infection.

ACKNOWLEDGMENTS

We are grateful to Olivier Dussurget and David Ribet for comments and for critical reading of the manuscript, Edith Gouin and Mélanie Hamon for help in the protease inhibitor activity tests, and all members of the Unité des Interactions Bactéries-Cellules laboratory for helpful discussions. We thank Titia de Lange for discussion, for advice, and for hosting A.S.-L.

This work was supported by the Pasteur Institute, INSERM (U604), INRA (USC2020), the Fondation Louis Jeantet, the European Research Council (Advanced grant 233348, MODELIST), the Fondation pour la Recherche Médicale (fellowship to F.S.), and the Fondation Le Roch Les Mousquetaires. P.C. is an HHMI senior international research scholar.

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