Overexpression of heat shock protein 70 restores the structural stability and functional defects of temperature-sensitive mutant of large T antigen at nonpermissive temperature

Yoshiaki Tabuchi,¹ Ryosuke Kuribayashi,² Ichiro Takasaki,¹ Takeshi Doi,¹ Hideki Sakai,² Noriaki Takeguchi,² Takashi Kondo,³ and Kenzo Ohtsuka⁴

¹Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Toyama 930-0194, Japan

²Department of Pharmaceutical Physiology, Faculty of Pharmaceutical Science, University of Toyama, Toyama 930-0194, Japan

³Department of Radiological Sciences, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan

⁴Laboratory of Cell and Stress Biology, Department of Environmental Biology, Chubu University, Aichi 487-8501, Japan

Abstract The effects of heat shock protein 70 (Hsp70), a molecular chaperone, on the degradation and functional alterations of a mutant large T antigen induced by a nonpermissive temperature were examined. In this study, mouse tracheal epithelial TM02-3 cells harboring temperature-sensitive simian virus 40 large T antigen and stable TM02-3 cells overexpressing human Hsp70 and/or Hsp40 were used. Although the temperature shift from 33°C (permissive temperature) to 39°C (nonpermissive temperature) induced increases in the endogenous chaperones including Hsp70 and Hsp40, degradation of the T antigen, activation of the p53-p21^{waf1} pathway, and an arrest of cell growth were observed in the mock cells. In contrast, these changes induced by the temperature shift were partially but significantly prevented in stable cells overexpressing human Hsp70 and/or Hsp40. A combination of Hsp70 and Hsp40 was the most effective, suggesting that Hsp40 may cooperate with Hsp70. Moreover, immunocytochemical observation indicated that human Hsp70 was expressed in the cytoplasm at 33°C, but it colocalized with T antigen in the nucleus at 39°C. These results suggest that overexpressed Hsp70 translocates from the cytoplasm to nucleus, and significantly restores the structural stability and functional defects of mutant large T antigen in the cells.

INTRODUCTION

It has been indicated that the use of immortal cell lines with cell-specific functions is of central importance in the development of cellular and molecular biology. Some viral and immortalizing genes have the ability to establish continuous proliferation in primary culture cells (Chang 1986). Previous reports have indicated that various immortal cell lines have been established using the simian

virus 40 large T (SV40LT) antigen (Paul et al 1988; Williams et al 1988). It has also been shown that cell immortalization caused by a mutant temperature-sensitive SV40LT (tsSV40LT) antigen accompanies more or less stable cell type-specific functions, and that the growth and differentiation of the cell lines can be controlled by temperature shift (Jat and Sharp 1989). In addition, transgenic animals bearing the mutant tsSV40LT antigen have been developed to establish conditionally immortalized cell lines from tissues that have proved difficult to culture in vitro (Noble et al 1995; Obinata 2001). Thus far, many kinds of conditionally immortalized cell lines that harbor the mutant tsSV40LT antigen were established by using

Correspondence to: Yoshiaki Tabuchi, Tel: +81-76-434-7187; Fax: +81-76-434-5176; E-mail: ytabu@cts.u-toyama.ac.jp.

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infecting primary culture cells with the mutant oncogene or transgenic animals harboring the mutant oncogene (Jat and Sharp 1989; Noble et al 1995; Obinata 2001; Tabuchi et al 2005b, 2006).

It is well known that wild-type SV40LT antigen induces immortalization by inactivation of functions of several tumor suppressor molecules, including retinoblastoma binding protein (pRB) and p53 (Levine 1997). In contrast, mutant tsSV40LT antigen encoded by the tsA58 allele forms complexes with pRB or p53 at the permissive temperature 33°C, but is rapidly conformation-changed and degraded and releases pRB or p53 from the complexes at the nonpermissive temperature 39°C (DeCaprio et al 1988; Levine 1997). Cell lines harboring the mutant oncogene, therefore, grow continuously at the permissive temperature, whereas growth arrest, apoptosis, and/or cell differentiation are induced at the nonpermissive temperature.

Heat shock proteins (HSPs) are highly conserved proteins occurring in almost all organisms and are induced by various stresses (Lindquist and Craig 1988; Smith et al 1998). Family members of the Hsp70 are central molecular chaperones that are involved in the folding and intracellular transport of newly synthesized proteins, the prevention of protein aggregation, and the active recovery of stress-induced nonnative proteins (Hartl and Hayer-Hartl 2002). In addition, Hsp40 interacts and cooperates with Hsp70 (Michels et al 1997; Hartl and Hayer-Hartl 2002). It is also recognized that an amino-terminal J domain of the SV40LT antigen can directly interact with Hsp70 family members (Sawai and Butel 1989; Srinivasan et al 1997; Sullivan and Pipas 2002). In conditionally immortalized cell lines harboring the mutant tsSV40LT antigen encoded by tsA58, the temperature shift from 33 to 39°C leads to inactivation and degradation of large T antigen and cell-growth arrest, and simultaneously induces chaperones including Hsp70 and Hsp40. However, the full effects of chaperones on the degradation and functional alterations of the mutant protein remain unknown. Among mutant SV40LT antigens, the localization-signal mutant large T antigen encoded by SV40 CT3 remains entirely in the cytoplasm of mouse cells (Lanford and Butel 1984). Jeoung et al (1991) previously reported that human Hsp70 complements the localization and functional defects of the cytoplasmic mutant SV40LT antigen in Swiss 3T3 mouse fibroblast cells.

In the present study, we used a conditionally immortalized mouse tracheal epithelial TM02-3 cell line, which is established from transgenic mice bearing the tsSV40LT antigen gene (tsA58) (Sugiyama et al 1998), and developed stable TM02-3 cells overexpressing human Hsp70 and/or Hsp40. The results obtained here demonstrate that Hsp70, which is overexpressed in nuclei, significantly

inhibits degradation and functional alterations of mutant large T antigen in tracheal epithelial cells.

MATERIALS AND METHODS

Characteristics of TM02-3 cells and cell culture

A mouse tracheal epithelial cell line, TM02-3, was developed from adult transgenic mice bearing tsSV40LT antigen gene (tsA58). The cells grew at a permissive temperature (33°C), but did not at a nonpermissive temperature (39°C). Large T antigen was expressed only in the nuclei at 33°C but disappeared at 39°C, indicating that TM02-3 cells show a temperature-sensitive growth phenotype reflected by the tsSV40 large T antigen (Sugiyama et al 1998). TM02-3 cells were cultivated in Dulbecco modified Eagle medium/Ham F-12 (DMEM/F12) (1:1) (Invitrogen, Tokyo, Japan) medium supplemented with growth factors (GFs) (2% fetal bovine serum, 2.0 mg/L insulin, 2.0 mg/L transferrin, 0.122 mg/L ethanolamine, 9.14 µg/L sodium selenite, and 10 ng/mL epidermal growth factor) in a collagen type I–precoated culture vessel at 33 or 39°C.

Establishment of stable cell lines

Human Hsp70 (Michels et al 1997) and human Hsp40 (Kobayashi et al 2000) were subcloned into pcDNA3.1/Hygro(+) (Invitrogen) and pRC/CMV (Invitrogen), respectively. Stable cell lines overexpressing Hsp70 alone, Hsp40 alone, and Hsp70 plus Hsp40 were developed using a standard method. For construction of mock cell lines, the cells were transfected with the corresponding empty vectors. The cloned cells were maintained in DMEM/F12 medium containing GFs and 0.5 mg/mL hygromycin and/or 0.5 mg/mL G418.

Analysis of cell growth

The cells were cultured at 33 or 39°C for 0–6 days. The cell number was counted using a hemacytometer.

Immunocytochemistry

The cells were fixed in methanol at room temperature for 7 minutes. After incubation with blocking solution (16% goat serum, 0.3% Triton X-100, 0.9% NaCl, and 20 mM Na phosphate, pH 7.4) at room temperature for 30 minutes, they were incubated with primary antibody at 4°C for 24 hours, and followed by incubation with secondary antibody at room temperature for 1 hour. Immunofluorescence images were visualized by using a laser scanning confocal microscope (LSM510; Carl Zeiss, Tokyo, Japan). Primary and secondary antibodies used were as fol-

lows: mouse monoclonal anti-large T antigen antibody (Oncogene Research Products, Cambridge, MA, USA); rabbit polyclonal anti-Hsp70 antibody (reacts with Hsp70 of mouse and human; MBL, Nagoya, Japan); Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA); Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting

The cells were washed once with phosphate-buffered saline (PBS) and placed into lysis buffer (50 mM NaCl, 1% Nonidet P-40, and 50 mM Tris-HCl, pH 8.0) containing the protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and homogenized by an ultrasonic disruptor. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described elsewhere (Laemmli 1970; Towbin et al 1979). Primary antibodies used were as follows: rabbit polyclonal anti-Hsp70 antibody; mouse monoclonal anti-Hsp40 antibody (react with Hsp40 of mouse and human; MBL); goat polyclonal anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-Hsc70 antibody (Santa Cruz Biotechnology); rat monoclonal anti-Hsp90 (MBL); rabbit polyclonal anti-Hsp110 antibody (MBL); mouse monoclonal anti-large T antigen antibody; mouse monoclonal anti-p53 antibody (Oncogene Research Products); rabbit polyclonal anti-p21^{waf1} antibody (Santa Cruz Biotechnology); mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (G3PDH) antibody (Organon Teknica, Durham, NC, USA). Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus; Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence (ECL) detection system. Bands of target proteins were quantified by densitometry using Multi-Analyst software (Japan Bio-Rad Laboratories, Tokyo, Japan). Fold induction was normalized by G3PDH.

Real-time quantitative polymerase chain reaction assay

Total RNA was extracted from the cells using the RNeasy Total RNA Extraction kit (Qiagen, Hilden, Germany). Then, RNA samples were treated with RNase-free DNase. Reverse transcriptase reaction was carried out on total RNA by using an oligo d(T)6 primer. TaqMan 5' nuclease fluorogenic real-time quantitative polymerase chain reaction (qPCR) was carried out according to our previous study (Tabuchi et al 2005a). Based on the database (GenBank accession no. U09507), primers and a probe were designed: mouse p21^{waf1} forward, reverse and probe, 5'-CATGAGCGCATCGCAATC-3' (position: 545–562), 5'-CTGGTGATGTCCGACCTGTTC-3' (645–625) and 5'-

Fam-TGCTCACTGTCCACGGGACCGAA-TAMRA-3' (573–595), respectively. TaqMan rodent G3PDH control reagent was purchased from Applied Biosystems (Tokyo, Japan). Fold induction was normalized by G3PDH.

Statistical analysis

Data are shown as means $\pm$ SD. Statistical analysis was carried out using Student's *t* test, and values of *P* < 0.05 were regarded as significant.

RESULTS

Establishment of stable cell lines overexpressing chaperones and the effects of culture temperatures on the chaperone expression levels

Stable mouse tracheal epithelial cell lines overexpressing human Hsp70 alone (TM70), human Hsp40 alone (TM40), and human Hsp70 plus Hsp40 (TM70+40) were established. To confirm the expression levels of Hsp70 and Hsp40 in chaperone-overexpressing cells at both permissive temperature (33°C) and nonpermissive temperature (39°C), we performed Western blot analysis to measure the protein levels. As shown in Figure 1, A, C, and D, at 33°C, the Hsp70 levels were markedly elevated in human Hsp70-overexpressing cells such as TM70 and TM70+40, with levels approximately 20-fold higher than those of TMmock cells (control cells). The Hsp40 levels were markedly elevated in human Hsp40-overexpressing cells such as TM40 and TM70+40 cells, with levels approximately 12-fold higher than those of TMmock cells. Culture at 39°C significantly increased the expression levels of endogenous Hsp70 or Hsp40 in TMmock and TM40 or TMmock and TM70 cells, with levels approximately 5- or 2-fold higher than those in TMmock cells at 33°C, respectively. On the other hand, no significant increase in expression levels of Hsp70 (endogenous plus human) or Hsp40 (endogenous plus human) was observed in TM70 and TM70+40 cells or in TM40 and TM70+40 cells, respectively. Stably expressed levels of human chaperones were markedly higher than those of chaperones induced by the nonpermissive temperature. Furthermore, the protein levels of several kinds of endogenous chaperones such as Hsp60, Hsc70, Hsp90, and Hsp110 were measured at both temperatures. The expression levels of Hsp60 and Hsc70 were constant among the chaperone-overexpressed cells. On the other hand, the level of Hsp90 or Hsp110 was significantly increased in TM40 and TM70+40 or TM70, TM40 and TM70+40 cells, respectively (Fig 1B,E–H). These results indicate that the expression levels of endogenous chaperones may be influenced by the overexpression of exogenous human Hsp70 and/or Hsp40 in the cells.

Fig 1. Levels of HSPs of chaperone-overexpressing cell lines at permissive and nonpermissive temperatures. The cells were cultured at permissive temperature (33°C) and nonpermissive temperature (39°C) for 3 days. SDS-PAGE and Western blotting were performed. (A, B) Signals were visualized by a luminescent image analyzer using an ECL system. (C–H) Bands were quantified by densitometry. Fold induction was normalized by G3PDH (C, Hsp70; D, Hsp40; E, Hsp60; F, Hsc70; G, Hsp90; H, Hsp110). Data represent means $\pm$ SD for 3–4 different experiments. * $P < 0.05$ vs TMmock at 33°C; # $P < 0.05$ (Student's *t* test).

Cell growth in chaperone-overexpressing cell lines at 33 and 39°C

The effects of culture temperatures on the cell growth of chaperone-overexpressing cell lines were examined. At

33°C, all cell lines proliferated constantly (Fig 2A). At 39°C, no cell proliferation was observed in TMmock cells. In contrast, significant increases in cell proliferation were observed in TM70, TM40, and TM70+40 cells at 39°C,

Fig 2. Cell growth of chaperone-over-expressing cell lines at 33 and 39°C. The cells were cultured at 33°C (A) and 39°C (B) for 0–6 days. The cell number was measured by a hemacytometer. The data represent means $\pm$ SD for 4–6 wells. * $P < 0.05$ vs TMmock cells at 39°C; # $P < 0.05$ (Student's *t*-test).

with levels being 45, 22, and 53% compared with those in TMmock cells at 33°C. Hsp70 and Hsp40 in combination appeared to be the most effective for restoration of cell growth (Fig 2B).

Subcellular localization of large T antigen and Hsp70 in chaperone-overexpressing cell lines at 33 and 39°C

The mutant tsSV40LT antigen is expressed in nuclei at 33°C but is rapidly inactivated and degraded at 39°C. At 33°C, immunocytochemical analysis showed relatively high expression levels of large T antigen in nuclei in all cell lines (Fig 3A,G,M,S). In contrast, at 39°C, although large T antigen had nearly disappeared in TMmock cells (Fig 3B), significant expression of large T antigen was detected in nuclei in chaperone-overexpressing cells such as TM70, TM40, and TM70+40 cells (Fig 3H,N,T). It has been reported that Hsp70 is present in the cytoplasm under normal conditions, but in the nucleus in response to heat shock or chemical stress (Pelham 1984; Welch and Feramisco 1984; Chughtai et al 2001; Quan et al 2004). In human Hsp70-nonexpressing cells (TMmock and TM40), expression levels of Hsp70 were very low at 33°C, but endogenous Hsp70 was induced in the nucleus at 39°C. Some colocalization of Hsp70 with T antigen in the nucleus was observed in Mock and TM40 cells (Fig 3C–E,O–R). In Hsp70-overexpressing cells (TM70 and TM70+40), relatively high expression levels of Hsp70 were observed in the cytoplasm at 33°C. In contrast, Hsp70 translocated from cytoplasm to nucleus and colocalized with T antigen at 39°C (Fig 3I–L,U–X).

Protein levels and function of large T antigen in chaperone-overexpressing cell lines at 33 and 39°C

The mutant tsSV40LT antigen is rapidly conformation-changed and degraded at nonpermissive temperatures. At 33°C, Western blot analysis indicated that relatively

high levels of large T antigen were maintained in all cell lines. In contrast, at 39°C, although the T antigen levels were dramatically decreased in TMmock cells (8% compared to that at 33°C), this decrease was significantly suppressed in chaperone-overexpressing cells such as TM70, TM40 and TM70+40 cells, with levels reaching approximately 70% compared to those of TMmock cells at 33°C (Fig 4A,B). It has also been indicated that the mutant large T antigen binds to p53, inactivates its function, and extends its half-life at 33°C, but is rapidly inactivated and releases p53 from complexes at 39°C (DeCaprio et al 1988; Levine 1997). At 33°C, relatively high levels of p53 were maintained in all cell lines (Fig 4A,C). In contrast, at 39°C, although p53 levels were dramatically decreased in TMmock cells (6% compared to that at 33°C), they were significantly increased in Hsp70-overexpressing cells such as TM70 and TM70+40 cells, reaching approximately 30–40% compared with those of TMmock cells at 33°C (Fig 4A,C).

We have recently shown that TM02-3 harboring mutant tsSV40LT antigen grows continuously at 33°C, and culture at 39°C induces inactivation of large T antigen and growth arrest, leading to activation of p53 and induction of p21^{waf1} (Sugiyama et al 1998; Tabuchi et al 2005a). It is well known that the promoter of the cyclin-dependent kinase inhibitor p21^{waf1} contains 2 elements recognizable by p53, and that binding of p53 to these regions up-regulates the transcription of p21^{waf1} (el-Deiry et al 1993). To evaluate the transcription and posttranscription activities of p53, the levels of transcript and protein for p21^{waf1} were investigated. At 33°C, relatively low levels of p21^{waf1} protein and mRNA were maintained in all cell lines (Fig 4D–F). At 39°C, remarkable elevation of protein and mRNA levels of p21^{waf1} was detected in TMmock cells, with levels approximately 15- and 7-fold higher than those at 33°C, respectively. These results suggest that activation of the p53-p21^{waf1} pathway occurs at the nonpermissive temperature condition. In contrast, these levels in cells overex-

Fig 3. Localization of large T antigen and Hsp70 in chaperone-overexpressing cell lines at 33 and 39°C. The cells were cultured at 33 and 39°C for 3 days. Immunofluorescence double-staining with antibodies against large T antigen (green; A, B, G, H, M, N, S, T) and Hsp70 (red; C, D, I, J, O, P, U, V) was performed. The merged patterns (yellow) of 2 signals are presented (F, L, R, X). Bar, 50 μm.

pressing Hsp70 alone or Hsp40 alone and Hsp70 plus Hsp40 were significantly decreased, with inhibition levels being approximately 40–60 and 80% compared to those in TMmock cells at 39°C, respectively. In particular, Hsp70 and Hsp40 in combination appeared to be the most effective for suppression of p21^{waf1} (Fig 4D–F).

DISCUSSION

It is well known that mutant tsSV40LT antigens are very useful in establishing conditionally immortalized cell lines with specific functions. Thus far, many kinds of conditionally immortalized cell lines have been established using infecting primary culture cells with a mutant oncogene or transgenic animals harboring a mutant oncogene (Jat and Sharp 1989; Noble et al 1995; Obinata 2001; Tabuchi et al 2005b, 2006). In almost all conditionally immortalized cell lines, when the culture temperature shifts from 33 to 39°C, conformational change and degradation of large T antigen and cell-growth arrest or cell differentiation are induced, with simultaneous induction of chaperones. The present results demonstrated that overexpressed exogenous human chaperone Hsp70, but not

endogenous induced chaperones, partially but significantly inhibited degradation and functional alterations of the mutant large T antigen induced by nonpermissive temperature in tracheal epithelial cell line TM02-3 harboring the tsSV40LT antigen.

HSPs are a group of evolutionarily highly conserved proteins and are induced by various stresses (Lindquist and Craig 1988; Smith et al 1998). HSPs including Hsp40 and Hsp70 act as molecular chaperones and are involved in the folding and intracellular transport of newly synthesized proteins, the passive prevention of protein aggregation, and active recovery of stress-induced nonnative proteins (Hartl and Hayer-Hartl 2002). Moreover, Hsp40 interacts and cooperates with Hsp70 (Michels et al 1997; Hartl and Hayer-Hartl 2002). Recent reports have indicated that Hsp70 and Hsp40 partially attenuated the accumulation of misfolded proteins formed by a mutant Huntington fragment (Muchowski et al 2000; Wacker et al 2004). In addition, it has been reported that Hsp70 and Hsp40 effectively suppress aggregate formation of truncated androgen receptor or mutant superoxide dismutase I in cultured neuronal cells (Kobayashi et al 2000; Takeuchi et al 2002). In the present study, overexpressed ex-

Fig 4. Levels of large T antigen, p53, and p21^{waf1} in chaperone-overexpressing cell lines at 33 and 39°C. The cells were cultured at 33 and 39°C for 3 days. (A–E) SDS-PAGE and Western blotting were performed. Signals were visualized by a luminescent image analyzer using an ECL system. (B, C, E) Bands were quantified by densitometry. Fold induction was normalized by G3PDH (B, T antigen; C, p53; E, p21^{waf1}). (F) Level of mRNA for p21^{waf1} was measured by using real-time qPCR. Fold induction was normalized by G3PDH. Data represent means $\pm$ SD for 3–4 different experiments. * P < 0.05 vs TMmock cells at 33°C; # P < 0.05 vs TMmock cells at 39°C; + P < 0.05 (Student's t -test).

ogenous human Hsp70 and/or Hsp40 significantly prevented the degradation and functional defects of the mutant large T antigen induced by nonpermissive temperature. In addition, the combination of Hsp70 with Hsp40 had a synergistic effect, as previously reported (Michels et al 1997; Kobayashi et al 2000; Takeuchi et al 2002). On the other hand, despite several kinds of endogenous chaperones such as Hsp40, Hsp70 and Hsp110 being induced by the temperature shift from 33 to 39°C in

TMmock cells, these induced chaperones could not prevent the degradation and functional alterations of the large T antigen. The expression levels of Hsp70 and Hsp40 of TM70+40 cells at 39°C were approximately 4- to 5-fold higher than those of TMmock cells at 39°C. These results suggest that appropriate amounts of chaperones may be necessary to inhibit such alterations induced by the temperature shift.

The mutant tsSV40LT antigen encoded by the tsA58

allele used here is expressed in nuclei at 33°C, but is rapidly misfolded and degraded at 39°C (DeCaprio et al 1988; Levine 1997). Under normal conditions, Hsp70 is present in the cytoplasm, whereas it is rapidly translocated to the nucleus in response to heat shock (Pelham 1984; Welch and Feramisco 1984; Chughtai et al 2001; Quan et al 2004). In the present study, either human Hsp70 or large T antigen was observed in the cytoplasm or nucleus at 33°C, respectively, and the colocalization of these proteins did not result in the detection of Hsp70-overexpressing cells (TM70 and TM70+40). Interestingly, Hsp70 was translocated from the cytoplasm to the nucleus and colocalized with T antigen at 39°C in the Hsp70-overexpressing cells. The SV40LT antigen consists of an amino-terminal J domain that can interact directly with Hsp70 family members (Sawai and Butel 1989; Srinivasan et al 1997; Sullivan and Pipas 2002). Recently, this protein was shown to be a DnaJ molecular chaperone (Sullivan and Pipas 2002). It has also been shown that Hsp40, like Hsp70, is observed in the cytoplasm or nucleus under normal or stress conditions, respectively (Hattori et al 1993). Furthermore, Hsp40 directly interacts with Hsp70 and its best-defined role is as a cochaperone for Hsp70 (Michels et al 1997; Hartl and Hayer-Hartl 2002). It therefore seems likely that the translocated Hsp70 and/or Hsp40 interact with misfolded large T antigen and refold it in the nuclei at a nonpermissive temperature. In the present study, Hsp70 and Hsp40 in combination appeared to be the most effective, but did not completely restore the structural stability and functional defects of the mutant large T antigen. It has been well known that the Hsp70/Hsp40 chaperone system is rapidly cycling (Hartl and Hayer-Hartl 2002). We speculate that large T antigens refolded by Hsp70/Hsp40 may partly unfold at the nonpermissive temperature. However, at present, few details are known about the mechanism by which Hsp70 or Hsp40 may influence the assembly of misfolded mutant large T antigens elicited by a nonpermissive temperature. Further studies are needed to clarify this mechanism.

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