

## Extensive Degradation of Mutant-Type D123 Protein is Responsible for Temperature-Sensitive Proliferation Inhibition in 3Y1tsD123 Cells

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**ABSTRACT.** A temperature-sensitive mutant of 3Y1, 3Y1tsD123, reversibly arrested in G1 phase of cell cycle at the restrictive temperature of 39.8°C, shows a single amino acid exchange in the D123 protein. In this study, we found that the D123 protein level in 3Y1tsD123, which was 1/8 of that in 3Y1 compared at the permissive temperature of 33.9°C, lowered to 1/4 after a shift to the restrictive temperature. During inhibition of protein synthesis with cycloheximide, the D123 protein level in 3Y1tsD123 decreased markedly depending on the incubation temperature, compared with that in 3Y1, indicating that the lowered levels of D123 protein in 3Y1tsD123 are due to its degradation. Unexpectedly, 2 stably temperature-resistant clones were isolated after transfection of SV-3Y1tsD123 (SV40-transformed 3Y1tsD123, which shows cell death instead of G1 arrest at the restrictive temperature) with the cDNA of the mutant-type (3Y1tsD123-derived) D123 protein. The D123 protein in both clones degraded extensively at both temperatures, suggesting that the overexpression of the mutant-type D123 protein exceeds its degradation. Both temperature-resistant clones contained higher levels of D123 protein at the restrictive temperature than did SV-3Y1tsD123 at the permissive temperature. We concluded that the lowered D123 protein level at the restrictive temperature induces the temperature-sensitive characteristics of 3Y1tsD123 and SV-3Y1tsD123.

**Key words:** D123 protein/3Y1/temperature-sensitive mutation/protein degradation/G1 arrest/cell death

In the temperature-sensitive mutants derived from the rat fibroblast line 3Y1, 3Y1tsD123 is reversibly arrested in the G1 phase of cell cycle at a restrictive temperature of 39.8°C (13). D123 cDNA (15) was cloned from an expression cDNA library by complementing the temperature-sensitive mutation in 3Y1tsD123. In 3Y1tsD123, a point mutation occurs in the D123 gene, which results in an amino acid exchange (A109 → V109) in the D123 protein composed of 336 amino acids (15). Over 80% of the D123 protein is located in cytoplasm (A. Okuda, unpublished data). The D123 protein level is lower in 3Y1tsD123 than in 3Y1 irrespective of incubation temperature (15). However, since 3Y1tsD123 can proliferate with a low level of D123 protein at the permissive temperature, more quantitative analysis is necessary to investigate the relation between the temperature-sensitive G1 arrest and

the decrease in the D123 protein level.

In this study, we first investigated whether the low level of the D123 protein in 3Y1tsD123 is caused by the instability of the protein due to the amino acid exchange. We then investigated whether the lowered D123 protein level is responsible for the temperature-sensitive G1 arrest of 3Y1tsD123.

### Materials and Methods

#### Cell culture

Rat diploid fibroblast line 3Y1-B clone 1-6 (9) (referred to as 3Y1); SV-3Y1-C66, an SV40-transformed derivative of 3Y1 (16) (referred to as SV-3Y1); 3Y1tsD123, a 3Y1-derived temperature-sensitive G1-cell-cycle mutant (13); and SV-3Y1tsD123-1, an SV40-transformed derivative of 3Y1tsD123 (14) (referred to as SV-3Y1tsD123) were used. Cells were routinely cultured with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum in 10% CO<sub>2</sub> incubators. Cultures were kept at 33.8±0.1°C (permissive temperature) when not specified, or 39.8±0.1°C (restrictive temperature).

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Abbreviation: HYB, hygromycin B.

### Mammalian expression plasmids

Mammalian expression plasmid of D123 cDNA of the wild-type (3Y1-derived) or the mutant-type (3Y1tsD123-derived) D123 protein was constructed using pcDE $\beta$ A vector (11) carrying a hygromycin B (HYB) marker.

### Transfection

To  $2.5 \times 10^5$  cells proliferating exponentially in a 3.3-cm dish, 4  $\mu$ g of plasmid DNA coprecipitated with calcium phosphate at pH 6.9 was added, followed by 15 h incubation at the permissive temperature in a 3% CO<sub>2</sub> incubator, as described by Chen and Okayama (3). After removal of the precipitate and additional 1-day incubation, cells were transferred to 8.5-cm dishes, followed by 2-day incubation under the permissive conditions. Temperature-resistant cell clones were isolated through 2-step selection, i.e., with HYB (100  $\mu$ g/ml) at the permissive temperature for 14 days, and at the restrictive temperature without HYB for an additional 10 days. The transfection efficiency was determined by the number of HYB-resistant clones developed during the first 14 days. The frequency of temperature-resistant clones was determined by the number of surviving clones after the subsequent 10 days.

### Preparation of monoclonal antibody to D123 protein

Recombinant human D123 protein was prepared using human D123 cDNA inserted into a pET vector as has been described (15). BALB/c mice were immunized by subcutaneously injecting 150  $\mu$ g of the recombinant protein emulsified in Freund's incomplete adjuvant 5 times at 2-week intervals. Spleen cells were collected 3 days after the last immunization. The spleen cells were fused with P3U1 cells, and cells producing the antibody to D123 protein were selected in HAT medium and ELISA as described (2). The culture medium of the selected cell line was used as an antibody to the D123 protein.

### Neutralization of antibody to D123 protein

Recombinant rat protein deleting the N-terminal 15 amino acid was prepared using the QIA expression system (Qiagen, Chatsworth, CA, USA) where a *kpnI-Eco47III* fragment of rat D123 cDNA was inserted into pQE-30 vector. The recombinant protein in the inclusion body was purified by SDS PAGE as described previously (15). The monoclonal antibody was neutralized by 3-times molecules of the recombinant rat D123 protein at 4°C for 12 h.

### Western blot analysis

Cells were solubilized with a sample buffer (50 mM Tris.Cl (pH 6.8), 2 mM EDTA, 2% SDS, 5% mercaptoethanol, 10% glycerol) and kept at -85°C until use. The lysate was boiled and electrophoresed through an 8% SDS-polyacrylamide gel, and transferred to nitrocellulose filter. D123 protein on the

filter was detected using the antibody to D123 protein and ECL Western Blotting Analysis System (Amersham International plc, Amersham Place, UK). The density of the D123 protein band was quantified by the NIH image program (NIH Image V1.56).

## Results

### Monoclonal antibody to D123 protein

D123 proteins in 3Y1 and 3Y1tsD123 cells were detected by Western blotting. As shown in Figure 1, the prepared monoclonal antibody to D123 protein detected a dense 46 kDa band in the lysate of 3Y1 cells (lane 4), but this was not the case using the monoclonal antibody blocked by rat D123 recombinant protein (lane 6). The 46-kDa band was faint in 3Y1tsD123 cells (lane 5). The amount of the protein of the 46-kDa band was estimated to be 4–8 times larger in a 3Y1 cell than in a 3Y1tsD123 cell (compare lane 5 with standard lanes 1–4). Similar results were obtained using another polyclonal antibody in the previous study (15). The doublet bands near 80 kDa was not detected by the polyclonal antibody (15).

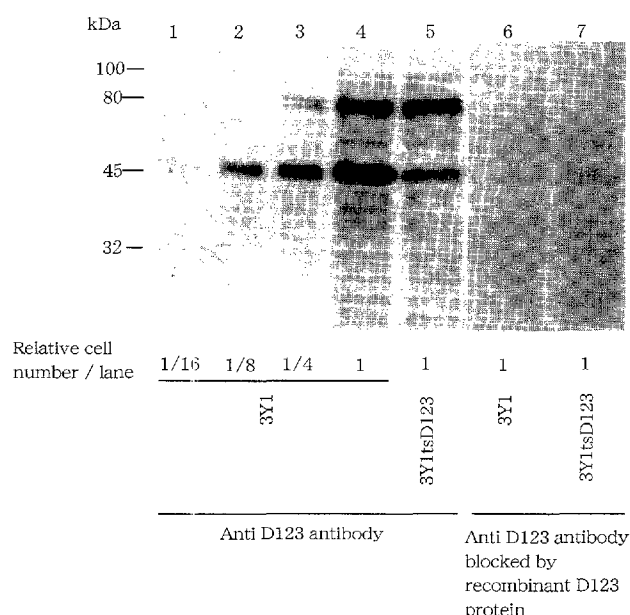


**Fig. 1.** Detection of D123 protein by monoclonal antibody. Lysates of 3Y1 and 3Y1tsD123 cells proliferating at 33.8°C were electrophoresed, and analyzed by Western blotting, using antibody to recombinant D123 protein and the antibody blocked by the D123 protein. To compare the D123 protein level between 3Y1 and 3Y1tsD123, lysates from varying numbers of 3Y1 cells were applied to lanes 1–4. The relative cell number 1 corresponds to  $10^5$  cells per lane.

### Instability of mutant-type D123 protein

As shown in Figure 2, in 3Y1tsD123 cells the amount of D123 protein decreased to as low as 1/8, when protein synthesis was inhibited by cycloheximide at the permissive temperature for 3 h (compare in the lower panel, lane 6 with standard lanes 2–5). Similar decrease was also observed at the restrictive temperature (lanes 7, 8 in the lower panel). It must be noted that the total D123 protein accumulated at the restrictive temperature was about 1/4 of that accumulated at the permissive temperature (compare in the lower panel, lane 7 with standard lanes 2–5). Such drastic decreases in the levels of the D123 protein were not seen in 3Y1 cells (upper panel).

### Very low frequency of temperature-resistant conversion of SV-3Y1tsD123, after transfection with mutant-type D123 cDNA

As shown in Table I, when SV-3Y1tsD123 cells were transfected with the 3Y1-derived cDNA of the wild-type D123 protein linked to the HYB-resistant transfection marker, over 80% of clones that expressed the HYB-resistance, were temperature-resistant. On the other hand, when SV-3Y1tsD123 cells were transfected with the 3Y1tsD123-derived cDNA of the mutant-type D123 protein, less than 3% of the clones that expressed the transfection marker were temperature-resistant. Although the frequency was very low, the appearance of temperature-resistant clones was unexpected, since transfection of cells carrying a defective mutation with the gene carrying the same mutation does not seem to overcome the mutation.

### Incomplete proliferating ability at restrictive temperature in ts2 and ts3, temperature-resistant clones obtained by transfection of SV-3Y1tsD123 with cDNA of mutant-type D123 protein

At the restrictive temperature, while 3Y1tsD123 cells are arrested at the G1 phase, SV-3Y1tsD123 cells die (14). The temperature-dependent cell death in SV-3Y1tsD123 is again seen in Figure 3.

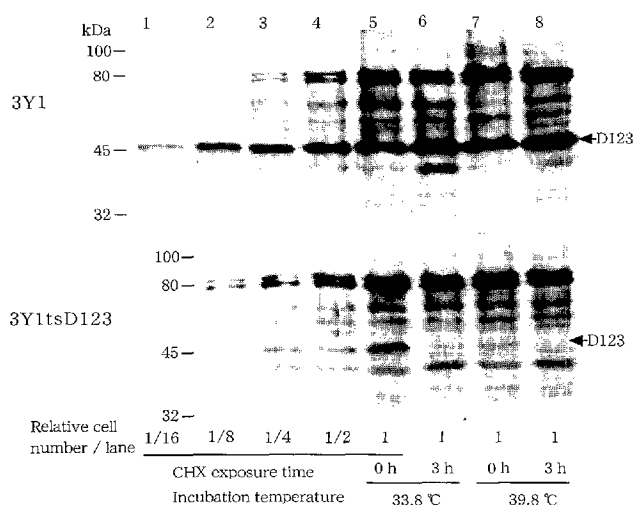


**Fig. 2.** Comparison of the degradation of D123 protein between 3Y1 and 3Y1tsD123. Duplicate cultures of 3Y1 and 3Y1tsD123 were prepared at 33.8°C or 39.8°C for 2 days. Then, at 0 h, one portion of the cultures was subjected to Western blotting analysis, and the other portion was exposed to 10  $\mu$ M cycloheximide (CHX) without changing the temperature for a further 3 h followed by Western blotting analysis. For quantitative comparison, lysates from varying numbers of cells prepared at 33.8°C were applied to lanes 1–5. The relative cell number 1 corresponds to  $2 \times 10^5$  cells per lane.

After transfecting SV-3Y1tsD123 cells with the cDNA of the wild-type or mutant-type D123 protein, colonies with HYB-resistant transfection marker were selected at the permissive temperature, and then temperature-resistant colonies were selected among them without reseedling. This mild selection allows picking up clones that are simply alive without proliferating activity at the restrictive temperature. As representative clones, we selected W24 and W26 among transfectants with the wild-type D123 cDNA, and ts2 and ts3 among transfectants with the mutant-type D123 cDNA. While W24 and W26 proliferate normally at the restrictive temperature, ts2 and ts3 were abnormal; on shifting up to the restrictive temperature the cell number increased 5-fold within 5 days, and then decreased (Fig. 3).

**Table I.** FREQUENCY OF TEMPERATURE-RESISTANT CLONES AFTER TRANSFECTION OF SV-3Y1tsD123 CELLS WITH CDNAs OF WILD- AND MUTANT-TYPE D123 PROTEIN

| Transfection dish number | Transfected with cDNA of | HYB-resistant clone/transfected cell (%) | Temperature-resistant clone/HYB-resistant clone (%) |
|--------------------------|--------------------------|------------------------------------------|-----------------------------------------------------|
| 1                        | Wild-type D123           | 0.13                                     | 85                                                  |
| 2                        | Wild-type D123           | 0.41                                     | 80                                                  |
| 3                        | Mutant-type D123         | 0.67                                     | 3                                                   |
| 4                        | Mutant-type D123         | 0.71                                     | 0                                                   |

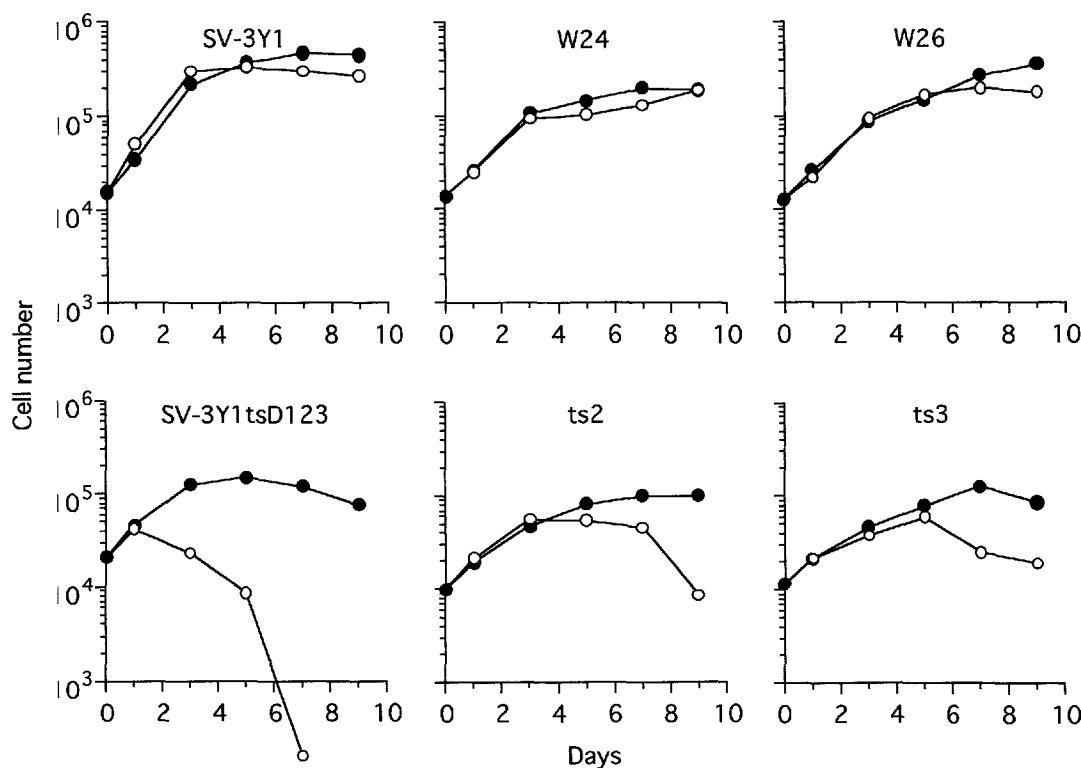


**Fig. 3.** Proliferation curves of temperature-resistant clones obtained by transfection of SV-3Y1tsD123 with wild-type (W24 and W26) and mutant-type D123 cDNA (ts2 and ts3). Cells were seeded in 1.9-cm<sup>2</sup> wells of 24-well multi dishes, incubated at 33.8°C for 1 day. Half of the dishes was shifted to 39.8°C at day 0 (○). The other half was kept at 33.9°C (●). Cell numbers were counted by Coulter counter at the indicated days.

#### **Higher D123 protein accumulation in ts2 and ts3 at the restrictive temperature than that in SV-3Y1tsD123 at the permissive temperature**

Figure 4 shows the D123 immunoblotting profiles of the lysates from SV-3Y1, SV-3Y1tsD123, W24, W26, ts2, and ts3 that grew at both permissive and restrictive temperatures. At the permissive temperature, the levels of 46-kDa D123 protein in W24, W26, ts2, and ts3 were comparable to or higher than that in SV-3Y1. At the restrictive temperature, the levels of 46-kDa D123 protein in W24, W26, ts2 and ts3 were higher than the level seen in SV-3Y1tsD123 at the permissive temperature. In W26 and ts2 the levels at the restrictive temperature were considerably lower than the respective levels at the permissive temperature. Another higher molecular-weight band of 50 kDa appeared in ts3 and faintly in ts2.

When ts3 cells reached a considerably high cell density at the restrictive temperature, they died (Figs. 3 and 5A). When ts3 cells began to die, they retained high D123 protein levels far above the levels observed in SV-3Y1tsD123 cells proliferating at the permissive tempera-

ture (Fig. 5B).

#### **Degradation rate of mutant-type D123 protein is temperature dependent in SV-3Y1tsD123 and ts2**

Figure 6 shows the degradation kinetics of D123 protein in SV-3Y1, SV-3Y1tsD123, ts2 and ts3. The mutant-type D123 protein degraded at both permissive and restrictive temperatures, in ts2 and ts3 as well as in SV-3Y1tsD123. On the other hand, degradation was not so marked in SV-3Y1, or in W24 and W26 (data not shown). These results clearly show that these transfected cell lines expressed the transfected cDNA.

The degradation rate was higher at the restrictive temperature than at the permissive temperature in SV-3Y1tsD123 and ts2. In ts3 significant difference was not seen. It must be noted that the higher molecular weight band (50 kDa) in ts3 also degraded rapidly, suggesting that this protein is a derivative of the D123 protein. The degradation of the 50-kDa protein was faster than that of 46-kDa protein in ts3 (Fig. 7).

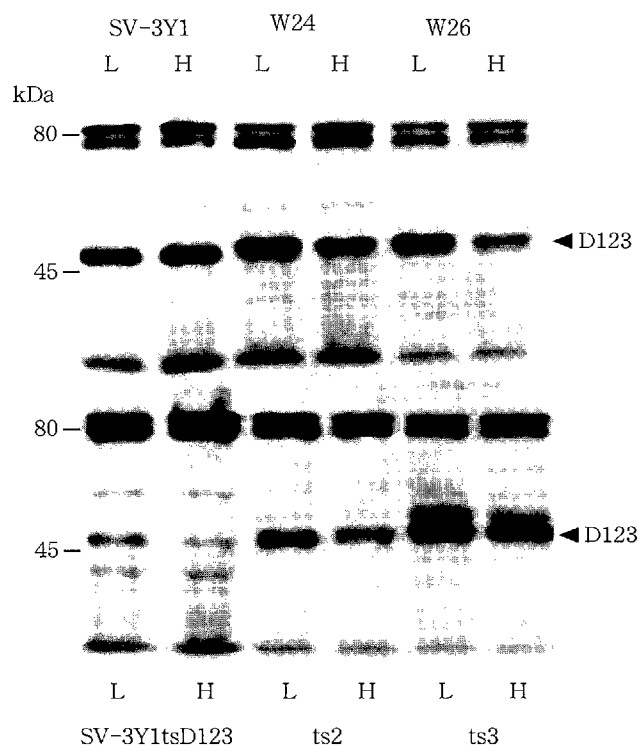


Fig. 4. D123 protein levels of temperature-resistant clones obtained by transfection of SV-3Y1tsD123 with wild-type (W24 and W26) and mutant-type (ts2 and ts3) D123 cDNA. Cells were kept at 33.8°C for 2 days (L), or shifted to 39.8°C for 2 days (H), and the lysate from  $10^5$  cells was applied to each lane.

## Discussion

The present study clearly showed that the low accumulation of D123 protein in 3Y1tsD123 and in SV-3Y1tsD123 cells observed at both permissive and restrictive temperatures is caused by the rapid degradation of the mutant-type D123 protein. On the other hand, ts2 and ts3, temperature-resistant clones of SV-3Y1tsD123 that were obtained by transfection with mutant-type D123 cDNA, maintained high levels of unstable D123 protein. The high production rate of the mutant-type D123 protein probably exceeds the degradation rate in those clones. The temperature-dependent degradation of D123 protein will cause temperature-dependent accumulation of D123 protein in SV-3Y1tsD123 and ts2.

Different from 3Y1tsD123 and SV-3Y1tsD123, ts2 and ts3 had another protein with a higher molecular weight of 50 kDa, detectable by the antibody against D123 protein. This protein also degraded rapidly. Therefore, the higher molecular-weight protein may be a modified product of the mutant-type D123 protein during the degradation process. In 3Y1tsD123 and SV-3Y1tsD123, this intermediate product will not be

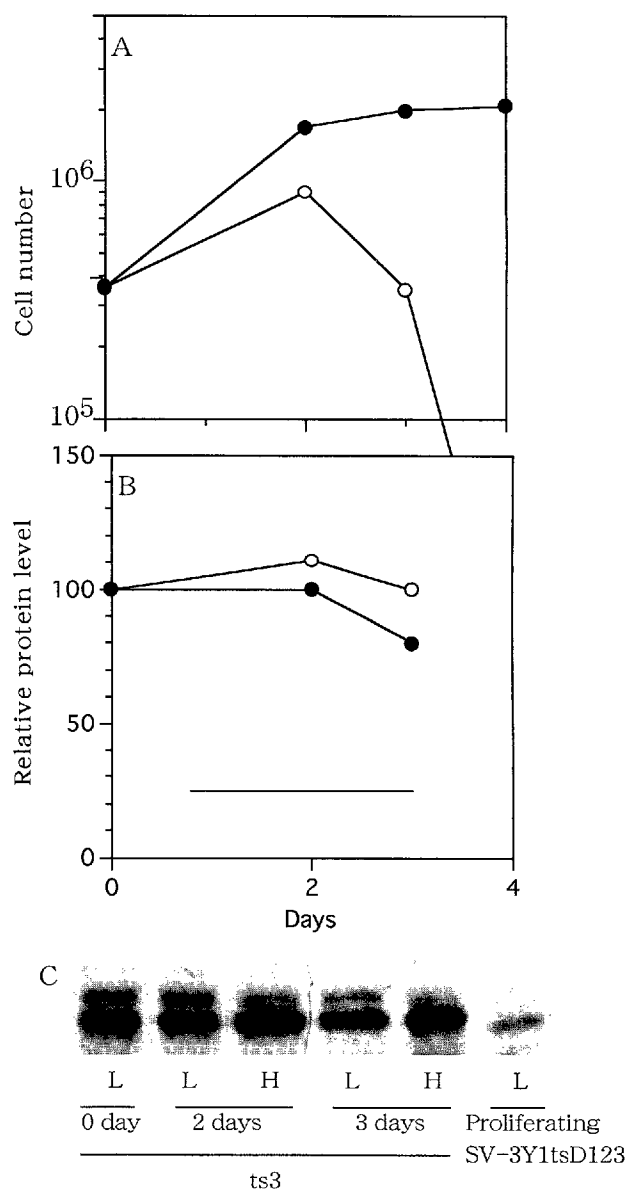


Fig. 5. No correlation was found between the killing of ts3 at restrictive temperature and D123 protein level. Cells were seeded in 21-cm<sup>2</sup> dishes, incubated at 33.8°C for 1 day. Half of the dishes was shifted to 39.8°C at day 0 (○, H). The other half was kept at 33.9°C (●, L). (A) Cell numbers were counted at the indicated days. (B) In parallel, lysates from  $10^5$  cells was analyzed for D123 levels, using Western blotting indicated in (C). D123 protein levels were determined by the densities of D123 protein bands (lower bands in (C)) using standard density curves of D123 protein bands obtained by varying the applied cell number as in lanes 1–4 in Figure 2. The relative D123 protein levels were normalized to 100 at 0 day. The horizontal line in the lower part of (B) indicates the D123 level in SV-3Y1tsD123 proliferating at 33.8°C.

detectable due to its low production rate that cannot cover its rapid degradation. In fact, the degradation of the 50-kDa protein was faster than the 48-kDa pro-

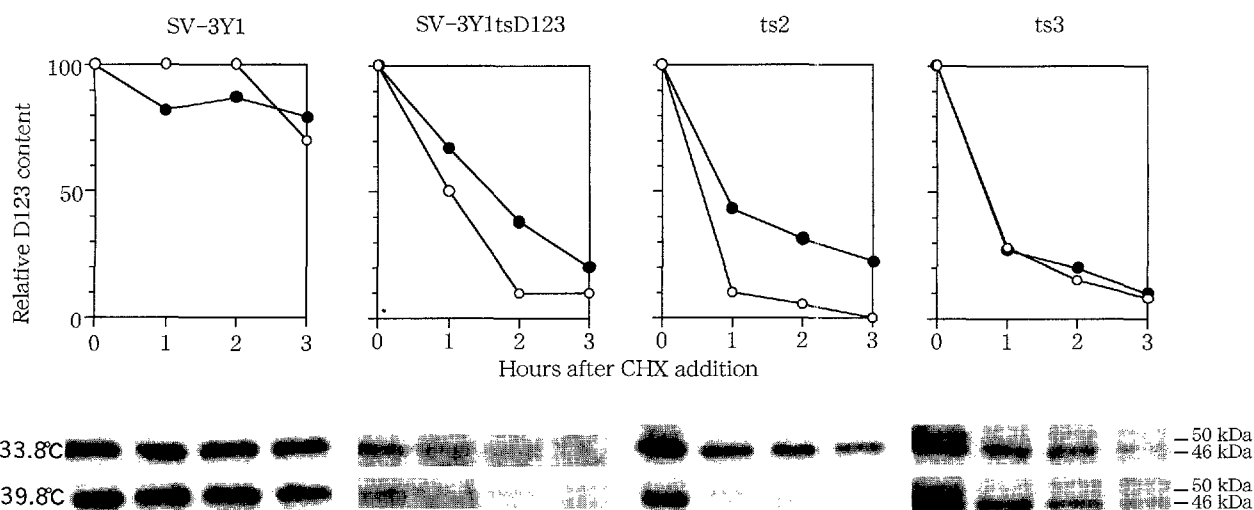


**Fig. 6.** The kinetics of D123 protein decay in SV-3Y1, SV-3Y1tsD123, ts2 and ts3. Cells were kept at 33.8°C for 2 days (●), or shifted to 39.8°C for 2 days (○). Ten  $\mu$ M cycloheximide (CHX) was then added at 0 h, incubation continued at the same temperature, and the lysates were prepared at the indicated times. The lysate of  $10^5$  cells was applied to each lane of the lower panel. Relative D123 protein levels in the upper panel were determined by the densities of D123 protein bands in the lower panel using standard density curves of D123 protein bands obtained by varying the applied cell number as in lanes 1–4 in Figure 2. The relative D123 protein levels were normalized to 100 at 0 h at each temperature.

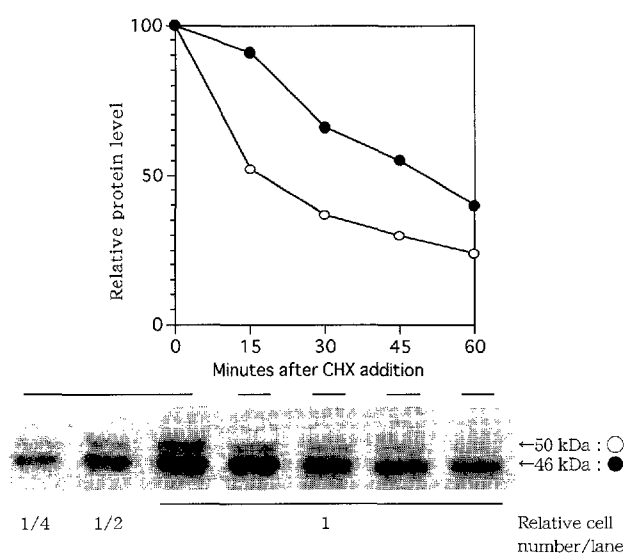


**Fig. 7.** The comparison of degradation rates of 2 types of D123 protein in ts3. Ten  $\mu$ M cycloheximide (CHX) was added to cells proliferating at 33.8°C, incubation continued at the same temperature, and the lysates were prepared at the indicated times. The lysate from  $10^5$  cells was applied to each lane of the lower panel. Relative D123 protein levels (●, 46 kDa; ○, 50 kDa) in the upper panel were determined by the densities of bands in the lower panel using the standard density curve of each band obtained by varying the applied cell number indicated in the lower panel. The relative D123 protein levels were normalized to 100 at 0 min.

tein in ts3. The modified product will not be the covalent intermediate between D123 protein and ubiquitin in the ubiquitin-proteasome protein-degradation process (reviews (1, 4, 7)). We inferred this by two findings; [1] the molecular weight difference between the 2 species of D123 protein (4 kDa) was less than that of ubiquitin (8.6 kDa), and [2] the higher molecular-weight species could not be detected by the anti-ubiquitin antibody (data not shown). However, this does not exclude the possibility that the mutant-type D123 protein degrades in the proteasome, because in some cases protein is degraded by proteasome without ubiquitination (8, 10). We recently observed that the degradation of D123 protein in ts3 is inhibited by lactacystin, a selective proteasome inhibitor (5, 12) (A. Okuda, unpublished data).

In 3Y1tsD123 and SV-3Y1tsD123, the D123 protein levels were extremely low at the restrictive temperature. On the other hand, the D123 protein levels in ts2 and ts3 at the restrictive temperature were much higher than the D123 protein level in SV-3Y1tsD123 at the permissive temperature. This was in parallel with their acquisition of temperature resistance. Therefore, the low D123 protein level is responsible for the temperature-sensitive behavior of 3Y1tsD123 and SV-3Y1tsD123. On the other hand, ts2 and ts3 proliferate incompletely at the restrictive temperature especially at high cell densities, compared to the clones of SV-3Y1tsD123 expressing additional wild-type D123 protein (W24 and W26). This suggests that the incomplete function of the mutated D123 protein at the restrictive temperature

is also responsible for the temperature-sensitive behavior of 3Y1tsD123 and SV-3Y1tsD123 at high cell densities.

SV40 large T antigen overcomes G1 arrests caused by various conditions, probably through inhibiting Rb or related protein function (review (6)). In contrast to temperature-sensitive arrest in G1 phase observed in 3Y1tsD123 cells, SV-3Y1tsD123 cells die at the restrictive temperature, without being arrested in a particular cell cycle phase (14). Therefore, we assumed that although large T antigen allows the cells to enter S phase without the function of D123 protein, the cells entering are destined to die. Abnormal entry into S phase without the function of D123 protein will cause cell death.

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