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A Hamster Temperature-Sensitive Alanyl-tRNA Synthetase Mutant Causes Degradation of Cell-Cycle Related Proteins and Apoptosis

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We have isolated a temperature-sensitive alanyl-tRNA synthetase mutant from hamster BHK21 cells, designated as ts ET12. It has a single nucleotide mutation, converting the 321st amino acid residue, 321Gly, to Arg. The mutation was localized between two RNA-binding domains of alanyl-tRNA synthetase. Thus far, we have isolated two temperature-sensitive aminoacyl-tRNA synthetase mutants from the BHK21 cell line: ts BN250 and ts BN269. They are defective in histidyl- and lysyl-tRNA synthetase respectively. Both mutants rapidly undergo apoptosis at the nonpermissive temperature, 39.5°C. ts ET12 cells, however, did not undergo apoptosis until 48 h after a temperature-shift to 39.5°C, while mutated alanyl-tRNA synthetase of ts ET12 cells was lost within 4 h. Loss of the mutated alanyl-tRNA synthetase was inhibited by a ubiquitin-dependent proteasome inhibitor, MG132, and by a protein-synthesis inhibitor, cycloheximide. Cell-cycle related proteins were also lost in ts ET12 cells at 39.5°C, as shown in ts BN250. In contrast, the mutated aminoacyl-tRNA synthetases of ts BN250 and ts BN269 were stable at 39.5°C. However, the defects of these mutants released EMAPII, an inducer of apoptosis at 39.5°C. No release of EMAPII occurred in ts ET12 cells at 39.5°C, consistent with the delay of apoptosis in these cells.

Kew words: apoptosis, ARS, EMAPII, protein-degradation.

Because it plays an essential role in the synthesis of proteins, the aminoacyl-tRNA synthetase should have appeared on Earth at the same time as proteins. Consistent with this idea, the structures of aminoacyl-tRNA synthetases are well conserved from prokaryotes to eukaryotes. Aminoacyl-tRNA synthetases attach a specific amino acid to their cognate tRNAs, thereby ensuring faithful translation of a genetic code. In addition to their enzymatic function, aminoacyl-tRNA synthetases regulate such cellular functions as tRNA export, ribosomal RNA synthesis, apoptosis, inflammation, and angiogenesis (*1–3*).

Conditional mutations, particularly temperature-sensitive mutations, have been valuable tools for clarifying genes essential for cell-proliferation (*4*, *5*). We have isolated a series of ts mutants for cell-proliferation from the hamster BHK21 cell line (*6–8*). These mutant cell lines were classified based on the ability of hybrid cells created by the fusion of different mutant cell-lines to grow at the nonpermissive temperature, 39.5°C. Recently isolated ts ET12 cells were found to be a novel ts mutant of the hamster BHK21 cell line. Subsequently, the gene responsible for the temperature-sensitive phenotype of ts ET12 cells was identified (*8*). It turned out to be the gene encoding the alanyl-tRNA synthetase (ARS). ts ET12 cells did not undergo apoptosis as rapidly as the previously isolated mutants defective in either histidyl- or lysyl-tRNA synthetase (HRS and KRS) (*9*). Temperature-sensitive mutants of mammalian aminoacyl-tRNA synthetases have been reported (*9–11*). These mutants can be suppressed by the addition of a cognate amino acid. Mutants of mammalian cultured cells defective in an aminoacyl-tRNA synthetase, thus, have been isolated as mutants that require a specific amino acid for cell-proliferation. However, the suppression of ts ET12 cells by alanine was weak, probably consistent with the fact that the mutated ARS was rapidly lost in tsET12 cells. Aminoacyl-tRNA synthetases form macromolecular protein complexes comprised of various auxiliary factors including p43/proEMAPII (*12*, *13*). In tsBN250 and ts BN269 cells, but not ts ET12 cells, EMAPII was rapidly released and p43 was degraded, consistent with the delay of apoptosis in ts ET12 cells. Taken together, our results suggest that a defect of aminoacyl-tRNA synthetase causes various biological phenotypes.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and ts⁺ Transformation—ts ET12, ts BN250, ts BN269, and wild-type (wt) BHK21 cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 10% CO_2 at 33.5°C, the permissive temperature. For the nonpermissive temperature, 39.5°C was used.

cDNAs carried on in pcDEBΔ (1 μg/92-mm dish) and as a control, pcDEBΔ vector were introduced into cells using the calcium phosphate co-precipitation method as described previously (*8*). Transfected cells were incubated at 33.5°C for 18 h, then washed with TD (Tris-buffered saline without Ca^{2+} and Mg^{2+}) and incubated for another 48 h. Transfected cells were then incubated

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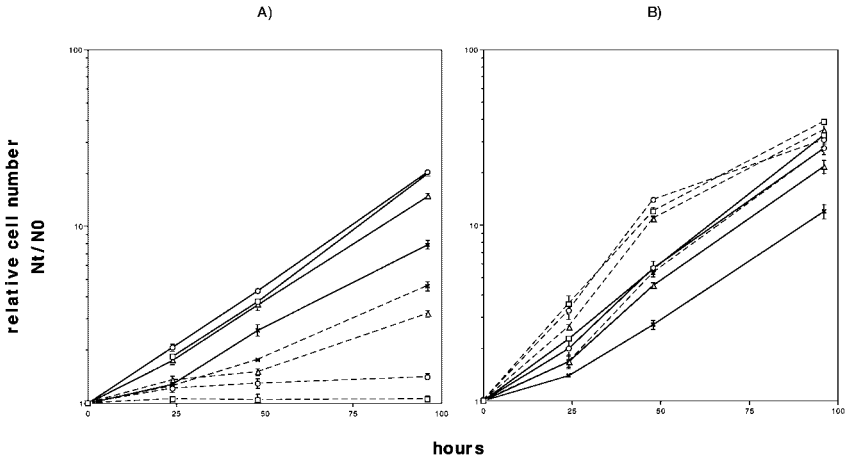


Fig. 1. **The growth of ts ET12 cells can be rescued by addition of a high concentration of alanine to DMEM medium.** Asynchronously growing ts ET12 cells (A) and as a control, wt BHK21 cells (B) were seeded at 1×10^5/92-mm dish and incubated at 33.5°C. After overnight incubation, medium was changed to fresh normal medium (squares) or medium containing (40 mM) (circles), 120 mM (triangles) or 240-mM (crosses) alanine, and incubation was continued at 33.5°C (solid line) or at 39.5°C (dotted line). At the indicated times, both adherent and floated cells were counted. At each time point, three dishes were counted, the average is plotted here, and the standard deviation (SD) was calculated.

either at 33.5°C in the presence of hygromycine (1.2 mg/ml) or at 39.5°C. Colonies appearing after incubation for 2 wk were fixed and stained for counting.

FACScan Analysis—Cells were removed from dishes with trypsin, washed twice with cold PBS, fixed with 50% methanol, treated with RNase A (50 μg/ml), and stained with propidium iodide (0.1% sodium citrate, propidium iodide 50 μg/ml) in preparation for FACScan analysis (Becton-Dickinson).

Southern Blot Analysis—Total cellular DNAs were digested with *Eco*RI, electrophoresed in a 0.8% agarose gel and transferred onto a nylon membrane. The filters were pre-hybridized at 42°C for 2 h in 5× SSC buffer containing 40% formamide, then hybridized with a ^{32}P-labeled human-ARS cDNA at 42°C overnight.

Isolation of cDNA Clones—Human ARS cDNA: Total cellular RNA prepared by the guanidine method as described previously (*14*) was reversibly transcribed using a first-strand cDNA synthesis kit (Amersham

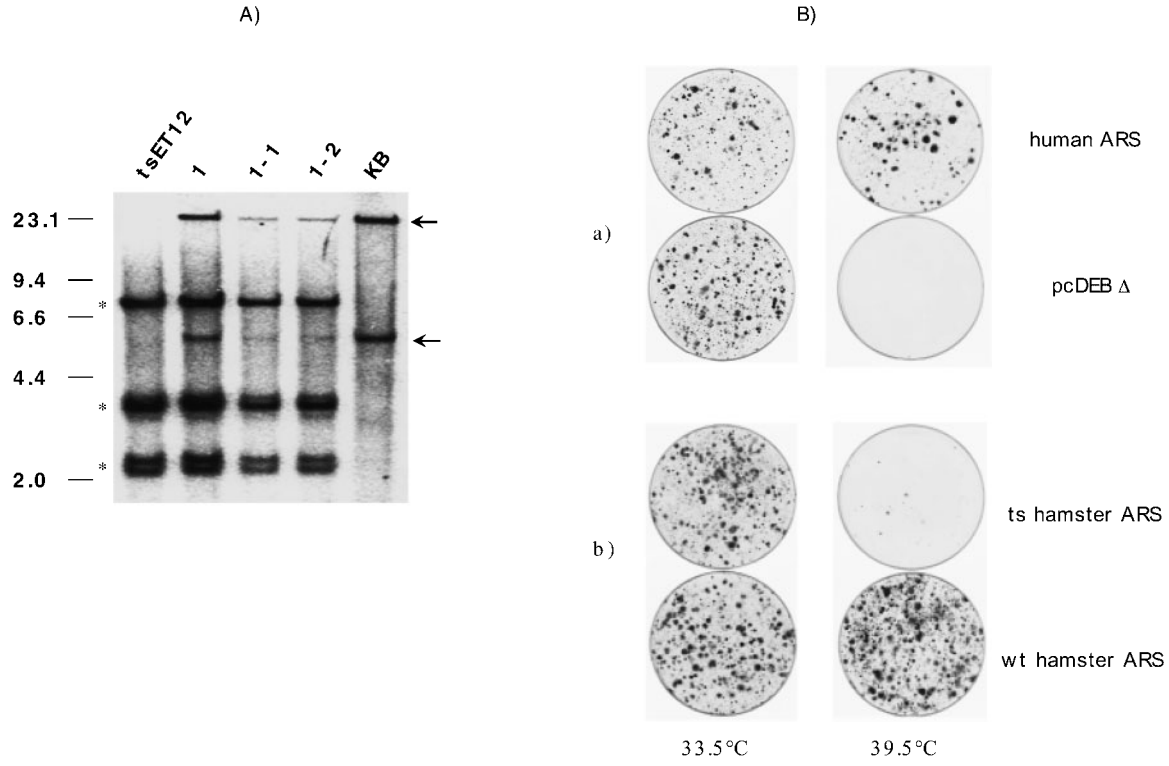


Fig. 2. **Human and hamster ARS gene rescued a temperature-sensitive lethality of ts ET12 cells.** (A) Human ARS gene conserved through ts$^+$ transformation of ts ET12 cells. Total cellular DNA (20 μg) extracted from the primary No. 1, the secondary No. 1-1 and 1-2 ts$^+$ transformants, and as controls from ts ET12 and human KB cells (KB), were digested with *Eco*RI, and analyzed by Southern hybridization using ^{32}P-labeled Human ARS cDNA as a probe. Asterisks indicate the DNA fragments of hamster *ARS* gene and arrows indicate the DNA fragments of human *ARS* gene. (B) Human ARS cDNA and the vector (pCDEBΔ) alone were transferred into cultures of ts ET12 cells (2×10^5/100-mm dish) as indicated (a), or with hamster wt and mutant ARS cDNAs as indicated (b). After incubation at 33.5°C for 36 h, cells were cultured in selection medium containing hygromycin (1.2 mg/ml) at 33.5°C or 39.5°C for about 2 weeks. Cells were fixed and stained with 0.5% crystal violet.

Fig. 3. **Alignment of ARS.** (A) The amino acid sequences of human, *S. cerevisiae* and *E. coli* ARS were derived from sequences with the following GenBank accession numbers: human, I60107; *S. cerevisiae*, NP_014980; *E. coli*, E85917; and hamster, AB097816. The color indicates the position of domains I-III conserved through the class II aminoacyl-tRNA synthetases, RNA-binding region for tRNA-acceptor stem (two α-helixes), and the DHHA1 domain shown in panel (B). (B) Model of ARS. The red characters indicate the position of the mutation site in the ARS of ts ET12 cells, and the red arrows indicate the position of the primers used for amplification of human and hamster ARS cDNA fragments with PCR.

Pharmacia Biotech) following the supplier's recommendations. Transcribed cDNAs were amplified with the KOD polymerase using the primer 1 (5′-ATG GAC TCT ACT CTA ACA GCA AGT-3′), and primer 5 (5′-AGA CAT AGG ACT GCT CCC AAG TG- 3′). The resulting DNA was further amplified with the Taq and 1/20 pfu polymerase using primers 1 and 2 (5′-TTT TCC TGT CCA GGA TGC GAC-3′), or primers 3 (5′-GCA TTT CCT GAG CTG AAG AAG GA-3′) and 4 (5′-ACT CAG TTC TTT ACA TCC CC GA-3′). The positions of these primers on ARS are shown by arrows in Fig. 3B. The resulting cDNA fragments were ligated at the *Ase*I site using pCR2.1 vector, then inserted into the *Bam*H1/*Xba*1 sites of pcDEBΔ vector.

wt hamster ARS cDNA: A cDNA library of wt BHK21 cells constructed in the λgt10 vector (*15*) was screened by using human ARS cDNA as a probe, and the resulting cDNA was subcloned into pZERO™-2 vector, and then pcDEBΔ vector.

Table 1. **Number of colonies/dish.**

Transfected DNA	33.5°C*	39.5°C
Human ARS	38.6 ± 5.8	36.6 ± 2.4
pcDEBΔ	116.0 ± 16.7	0 ± 0
ts Hamster ARS	118.4 ± 13.7	3.2 ± 1.9
wt Hamster ARS	132.0 ± 11.0	108.0 ± 6.3

Cell numbers were counted based on the average of five dishes and the standard deviation was calculated. *The number of colonies appearing in the presence of hygromycin (1.2 mg/dish).

ts mutated ARS cDNA: ts ET12-ARS cDNA was prepared by RT-PCR as described above using the pfu-turbo polymerase (Invitrogen, CA) together with primer 6 (5′ CCC TGA GGT GCC TTT CAA GATG 3′) and primer 7 (GAC AGG CTT AGC TGC CTC AATC), or primer 8 (5′ ATG CTG GAC ATT TAT GCT ATCGA 3′) and primer 9 (5′ CAG GTC CAA GGT ATG CTC CAAG). The resulting DNA fragments were amplified with primers 7 and 10 (5′ GCA TCT GAG GGA GCA GTTC 3′), and cloned into pZERO™-2 vector, and then into pcDEBΔ vector. The positions of these primers on ARS are shown by arrows in Fig. 3B.

Immunoblot Analysis—Cells were lysed in buffer containing 62.5 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, and 10% glycerol. After electrophoresis on a 5–20% SDS–polyacrylamide slab gel (SDS-PAGE) (ATTO, Japan), proteins were transferred to a polyvinylidene difluoride membrane (PVDF) (Millipore, Mass) in Tris-glycine buffer (0.375 M Tris-HCl, pH 7.5, 0.192 M glycine, 20% methanol). The resulting membrane was blocked with 3% solution of nonfat milk in TBS buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 0.2% Tween 20) for 2 h at room temperature or overnight at 4°C, then treated with the indicated antibodies in TBS buffer for 1 h at room temperature or overnight at 4°C with shaking. Proteins were visualized with the ECL Western blotting protocol (Amersham Pharmacia Biotech) using a horseradish peroxidase-conjugated secondary antibody.

Antibodies used in this paper were as follows: rabbit anti-hamster ARS (raised against the GST-fused ARS-peptide 651–969 in rabbit); anti-human Cdk4 (PharMingen); anti-human Cyclin D1(BD Biosciences); anti-human Cyclin D3 (Santa Cruz); anti-mouse p21(Santa Cruz); anti-hamster Cyclin A (*11*); anti-hamster Cdc2 (*16*); anti-Cyclin B1 (*17*); anti-PCNA (Santa Cruz); anti-α-tubulin (Sigma); anti-human histidyl-tRNA synthetase (HRS) (US Biological); anti-human lysyl-tRNA synthetase (KRS) (given by Dr. K. Shiba) (*18*). The following antibodies were provided by Dr. S. Kim (*13*): anti-isoleucyl-tRNA synthetase (IRS); anti-methionyl-tRNA synthetase (MRS); anti-arginyl-tRNA synthetase (RRS); anti-leucyl-tRNA synthetase (LRS); anti-tryptophanyl-tRNA synthetase (WRS); anti-glutamyl-prolyl-tRNA synthetase (EPRS); anti-tyrosyl-tRNA synthetase (YRS) and anti-p43.

Gel Filtration Chromatography—Cells were harvested, washed twice in cold PBS, then subjected to freezing at –80°C and thawing in buffer A [20 mM HEPES-KOH buffer, pH 7.4, containing 0.5 mM spermine, 130 mM KCl, 1% thiodiglycol, 0.5 mM EDTA, 2 mM $CaCl_2$, and protease inhibitor cocktail (1 tablet/50 ml solution)] (Roche) as described (*19*). They were then incubated on ice for 15 min with occasional stirring, followed by centrifugation at 10, 000 ×*g* for 30 min. The resulting supernatant was applied to a column of Sephacryl S300 (separation range, 10–1,500 kDa) in an FPLC director™ version 1.10 system (Pharmacia Biotech) that had been equilibrated with 50 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 0.2 mM dithiothreitol, 0.2 mM EDTA, and 100 mM NaCl. The fractionated proteins were resolved by SDS-PAGE and subjected to immunoblotting analysis with the indicated antibodies.

RESULTS

Lethality of ts ET12 Cells Was Rescued by Addition of Alanine—A new series of temperature-sensitive (ts) mutants was isolated from wt BHK21 cells using the reported method (*6–8*). The ts lethality of aminoacyl-tRNA synthetase mutants can be rescued by the addition of a cognate amino acid (*9–11*).

Based on this fact, a series of essential amino acids was added to the medium in order to screen mutants defective in an aminoacyl-tRNA synthetase, as the first examination of newly isolated ts mutants. The addition of alanine rescued the ts lethality of ts ET12 cells in a dose-dependent manner (Fig. 1). The concentration of cognate amino acid required to rescue the ts phenotype of ts ET12 was higher than that required for the previously isolated ts BN250 and ts BN269, which are defective in HRS and KRS, respectively (*9*, *11*). In parallel, total human DNAs were extracted from human KB cells and transfected into ts ET12 cells. From the resulting ts$^+$ transformant of ts ET12 cells, designated as No. 1, which grew at 39.5°C, total cellular DNAs were extracted and transferred into ts ET12 cells to obtain the secondary ts$^+$ transformants No. 1-1 and No. 1-2. The finding that alanine rescued the ts lethality of ts ET12 cells indicated that the hamster gene encoding ARS might be defective in ts ET12 cells. To confirm this, total DNAs were extracted from ts ET12 cells, ts$^+$ transformants, No. 1, No. 1-1, and No. 1-2, and KB cells and analyzed by Southern-blot hybridization using a cDNA encoding the human ARS as a probe. As shown in Fig. 2A, the DNA fragments of the human ARS were conserved through the first and second ts$^+$ transformations of ts ET12 cells. The cDNA encoding human ARS, which was amplified by PCR, rescued the ts lethality of ts ET12 cells (Fig. 2B, Table 1).

ts ET12 Cells Have a Single Point Mutation in the Hamster ARS—The next question is whether the ts ET12 cell line carries a mutation in the hamster ARS. To address this question, the cDNA encoding the hamster ARS was isolated from the cDNA library of parental wt BHK21 cells by using a cDNA encoding human ARS as a probe. The resulting hamster ARS cDNA fragments were sequenced, and hamster ARS was found to be 95.9% identical at the amino acid level with human ARS (deposited as No. AB097816, GenBank). Subsequently, the cDNA of the hamster ARS was amplified from ts ET12 cells as described in "MATERIALS AND METHODS." Compared with ts ET12-ARS, glycine of the 321st codon in the amino acid sequence of the wt hamster ARS was found to be changed to arginine, which is localized between two RNA-binding domains of ARS (Fig. 3). When transfected, the cDNA encoding the wt hamster ARS rescued the ts lethality of

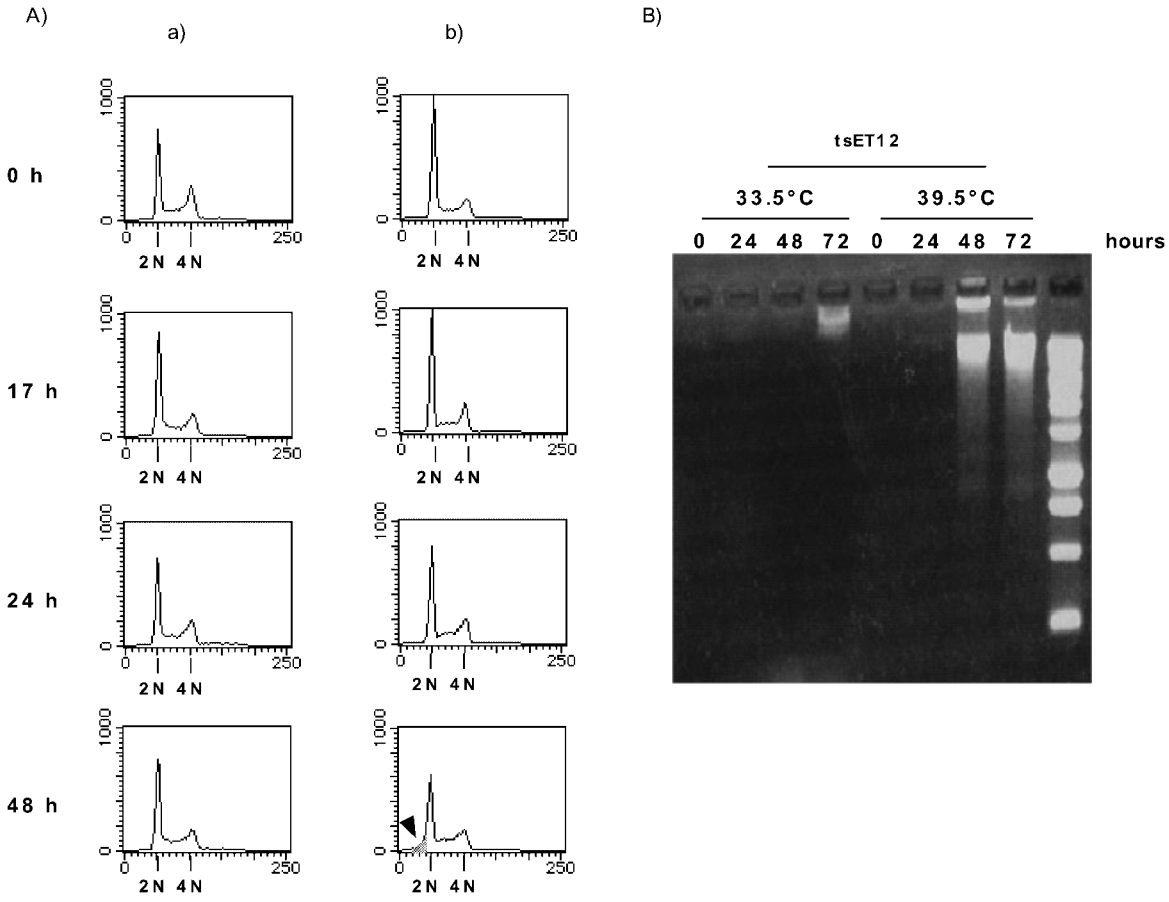


Fig. 4. **Apoptosis in ts ET12 cells.** (A) Cultures of wt BHK21 (a) and ts ET12 cells (b) (5×10^5/92-mm dish) were incubated at 39.5°C. At the indicated times (h: hour), cells were prepared for FACScan analysis. Cells undergoing apoptosis are marked as a shaded area as indicated by an arrowhead in the figure. The vertical axes show cell numbers in arbitrary units. The horizontal axes show DNA content. (B) DNA fragmentation of ts ET12 cells at 39.5°C. Cultures of ts ET12 cells (5×10^5/92-mm dish) were incubated either at 33.5°C or at 39.5°C. At the indicated times, cellular DNA was electrophoresed on 2% agarose gel, then stained with ethidium bromide. The right column shows molecular-size markers of DNA.

ts ET12 cells, but the cDNA encoding the ts mutated hamster ARS, as expected, did not (Fig. 2B, b and Table 1).

Apoptosis Was Induced in ts ET12 Cells, but Its Appearance Was Very Slow Compared to the Defect of HRS or KRS—The aminoacyl-tRNA synthetase mutants of BHK21 cells that were previously identified rapidly underwent apoptosis at 39.5°C, the nonpermissive temperature (*9*). Based on these findings, we examined whether ts ET12 cells underwent apoptosis at 39.5°C. Cultures of wt BHK21 and ts ET12 cells were prepared at 33.5°C, then incubated at 39.5°C. At the indicated times, cells were subjected to fluorescence-activated cell sorting (FACS) analysis. As shown in Fig. 4A, ts ET12 cells containing a smaller amount of DNA than cells in the G1-phase started to accumulate after incubation at 39.5°C for 48 h. At the same time, the cellular DNA fragmentation was observed in ts ET12 cells at 39.5°C, but not at 33.5°C (Fig. 4B), indicating that ts ET12 cells underwent apoptosis after incubation at 39.5°C for 48 h. This finding contrasts with the cases of ts BN250 and ts BN269 cells, which underwent apoptosis at the permissive temperature upon depletion of the cognate amino acid (*9*).

Cell Cycle Related Proteins Were Degraded in ts ET12 Cells at 39.5°C—Either depletion of a cognate amino acid or the defect of tRNA synthetase was thought to cause the accumulation of aberrantly folded proteins, which might enhance apoptosis. In this regard, we examined whether the mutated ARS was stable at 39.5°C. Cultures of ts ET12 cells and, as a control, wt BHK21 cells, were prepared at 33.5°C. Half of each culture was then incubated at 33.5°C, and half at 39.5°C. Total cell-extracts were prepared at the times indicated in Fig. 5A and subjected to the immunoblotting analysis using antibodies against the indicated proteins. The amount of ARS rapidly decreased in ts ET12 cells after the temperature-shift to 39.5°C. In contrast, the amount of ARS was not changed in wt BHK21 cells at 39.5°C, suggesting that the mutated ARS became unstable in ts ET12 cells at 39.5°C. Notably, other proteins that are involved in the cell cycle regulation became unstable in ts ET12 cells at 39.5°C, similar to the mutated ARS. For example, the amounts of Cyclin D1, Cyclin D3, p21, Cdc2 and Cyclin B1 were significantly reduced in ts ET12 cells after the temperature-shift to 39.5°C (Fig. 5A). These proteins are reported to be degraded by ubiquitin-dependent proteasomes (*20–23*). To confirm this, an inhibitor of ubiquitin-dependent pro-

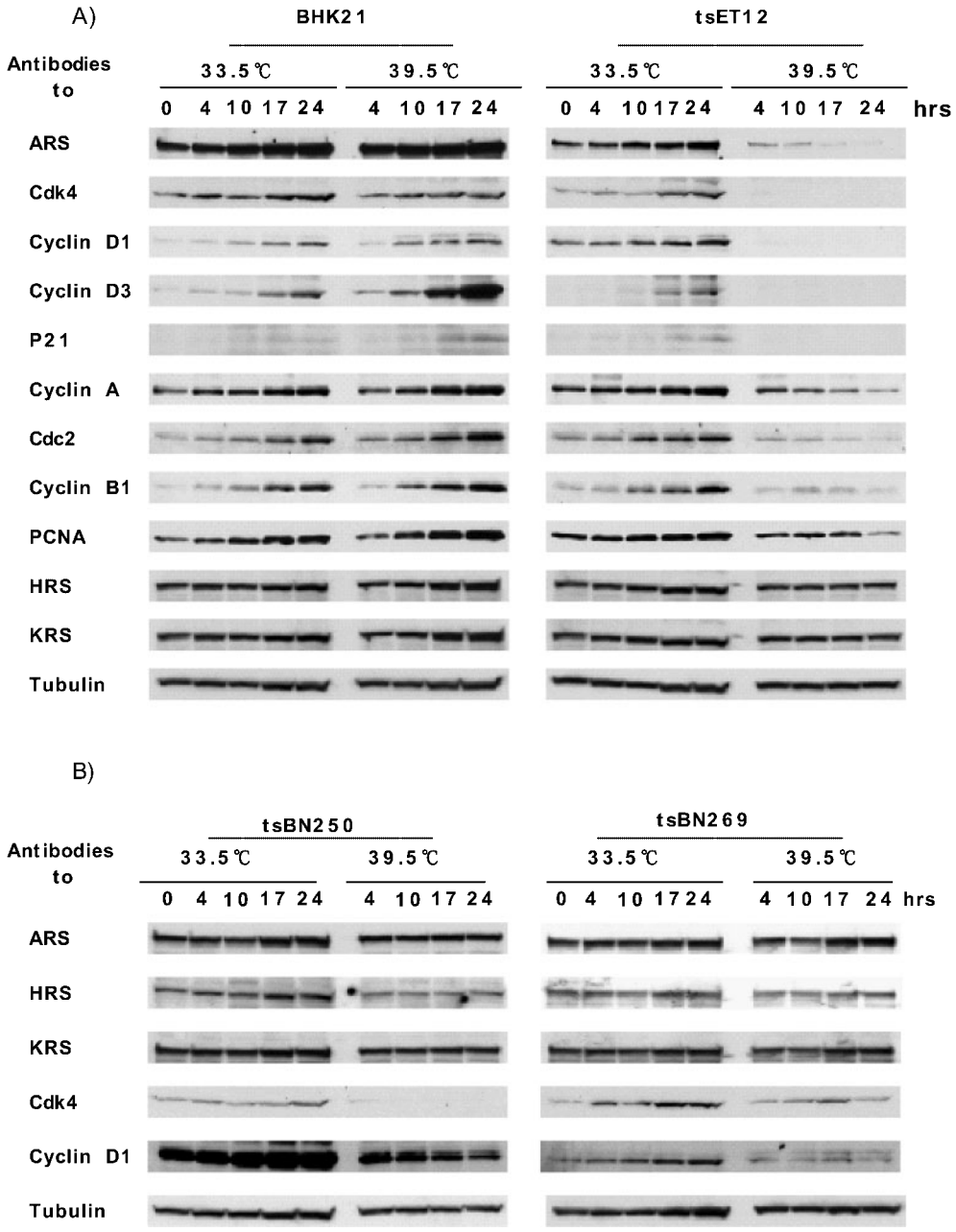


Fig. 5. **The stability of cellular proteins.** Cultures (5×10^5/92-mm dish) of wt BHK21 and ts ET12 cells (A), and ts BN250 and ts BN269 cells (B) were incubated at either 33.5°C or 39.5°C. At the indicated times, cells were collected and lysed by addition of 250 μl of sample buffer as described in "MATERIALS AND METHODS." After separation by SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane and subjected to immunoblotting analysis using antibodies to the indicated proteins.

teasomes, MG132, was added to cultures of ts ET12 cells, which were then incubated at 39.5°C. As shown in Fig. 6A, the amount of ARS did not decrease in the presence of MG132, compared to the case of addition of DMSO, which was used to dissolve MG132 in medium. In addition to ARS, both Cdk4 and Cyclin D1 were stabilized in ts ET12 cells at 39.5°C by the addition of MG132 (Fig. 6A), proving that cell cycle related proteins including the mutated ARS was degraded by ubiquitin-dependent proteasomes in ts ET12 cells at 39.5°C.

To examine whether the ubiquitin-dependent proteasomes were activated in ts ET12 cells at 39.5°C, we utilized the green fluorescent protein (GFP)-based substrates that allow rapid quantification of ubiquitin-proteasome-dependent proteolysis (*24*). Two N-end rule substrates of jellyfish GFP proteins were expressed in ts ET12 cells. As shown in Fig. 6C, at both permissive and non-permissive temperatures, jellyfish GFP proteins were not degraded, in contrast to the previous report (*24*). Under the same conditions, the mutated ARS was degraded and stabilized by the addition of MG132 (Fig. 6C). These results suggested that the ubiquitin-dependent proteasomes were not significantly activated by the defect of ARS.

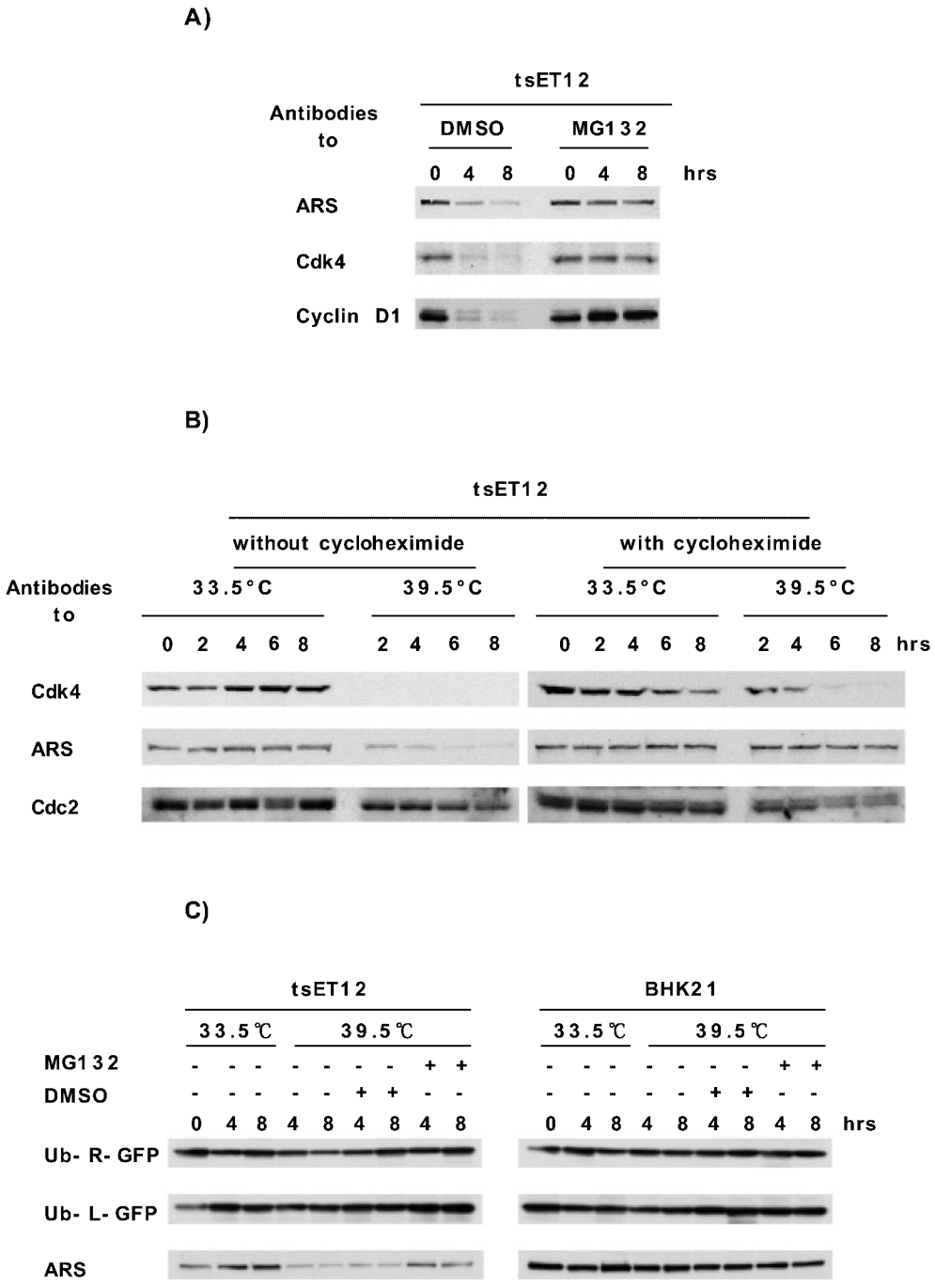
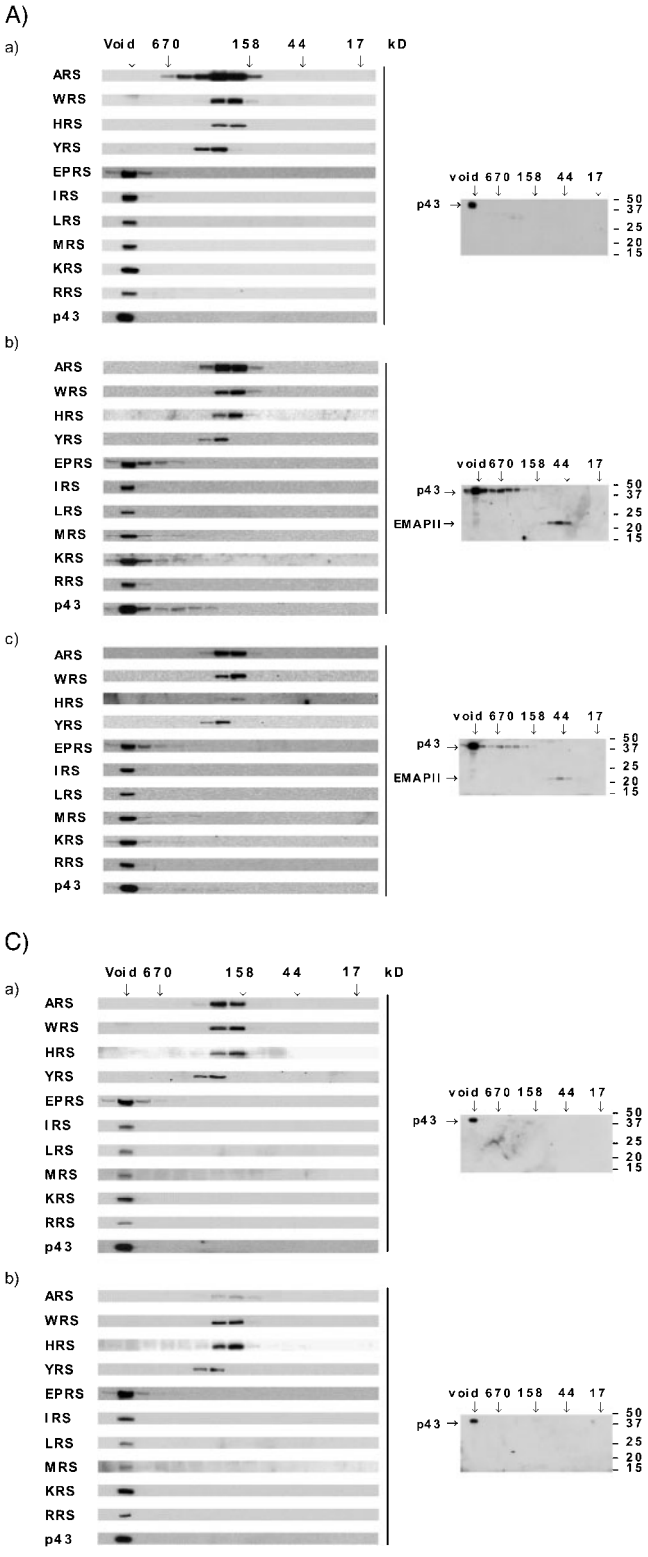


Fig. 6. **Ubiquitin-dependent protein degradation was activated in ts ET12 cells.** Cultures of ts ET12 cells (5×10^5 cells/92-mm dish) were incubated at 39.5°C in the presence of DMSO alone or 50 μM MG132 dissolved in DMSO. At the indicated times, cells were harvested, lysed and analyzed by immunoblotting using antibodies against the indicated proteins. (B) Cultures of ts ET12 cells were incubated at 33.5°C or 39.5°C in the presence or absence of 10 μg/ml cycloheximide. At the indicated times, cells were harvested, lysed and analyzed by immunoblotting using antibodies against the indicated proteins. (C) Cultures of ts ET12 cells and BHK21 cells which were transiently transfected with Ub-R-GFP or Ub-L-GFP vectors were incubated for two days, and then at 33.5°C or 39.5°C in the presence of DMSO alone or 50 μM MG132 dissolved in DMSO. At the indicated times, cells were harvested, lysed and analyzed by immunoblotting using antibodies against the indicated proteins.

In addition to MG132, a protein synthesis inhibitor, cycloheximide, also blocked the degradation of the mutated ARS in ts ET12 cells at 39.5°C (Fig. 6B). Curiously, the mutated HRS and KRS of ts BN250 and ts BN269 cells were not broken down by the temperature-shift to 39.5°C (Fig. 5B).

EMAPII Was not Released in ts ET12 Cells—Recently, the C-terminal region of human YRS has been reported to be homologous to the endothelial monocyte-activating polypeptide II (EMAPII) (*25*). p43, the auxiliary component of aminoacyl-tRNA synthetase complex, was subsequently reported to be identical to pro-EMAPII (*26*).



Thus, if EMAPII appeared upon cleavage of p43 or YRS, due to the mutated aminoacyl-tRNA synthetase, cells which have a defect in an aminoacyl-tRNA synthetase could undergo apoptosis. The efficiency of the aminoacyl-tRNA synthetase mutants undergoing apoptosis might reflect whether or not p43 was cleaved due to the mutation, because HRS, KRS and ARS have no homologous

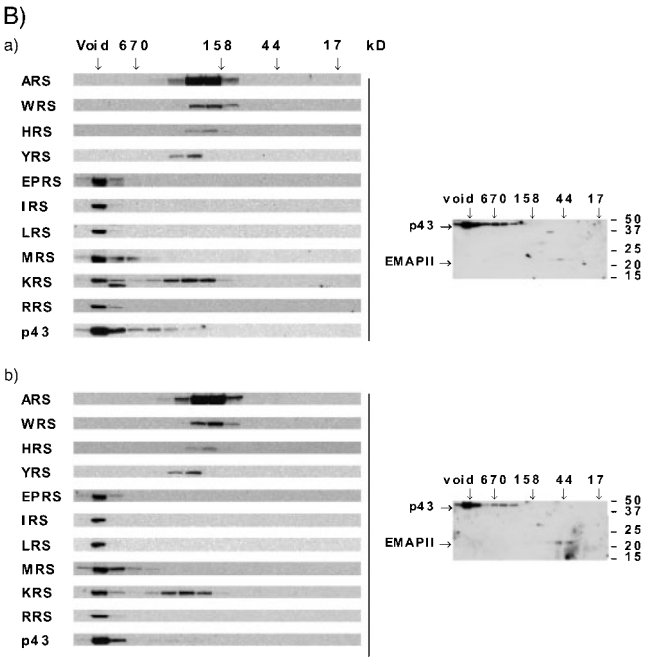


Fig. 7. **Complexes of aminoacyl-tRNA synthetases.** (A) Cultures of wt BHK21 (a) and ts BN250 (b and c) (5×10^6/150-mm dish) were incubated either at 33.5°C (b) or at 39.5°C (a, c). After 24 h, total cellular proteins (0.5 mg/ml) were extracted, applied to the gel chromatography of Sephacryl S-300. Resulting fractions were resolved in SDS-PAGE and subjected to immunoblotting analysis with antibodies against the indicated aminoacyl-tRNA synthetases and p43. (B) Cultures of ts BN269 cells (5×10^6/150-mm dish) were incubated either at 33.5°C (a) or at 39.5°C (b). After 24 h, total cellular proteins (0.5 mg/ml) were extracted and processed as described above. (C) Cultures of ts ET12 cells (5×10^6/150-mm dish) were incubated either at 33.5°C (a), or at 39.5°C (b). After 24 h, total cellular proteins were extracted and processed as described above. The inserted figures indicate the long exposure of p43. The position of molecular mass is shown in the right column. Arrows indicate the position of p43 and EMAPII.

domain to EMAPII. To address this question, a series of cultures of wt BHK21, ts ET12, ts BN250, and ts BN269 cells were prepared at 33.5°C and half of each culture was incubated at 33.5°C and half at 39.5°C. After incubation for 24 h, total cell extracts were prepared and subjected to gel chromatography on Sephacryl S-300. The resulting fractions were resolved by SDS-PAGE and subjected to immunoblotting analysis using the indicated antibodies (Fig. 7). In wt BHK21 cells incubated at 39.5°C, KRS and p43 were fractionated in the void fraction with molecular mass of more than 670 kDa, while ARS and HRS were fractionated between 670 kDa and 158 kDa, as previously reported (*13*). As expected, p43 was broken down and EMAPII appeared in ts BN250 and ts BN269, both of which rapidly underwent apoptosis (Fig. 7, A and B). In ts BN250, EMAPII appeared even at 33.5°C, consistent with the earlier report that this mutant underwent apoptosis at 33.5°C (*9*). In ts ET12, in contrast, p43 was not broken down, while the complex of ARS disappeared after the temperature-shift to 39.5°C (Fig. 7C). Thus, the ratio of tsET12 cells showing apoptosis seemed to be correlated with the cleavage of p43 and the appearance of EMAPII.

DISCUSSION

Thus far, we have identified the mutated genes of 11 ts mutants of the hamster BHK21 cell line (*8*, *9*, *11*, *27*, unpublished results). Five of them are localized on the X chromosome. The isolation of these mutants is consistent with the argument that a recessive mutation could appear by a single-step mutation from a haploid dose of cells, since the mammalian X chromosome is expressed in a haploid dose. Five of them, however, have a mutation on the autosomal chromosomes. These are ts BN2, ts BN250, ts BN269, and ts BN7 (*8*, *9*, *11*), in addition to the presently identified ts ET12 cell line. All of these mutants are recessive, since hybrid cells created by fusion of different mutant cell-lines can survive at 39.5°C. We previously thought that the mutants of mammalian cultured cell lines, which rapidly enter apoptosis following a temperature-shift to 39.5°C, could be preferentially concentrated by the FuDR method, since these mutants, except for ts ET12 cells, rapidly underwent apoptosis at 39.5°C, the nonpermissive temperature (8, unpublished results). ts ET12 cells did not undergo apoptosis rapidly. Thus, there must be the other reasons for the isolation of autosomal mutants from mammalian cultured cell-lines. It has been thought that the mutation of genes localized on the autosomal chromosomes could be obtained if those genes are functional hemizygotes (*28*). If one of alleles is deleted on the autosomes, only the mutated gene should be amplified. The same nucleotide change as that found in the cDNA of ARS was found when the genomic DNAs of ts ET12 cells were amplified by PCR (unpublished results).

We found that the amount of mutated ARS decreased in ts ET12 cells following the temperature-shift to 39.5°C. A similar phenomenon has been observed in ts BN2 and ts BN7 cells. ts BN2 cells are defective in RCC1, the guanine nucleotide exchange factor of a small G protein, Ran (*16*), and tsBN7 cells are defective in DAD1, a subunit of the oligosaccharyltransferase (*29*). In the present work, the degradation of mutated proteins was suggested to be carried out by the ubiquitin-dependent proteasomes, since an inhibitor of the ubiquitin-dependent proteasomes, MG132, prevented the degradation of the mutated ARS. Not only the mutated ARS, but also the other proteins that are supposed to be degraded by the ubiquitin-dependent proteasomes (*20–23*), were lost in ts ET12 cells upon incubation at 39.5°C. These proteins were also stabilized by the addition of MG132. Since Cdk4 was also lost in ts BN250 following the temperature-shift to 39.5°C, the degradation of proteins might be one of the phenotypes of the aminoacyl-tRNA synthetase defects. Anyway, the finding that the amount of mutated ARS was reduced at 39.5°C may explain why the cell-proliferation of ts ET12 cells was suppressed by the addition of alanine in a dose-dependent manner. A concentration of alanine that is 60-fold higher than in normal medium is required for the significant recovery of cell proliferation at 39.5°C. In contrast, a 10-fold concentration of a cognate amino acid is enough to restore the cell-proliferation of ts BN250 and ts BN269 (*9*, *11*), in both of which the mutated aminoacyl-tRNA synthetases were not degraded. The degradation of the mutated ARS in ts ET12 cells was not prevented by the addition of alanine (unpublished data). We, therefore, reasoned that the higher dose of alanine might be required for activating an enzymatic activity of the residual mutated ARS.

It is notable that cycloheximide, a protein synthesis inhibitor, showed a tendency to prevent the degradation of the mutated ARS, in addition to Cdk4, in ts ET12 cells. Previously, we have found that cycloheximide inhibits the apoptosis of ts BN7 cells at 39.5°C (*29*), and also the apoptosis of ts BN269 at 33.5°C induced by depletion of a cognate amino acid (*9*). In ts BN7 cells, a subunit of the oligosaccharyltransferase DAD1, which is the mammalian homologue of yeast Ost2p (*30*), is defective. The oligosaccharyltransferase is involved in protein glycosylation, which plays a key role in forming the overall conformation of macromolecules (*31*). Thus, the inhibition of protein synthesis may prevent the accumulation of non-glycosylated proteins that might be aberrantly folded. Furthermore, the inhibition of N-linked glycosylation causes apoptosis (*32*). Thus, a proper protein folding seems to be important in order to prevent apoptosis. We think that in ts ET12 cells, cycloheximide inhibited the accumulation of aberrantly folded proteins that might be produced by the mutation of ARS. The accumulation of the abnormally folded proteins might induces ER stress, which induces apoptosis (*33*). Cycloheximide, thus, could reduce ER stress by inhibiting the accumulation of aberrantly folded proteins. However, in ts BN250, cycloheximide activates the apoptosis induced at 33.5°C by depletion of a cognate amino acid (*9*). The effect of cycloheximide on apoptosis and protein-degradation, thus, remains to be further investigated. In ts ET12 cells, cycloheximide might prevent protein-degradation by preventing the synthesis of aberrantly folded proteins.

Aminoacyl-tRNA synthetases are reported to exist in two forms: a giant complex form of over 1000 kDa, to which KRS belongs; and a smaller form of about 100–200 kDa, to which HRS and ARS belong. In both ts BN250 and tsBN269 cells, the aminoacyl-tRNA synthetase complexes of the mutated HRS and KRS were degraded and EMAPII was released at 39.5°C, consistent with the rapid appearance of apoptotic cells. In contrast, all forms of the aminoacyl-tRNA synthetase complexes seemed to be stable at 39.5°C in ts ET12 cells, except for loss of the ARS complex. Since p43 was not cleaved in ts ET12 cells, we reasoned that ts ET12 cells undergo apoptosis differently from ts BN250 and ts BN269 cells. In view of the potential of mammalian aminoacyl-tRNA synthetases to act as sophisticated multifunctional proteins regulating various cellular procedures, the difference in apoptosis among three aminoacyl-tRNA synthetase mutants should not be surprising. Our present results indicate that while we thought we knew a lot about aminoacyl-tRNA synthetases as "old" proteins, in actuality, we are a long way from clarifying their functions completely. In this paper, we have expanded the function of ARS by linking it with the ubiquitin-dependent protein degradation and the EMAPII release.

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