Degradation of a Nuclear-localized Protein in Mammalian COS Cells, Using *Escherichia coli* β-Galactosidase as a Model Protein*

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To investigate the mechanism of degradation of proteins localized in the nucleus, we constructed genes encoding modified *Escherichia coli* β-galactosidases and expressed them in mammalian COS cells. When the β-galactosidase with a nuclear localization signal from SV 40 T antigen was expressed in COS cells, the β-galactosidase polypeptide was localized in the nuclei and was stable for at least 4 h. When 16 amino acid residues were deleted from the C-terminal end, the β-galactosidase polypeptide was also observed in the nuclei but it was degraded rapidly, with a half-life of 1.6 h. When the nuclear localizing signal was replaced with a mutant sequence, which lacks nuclear targeting activity, the β-galactosidase polypeptides were present throughout the cells rather than in the nuclei. The β-galactosidase polypeptide with the complete C terminus was stable and the cytoplasmic truncated polypeptide was degraded at the same rate as the nuclear C terminus truncated polypeptide. The β-galactosidase polypeptides with the complete C terminus were present as a tetramer as reported previously and had β-galactosidase activity, but the C terminus truncated polypeptides were present as monomer and had no enzyme activity, indicating that C terminus truncated β-galactosidase is malfolded. Together, the results suggest that a nuclear-localized malfolded protein is degraded as rapidly as a cytoplasmic malfolded protein.

Most of the genetic information is in the nucleus, and output of the information is regulated by many nuclear proteins. Regulation by such proteins seems to involve degradation of the proteins themselves (Hochstrasser and Varchavsky, 1990; Scheffner et al., 1990), suggesting that the degradation of nuclear proteins is a very important issue. There are many reports about degradation of cytoplasmic proteins. However, the degradation of nuclear proteins could be quite different from the degradation of cytoplasmic proteins, because the nucleus is surrounded by the nuclear membrane and transport of components between the nucleus and the cytoplasm is limited (reviewed by Nigg et al., 1991).

Several nuclear proteins are reported to have very short half-lives, including some nuclear oncoproteins, and certain nuclear proteins are stable (Mihara et al., 1988; Begum et al., 1991). There are a few reports of studies of the degradation of nuclear proteins. Recently it was suggested that the ubiquitin pathway may play a role in the degradation of some nuclear oncoproteins (Ciechanover et al., 1991) and of yeast α2 repressor (Hochstrasser et al., 1991). However, the structural features of nuclear short-lived proteins that are recognized by the proteolytic pathway are not clear.

To investigate how structural features influence degradation of polypeptides in the nucleus, we expressed some modified *Escherichia coli* β-galactosidases as model molecules in mammalian COS cells. Since a single β-galactosidase polypeptide of M, 116,000 is too large to pass through the nuclear pores by diffusion (reviewed by Nigg et al., 1991), we can distinguish nuclear events from cytoplasmic ones. Since *E. coli* β-galactosidase is a prokaryotic protein and eukaryotic cells do not have the same enzyme, the β-galactosidase is not expected to have any specific interactions with components in mammalian cells. Therefore, we can analyze the proteolytic pathway using the large prokaryotic proteins more simply than using eukaryotic proteins. To target the β-galactosidase to the nuclei, the protein was tagged with an SV40 T antigen nuclear localization signal (Kalderon et al., 1984; Rihs and Peters, 1989). While the intact β-galactosidase was very stable in the nuclei, the β-galactosidase polypeptide missing 16 C-terminal amino acid residues was degraded rapidly, presumably because it is malfolded and fails to form tetramers.

**EXPERIMENTAL PROCEDURES**

**Materials**

Anti-β-galactosidase rabbit IgG was purchased from Rockland, Inc. Fluorescein iso-thiocyanate-conjugated anti-rabbit IgG and peroxidase-conjugated anti-rabbit IgG were purchased from Organon Teknika N.V.-Cappel Products. Anti c-Myc protein rabbit serum was purchased from Medac Gesellschaft fur Klinische Spezialpraparate mbh. Prestained SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories. The low range molecular weight standards contain phosphorylase B (M, 106,000), bovine serum albumin (M, 80,000), ovalbumin (M, 49,500), carbonic anhydrase (M, 32,500), soybean trypsin inhibitor (M, 27,500), and lysozyme (M, 18,500). The high range molecular weight standards contain myosin (M, 205,000), β-galactosidase (M, 116,500), bovine serum albumin (M, 80,000), and ovalbumin (M, 49,500). pRC/CMV was purchased from Invitrogen Corp.

**Methods**

**DNA Constructs**—The plasmids encoding modified β-galactosidases were constructed as described below. A PstI fragment containing LacZ from pMC1871 (Casadaban et al., 1983) was cloned into a PstI site of pCDM8 (Seed, 1987) to yield pLacZCDM8. Oligonucleotides (5'-TCTAGAAGCTTACCATGGGTTGGCCGCGGCCG-CTT-AGTGATGATGATGATGATGCCCGACTCTCAACATGATCTC-TCCAAA and 5'-GGGGTCTTCTACCTTTCTCTTCG/TTTTTT-GGAGGTAATGTT) were annealed at 70 °C for 20 min, filled-in using DNA polymerase (Klenow), and digested with HindIII. The resulting oligonucleotides is 97 bp long, encoding 31 amino acid residues starting from the initiation methionine as described (see Fig.

1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; bp, base pair(s); PBS, phosphate-buffered saline; N-βgal, nuclear β-galactosidase; C-βgal, cytoplasmic β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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pH 7.2, and permeablized with 0.2% Triton and 2.75 volumes of 1% Triton X-100 solution (1% Triton X-100, after further incubation as described in the figure legends. The cells were rinsed with phosphate-buffered saline (PBS), and then resuspended in SDS-PAGE sample buffer (Laemmli, 1970). The cells were lysed with 10% dimethyl sulfoxide in PBS for 1 min. After further incubation in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum for 2 h, the cells were collected by trypsin and EDTA and divided into several dishes. Cells were used for assays 17 h to 3 days after transfection.

Indirect Immunofluorescence—Immunofluorescence staining was performed on COS cells that were plated onto cover slips. The cells were fixed with 4% paraformaldehyde, 0.1 M NaCl, 50 mM HEPES, pH 7.2, and permeabilized with 0.2% Triton X-100 in PBS. After exposure to rabbit anti-β-galactosidase IgG, the cells were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.

Immunoprecipitation—One day after transfection of plasmids, cells were rinsed once with PBS on dishes and harvested with rubber policemen. Cells were collected by centrifugation and suspended in 1% SDS solution (1% SDS, 50 mM Tris, pH 7.6, 30 mM dithiothreitol) and disrupted by vortexing followed by boiling for 10 min. The extracts were diluted 5-fold by adding 1.25 volumes of 4% bovine serum albumin in PBS and 2.75 volumes of 1% Triton X-100 solution (1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.6) and centrifuged at 350,000 × g for 30 min at 4 °C. The supernatants were counted on a liquid scintillation counter. Supernatant samples containing equal amounts of ¯35S were immunoprecipitated sequentially with anti-β-galactosidase antibody or anti-C-Myc protein serum. After incubation at 4 °C for 1 h with gentle mixing, protein A-Sepharose was added and the samples were incubated at 4 °C for 2 h. The Sepharose was washed three times with 0.1% Triton X-100 (1×), 50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 30 mM dithiothreitol, and once again with buffer A and then resuspended in SDS-PAGE sample buffer (Laemmli, 1970). Immunoprecipitated samples were subjected to SDS-PAGE using a 7.5% polyacrylamide gel and fluorographed. The gel was dried and exposed on Kodak X-Omat SB-5 film at −80 °C. To quantify the bands, densitometric scanning of the fluorogram was performed with a Shimazu CS-9000.

RESULTS

Intact or C-terminal Truncated β-Galactosidase with a Nuclear Localization Signal—Nuclear proteins usually contain a signal, a short stretch of amino acid residues, which directs their intracellular transport to the nucleus. The best characterized example of a nuclear localization sequence that of the Simian virus 40 large tumor antigen (SV40 T antigen) (Kalderon et al., 1984; Rihs and Peters, 1989). Thus we used this nuclear localization signal to target β-galactosidase to the nuclei.

A synthetic oligonucleotide encoding a short sequence of SV40 T antigen was fused to the structural gene encoding β-galactosidase at its N-terminal end (N-βgal). For a control, we also fused a synthetic oligonucleotide encoding a mutant SV40 T antigen sequence to the β-galactosidase gene (C-βgal). The mutant antigen had a substitution of lysine 128 by threonine, abolishing nuclear targeting activity.

We also constructed genes encoding β-galactosidase missing a short C-terminal amino acid sequence. A stop codon was introduced, and the resulting genes encode β-galactosidase polypeptides missing 16 amino acids at the C terminus and having the wild type (N-βgal-D) or mutant (C-βgal-D) nuclear localization signal at the N terminus. The β-galactosidase polypeptide has more than 1000 amino acid residues, thus as described below (see Fig. 3), the 16 amino acids were not affect the mobility of β-galactosidase on SDS-PAGE. The primary structure of the modified β-galactosidase polypeptides we constructed are indicated in Fig. 1.

Expression and Subcellular Localization of the Modified β-Galactosidase Polypeptides in COS Cells—The genes described above were engineered in plasmids containing the SV40 origin of replication, which allowed their overexpression after transfection into COS cells (Gluzman, 1981). The cells were lyzed 3 days after transfection of plasmids and polypeptides were analyzed on SDS-PAGE and subjected to Western blots using anti-β-galactosidase antibody. The cell lysates from the cells expressing N-βgal, C-βgal, N-βgal-D, and C-βgal-D contain protein bands with the expected molecular mass of E. coli β-galactosidase (see Fig. 3). The subcellular localization of the β-galactosidase was visualized 17 h after transfection by indirect immunofluorescence staining with anti-β-galactosidase antibody. As shown in Fig. 2, N-βgal and N-βgal-D are localized in the nucleus of every COS cell expressing β-galactosidase, while C-βgal and C-βgal-D are present throughout the cells and are not localized in the nuclei.

Stability of the β-Galactosidase Polypeptides—Western blotting analysis shows that the amounts of the modified β-galactosidase proteins expressed in COS cells differ greatly:

Fig. 1. Primary structures of β-galactosidases used in this study. Nuclear localization signal (NLS, □) or mutant NLS (□) was fused to β-galactosidase. The long rectangular boxes indicate β-galactosidase residues. Note that N-βgal-D and C-βgal-D are missing 16 amino acids indicated in the C-terminal end. The amino acid sequences of NLS and mutant NLS are indicated in the inset. Residues in the NLS from SV40 T antigen are underlined. The position of the mutation that abolishes the nuclear targeting activity is indicated by an asterisk.
of the accumulated protein may result from differences in the stabilities of the proteins.

To measure the stability of the β-galactosidase polypeptides directly, a pulse-chase experiment was performed. Seventeen h after transfection of plasmids, COS cells were pulse-labeled for 30 min with [35S]methionine and chased with fresh unlabeled medium for various times. The cell lysates were immunoprecipitated with anti β-galactosidase antibody and analyzed by SDS-PAGE. As shown in Fig. 4A, the intensity of the bands of N-βgal (lanes 1–4) and C-βgal (lanes 9–12) did not decrease during the 4-h chase, indicating that β-galactosidase with its complete C-terminal end is a very stable protein, even if it is in the nuclei. However, the intensity of bands of N-βgal-D (lanes 5–8) and C-βgal-D (lanes 13–16) decreased during the chase, and after 4 h of the chase only week bands of N-βgal-D (lane 8) and C-βgal-D (lane 16) were observed, indicating that N-βgal-D and C-βgal-D are degraded rapidly and the C terminus truncated polypeptides are unstable both in the nuclei and the cytoplasm. When the x-ray film was overexposed, a faint band of approximately 98 kDa appears in the cells expressing N-βgal-D (Fig. 4B) and C-βgal-D (data not shown), while the 116-kDa β-galactosidase band disappears during the chase.

Quantitative analysis of the results was performed using a densitometer. As shown in Fig. 5, N-βgal-D and C-βgal-D were degraded with half-lives of approximately 1.6 h, while N-βgal and C-βgal slightly increased. Because the results were normalized by the radioactivity remaining in the cells (see “Experimental Procedures”) and whole cell proteins are degraded during the chase, the increasing amounts of N-βgal and C-βgal suggest that N-βgal-D and C-βgal are more stable than average proteins.

To confirm that “nuclear-localized” N-βgal-D is degraded, cells expressing the β-galactosidases were treated with cyclo-
Degradation of a Nuclear-localized Protein

**Table I**

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<thead>
<tr>
<th>Protein</th>
<th>Time after CH treatment</th>
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<tr>
<td></td>
<td>0 h</td>
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<tr>
<td>N-β-gal</td>
<td>154</td>
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<tr>
<td>(99%)</td>
<td>(104%)</td>
</tr>
<tr>
<td>N-β-gal-D</td>
<td>80</td>
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<td>(55%)</td>
<td>(12.5%)</td>
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**FIG. 5.** Quantitative analysis of degradation of β-galactosidase polypeptides and c-Myc protein. β-Galactosidases and c-Myc protein were immunoprecipitated and analyzed by SDS-PAGE, fluorography, and densitometric scanning as described under “Experimental Procedures.” The intensity of β-galactosidase or c-Myc protein immunoprecipitated from cells just after pulse-labeling was taken as 100%. N-β-gal (●), C-β-gal (■), N-β-gal-D (○), C-β-gal-D (□), c-Myc protein (×).

FIG. 6. Gel electrophoresis of the β-galactosidase proteins under nondenaturing conditions. Two days after transfection of plasmids, cells were collected and extracted with 0.1% Triton X-100. The extracts (lanes 1–4) were run on alkaline electrophoresis and transferred to nitrocellulose. β-Galactosidases were detected with anti-β-galactosidase antibody as described under “Experimental Procedures.” COS cells were expressing N-β-gal (lanes 1 and 5), C-β-gal (lanes 2 and 6), N-β-gal-D (lane 3), C-β-gal-D (lane 4). Samples in lanes 5 or 6 (N-β-gal-D, C-β-gal-D, respectively) were treated with 9 M urea before electrophoresis.

heximide and examined by fluorescence microscopy after indirect immunofluorescence staining with anti-β-galactosidase antibody (Table I). In the case of N-β-gal, the number of cells stained did not change during cycloheximide treatment for at least 10 h. However, in the case of N-β-gal-D, the number of stained cells decreased with time after addition of cycloheximide. After 4 h in cycloheximide the number of stained cells was 55% of that before the treatment, and after 10 h few cells were stained. Throughout the cycloheximide treatment the nuclei were specifically stained. These results suggest that nuclear-localized N-β-gal-D is degraded rapidly, while N-β-gal is very stable in the nuclei. The 4 h required to decrease the number of N-β-gal-D-expressing cells stained with the antibody by half is longer than a half life of the polypeptide (Fig. 5). This is expected, because we may score a nucleus as stained even if the amount of β-galactosidase decreases to well below half the original value.

C-Myc protein is known to be a nuclear short-lived protein. For comparison, we determined the half-life of c-Myc protein in our experimental system. The results indicated that c-Myc protein was also degraded rapidly, with a half-life of approximately 30 min (Fig. 5), which is consistent with results using other cell lines (Hann and Eisenman, 1984; Ramsay et al., 1984; Beimling et al., 1985; Hann et al., 1985; Luscher and Eisenman, 1988). This suggests that our experimental system is not unusual. As shown in Fig. 5, the degradation profiles for c-Myc protein and N-β-gal-D are slightly different. c-Myc protein was initially degraded more rapidly than N-β-gal-D and the degradation rate for c-Myc protein apparently decreased with longer chase times, while N-β-gal-D was degraded with first order kinetics.

N-β-gal-D and C-β-gal-D Do Not Have the Correct Quaternary Structure and Lack Enzyme Activity—It is known that β-galactosidase exists as a tetramer, and there is a report in which the polypeptide missing only 10 amino acid residues at the C terminus is present as a monomer (Fowler et al., 1966). To evaluate the molecular form of the modified β-galactosidase proteins expressed in COS cells, the β-galactosidases were extracted in 0.1% Triton X-100 and were electrophoresed without SDS, transferred to nitrocellulose, and detected with anti-β-galactosidase antibody. As shown in Fig. 6, N-β-gal-D and C-β-gal-D migrated faster than N-β-gal and C-β-gal. When cell extracts containing N-β-gal and C-β-gal were treated with 9 M urea before electrophoresis, the polypeptides migrated as fast as N-β-gal-D and C-β-gal-D did. N-β-gal and C-β-gal were also detected after gel filtration chromatography. N-β-gal and C-β-gal eluted at M, 480,009 with the same retention time as commercial E. coli β-galactosidase (data not shown). These results indicate that N-β-gal and C-β-gal are present as tetramers and N-β-gal-D and C-β-gal-D are present as monomers.

The enzyme activity of the modified β-galactosidase proteins in COS cells was tested using X-gal. Staining of COS cells containing the same amount of β-galactosidase polypeptide, N-β-gal or N-β-gal-D, indicated that N-β-gal-D did not have β-galactosidase activity, while the N-β-gal did (data not shown). Together, these results suggested that N-β-gal-D is malfolded.

**DISCUSSION**

In this study, we expressed modified β-galactosidases in mammalian COS cells. When N-β-gal was expressed in COS cells, the polypeptide was located in nuclei and was very stable. When N-β-gal-D was expressed in COS cells, the polypeptide was located in nuclei, but the polypeptide was degraded rapidly. The nuclear-localized N-β-gal-D disappeared after polypeptide synthesis was blocked, while the N-β-gal
remained. These results indicate that nuclear localized N-βgal-D is degraded and nuclear-localized N-βgal is stable.

What structural feature is a signal for the degradation of N-βgal-D? N-βgal-D lacks the 16 C-terminal amino acid residues, while N-βgal has the complete C terminus. Therefore, one possibility is that the C-terminal amino acid sequence contains a signal for stabilization. However, when α2 repressor, a yeast short-lived nuclear protein, was fused to the N terminus of β-galactosidase, the resulting fusion protein is localized in the nuclei (Halli et al., 1984) and degraded rapidly (Hochstrasser et al., 1990), suggesting that the C-terminal portion of β-galactosidase does not contain a stabilization signal. In this paper we suggest that N-βgal-D was not present in a correct quaternary structure and lacked enzyme activity, suggesting that N-βgal-D was malformed. These results suggest that malformation can be a signal for degradation of a nuclear localized protein.

Adenovirus E1A gene product is a nuclear oncprotein and has a short half-life. It has been shown that the N-terminal tetrapeptide of E1A is required for rapid degradation (Slavicek et al., 1988; Simon and Richter, 1990), suggesting that N-terminal residues could be a signal for rapid degradation of nuclear proteins. In our study, however, the rapidly degraded N-βgal-D has exactly the same amino acid sequence in the N-terminal end as N-βgal does. Therefore, the N-terminal portion of N-βgal-D does not have a rapid degradation signal. Taken together, the results suggest that there are two types of signals for rapid degradation of nuclear proteins: 1) N-terminal amino acid composition and 2) global features of the protein. For yeast α2 repressor it was reported that there are two degradation signals in the molecule (Hochstrasser and Varshavsky, 1990). These signals are in long amino acid stretches unlike the E1A degradation signal. These signals may be classified in the category of global features. It has been suggested that N-terminal amino acid composition and global features act as degradation signals for cytoplasmic proteins (reviewed by Rechsteiner et al., 1987; Bachmair and Varshavsky, 1989; Bachmair et al., 1988; Katznelson and Kulk, 1985). Therefore, the degradation signals of nuclear-localized proteins are consistent with those of cytoplasmic proteins.

In the reports mentioned above (Slavicek et al., 1988; Simon and Richter, 1990; Hochstrasser and Varshavsky, 1990), it is not clear whether the proteins with the degradation signals were degraded after reaching the nucleus or before. However, our work indicates clearly that N-βgal-D is degraded after reaching the nucleus.

Where is N-βgal-D degraded? It has been reported that there is proteolytic machinery in the nuclei (Tanaka et al. 1989). This machinery might take charge of the degradation of N-βgal-D. Or N-βgal-D might be taken from the nucleus to the cytoplasm and degraded by a proteolytic pathway in the cytoplasm. Further work is required to distinguish these possibilities.

We detected a 98-kDa polypeptide while chasing pulse-labeled COS cells expressing N-βgal-D or C-βgal-D. In E. coli, a 90-kDa polypeptide was identified as an intermediate in the degradation of β-galactosidase missing several C-terminal amino acids (McKnight and Fried, 1981; Wang and Fried, 1987). In the mammalian cells, the 98-kDa polypeptide might be an intermediate in the degradation of β-galactosidase, although there are few cases in which degradation intermediates are detected.

We used modified E. coli β-galactosidase as a model protein to study degradation of nuclear localized protein. This may avoid some complexity due to the interaction of a native eukaryotic nuclear protein with other cellular components. The degradation rate for c-Myc protein apparently decreased at longer chase times, which has also been observed by others (Luscher and Eisenman, 1988). However, the degradation rate for N-βgal-D did not change. The degradation profile of N-βgal-D may reflect only the nature of the degradation, while degradation profile of c-Myc protein may reflect the nature of the degradation and the interaction of c-Myc with nuclear components. This work is the first to investigate the turnover rate of nuclear protein using a mammalian cell transient expression system. This experimental system should be useful for obtaining more information about the machinery for degradation of short-lived nuclear proteins.

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REFERENCES