Proc. Nat. Acad. Sci. USA
Vol. 71, No. 12, pp. 4732–4736, December 1974

Selective Degradation of Abnormal Proteins in Mammalian Tissue Culture Cells

(protein degradation/hypoxanthine-guanine phosphoribosyltransferase/immunoprecipitation/somatic cell mutants)

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Communicated by Eugene P. Kennedy, September 16, 1974

ABSTRACT **The degradation rates of several missense mutants of hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) in mouse L cells are compared to those of the wild-type enzyme. Although the rates of total protein breakdown in the mutant cell lines are identical to that of the parental L cell line, defective molecules of hypoxanthine-guanine phosphoribosyltransferase present in the mutant cell lines are degraded much faster than the wild-type enzyme. The level of defective phosphoribosyltransferase molecules present in the mutant cell lines is inversely proportional to the breakdown rate. This observation indicates that the major factor determining the concentrations of the defective phosphoribosyltransferases is their specific degradation rate. These results strongly support the hypothesis that abnormal proteins are selectively degraded in mammalian cells.**

Proteins in eukaryotic and prokaryotic cells are in a dynamic state, continuously being synthesized and degraded (1–3). To replenish the degraded proteins, the cell expends considerable metabolic energy. It is still unclear what underlying physiological advantage is conferred by continuous protein turnover. It may provide a means of preventing the accumulation of abnormal and possibly deleterious proteins (1, 2). If the protein degradative machinery of the cell could selectively remove defective proteins, then the pool of abnormal proteins would be reduced relative to normal proteins.

Abnormal proteins can arise in several ways. Proteins may age during the course of normal cellular functioning (4–6). Errors in protein synthesis at the transcriptional and translational level can generate abnormal proteins (7). Diploid cells may have the special requirement for eliminating abnormal proteins produced by recessive mutations in one of the two alleles.

There are several observations in *Escherichia coli* and mammalian cells that are compatible with the hypothesis that proteolytic degradation functions selectively to eliminate abnormal proteins. Polypeptide fragments produced by deletions or nonsense mutations in *E. coli* are degraded more rapidly than the corresponding normal proteins (8–12). Bacterial strains that contain mutations causing increased errors in translation, such as RAM and missense suppressor mutations, exhibit increased rates of total protein breakdown (13). Cells that have incorporated certain amino acid analogues *in vivo* also have higher rates of total protein degradation (8, 13, 14). Indications that abnormal hemoglobins may be degraded faster have also been reported (15, 16).

In this communication we examine the degradation rates of several missense mutants of hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8; IMP: pyrophosphate phosphoribosyltransferase) in mouse L cells. This system has a number of distinct advantages for studying the model of selective catabolism of abnormal proteins. First, under normal growth conditions HGPRT is a nonessential enzyme, so loss of the enzymatic activity does not perturb the physiology of the cell. (This approach avoids the use of toxic agents such as amino-acid analogues or puromycin to induce the synthesis of abnormal proteins. Such agents may cause pleiotropic effects that could alter proteolytic rates.) Second, HGPRT is a minor component of the total protein pool (i.e., about 1/5000). Thus, large changes in the rate of degradation of HGPRT do not affect the total rate of proteolysis of the cell. Third, the mutants examined in this study have all lost enzymatic activity but retain their immunological reactivity with antiserum directed against purified wild-type HGPRT [i.e., they are positive for crossreacting material (CRM^+)] (17). With HGPRT, immunological reactivity is a fairly sensitive criterion for structural integrity. For example, heating wild-type HGPRT inactivates the antigenic determinants and enzymatic activity at exactly the same rate (17). Since the mutant proteins are known to be structurally similar to wild type proteins, these experiments are a stringent test of the cells' ability to detect and selectively degrade abnormal proteins.

Although the total proteolytic rates in the mutant cell lines are identical to that of the parental cell line, the defective HGPRT molecules in the mutant cell lines are degraded faster than wild-type enzyme. The amount of mutant protein present in the cell is inversely proportional to its degradation rate. This observation indicates that selective proteolysis is the major factor determining the concentration of these abnormal proteins in the cell.

MATERIALS AND METHODS

Materials. The preparation and characterization of antiserum against HGPRT is described in ref. 17. Antibody against rabbit IgG prepared in goat was the generous gift of Dr. M. C. Rechsteiner. [^{3}H]Leucine (50 Ci/mmol) and [^{3}H]-lysine (47 Ci/mmol) were purchased from Schwarz/Mann. [^{35}S]Methionine (100 Ci/mmol) was obtained from New England Nuclear Corp. Triton X-100 was a product of Rohm

Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; CRM, crossreacting material; $NaDodSO_4$, sodium dodecyl sulfate; phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO , 1.5 mM KH_2PO_4 (pH 7.5).

and Haas Co., Independence Hall West, Philadelphia, Pa. DE-81 filter discs were purchased from Whatman. Acrylamide and *N,N'*-methylene bisacrylamide were obtained from Eastman Kodak. Ultrapure sodium dodecyl sulfate ($NaDodSO_4$) was purchased from Pierce Chemical Co., P. O. Box 1A, Rockford, Ill.

Cell Line and Culturing Methods. Wild-type L cells (L^+) were obtained from J. Littlefield. The $HGPRT^-$ mouse L cell mutants examined in the present study were those derived by Sharp *et al.* (18). These mutants were selected for resistance to a combination of 8-azaguanine and 6-thioguanine after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. The medium and conditions used for cell culture have been described (18).

Cell Extracts were prepared in a 4° room with all buffers at 0–4°. The wild-type and mutant cell lines were harvested from monolayer cultures grown on 60-mm plastic plates (Falcon). The plates were washed three times with 5 ml of cold phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.5)] and drained for about 5 min. One-quarter milliliter of extraction buffer [10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 30 mM KCl, 0.5% Triton X-100, and 0.1 mM dithiothreitol] was added and the plates were gently shaken every 5 min for 20 min. They were then tilted for 5 min, followed by removal of the cell extract with a pasteur pipette. This method produces holes in the cellular membrane, releasing the cytoplasmic proteins. Most of the cellular membrane and nuclei remain attached to the plate.

The extracts were centrifuged at 1000 × *g* for 20 min to remove residual cellular debris and nuclei. This method of extraction gave higher recoveries of HGPRT than freeze-thawing or homogenization methods.

Radioimmune-Precipitation. The L^+ and mutant cell lines were grown on 60-mm plates in Eagle's minimal essential medium plus 10% fetal calf serum to about 75% confluency. The medium was then removed. Plates were washed with 5 ml of phosphate-buffered saline, and 1.5 ml of minimal essential medium containing [^{3}H]leucine (82 μCi/ml) and [^{3}H]-lysine (82 μCi/ml) or [^{35}S]methionine (82 μCi/ml) instead of the respective nonradioactive amino acids was added. The specific activity of the radioactive amino acids ([^{3}H]leucine, 10 Ci/mmol; [^{3}H]lysine, 9.4 Ci/mmol; [^{35}S]methionine, 20 Ci/mmol) was adjusted so that the rate of protein synthesis was not limited by the concentration of these amino acids. The cells were incubated in the radioactive medium for 3 hr at 37° followed by three 5-ml washes with phosphate-buffered saline at 4°. (Washing with cold buffer was found to more effectively remove unincorporated radioactive amino acids.) Five milliliters of warm minimal essential medium plus 10% fetal calf serum was then added, and incubation continued at 37° for the designated times. The cells were again washed three times with 5 ml of cold phosphate-buffered saline, and extracts were prepared as described above. About 3.5 mg of an $HGPRT^-$ CRM^- cell extract was added to compete with radioactively labeled non-HGPRT protein. Antiserum against HGPRT (42 μg) was then added, followed by incubation at 4° for 12 hr. Antibody against rabbit IgG prepared in goat (1.5 mg) was added, and incubation continued for an additional 4 hr at 4°. The resulting precipitates were sedimented by centrifugation at 1000 × *g* for 20 min. The supernates were removed. The precipitates were washed with 400 μl of buffer [25 mM Tris (pH 7.5) 3.5 mM $MgCl_2$, 500 mM KCl, and 2% Triton X-100] and resedimented by centrifugation at 1000 × *g* for 20 min. This procedure was repeated, followed by a final wash with 400 μl of buffer[50 mM Tris·HCl (pH 7.5) and 7 mM $MgCl_2$], and the precipitates were sedimented as before. The immunoprecipitates were then processed and subjected to electrophoresis as described in the next section.

$NaDodSO_4$–Urea Gel Electrophoresis. The stacking gel (3.3% acrylamide and 0.08% *N,N'*-methylene bisacrylamide) contained 125 mM Tris·HCl (pH 6.8), 0.1% $NaDodSO_4$, and 6 M urea. The separating gel (11.1% acrylamide and 0.27% *N,N'*-methylene bis-acrylamide) contained 150 mM Tris (pH 8.7), 0.1% $NaDodSO_4$, and 6 M urea. The running buffer was 25 mM Tris base, 192 mM glycine, 0.1% $NaDodSO_4$, and 6 M urea. The sample-loading buffer contained 50 mM Tris·HCl (pH 6.8), 1.6% $NaDodSO_4$, 5.4 M urea, 18% glycerol, 0.001% bromphenol blue, and 5% 2-mercaptoethanol. *N,N,N',N''*-Tetramethylethylenediamine and persulfate were adjusted to give a 10- to 15-min gelling time. Samples, prepared by immunoprecipitation, were dissolved in 100 μl of sample-loading buffer and heated at 100° for 5 min. The sample was loaded onto the gel with a Hamilton syringe. The gels (6 × 90 mm) were subjected to electrophoresis at a constant current of 3.5 mA per gel. The bromphenol blue reached the end of a 9-cm gel after about 8 hr.

All gels were internally calibrated with fluorescein isothiocyanate (isomer I)-labeled molecular weight standards. The fluorescein isothiocyanate labeling was done by the methods of Kawamura (19). The positions of the molecular weight standards were determined by visual examination under UV illumination. The fluorescein isothiocyanate-labeled proteins were shown to electrophorese with their unlabeled counterparts on $NaDodSO_4$–urea gels.

RESULTS

Detection of Wild-Type and Mutant HGPRT in Cell Extracts. Antiserum against HGPRT was used to isolate radioactively labeled HGPRT molecules from L^+ and $HGPRT^-$ mutant cell extracts labeled with [^{3}H]aminoacids. An excess of antiserum against HGPRT was allowed to react with the extracts overnight, followed by the addition of antibody against rabbit IgG prepared in goat to insure complete precipitation of HGPRT (the specificity of the antiserum against HGPRT is described in detail in ref. 17). The radioactively labeled immunoprecipitate was then examined by electrophoresis on calibrated $NaDodSO_4$–urea polyacrylamide gels. We have shown in a previous communication that HGPRT is composed of subunits with a molecular weight of 27,000 (20). The results of representative radioimmunoprecipitations of extracts prepared from L^+, an $HGPRT^-$ CRM^+ cell line, and an $HGPRT^-$ CRM^- cell line are illustrated in Fig. 1. The CRM activity in the mutant cell lines was determined by the standard precipitation-inhibition assay in which CRM is detected if it can compete with wild-type HGPRT for sites on the antibody and thus displace enzymatic activity into the supernate (17). Fig. 1a shows the pattern of radioactivity after electrophoresis on $NaDodSO_4$–urea polyacrylamide gels of the immunoprecipitate from wild-type L cells (L^+) labeled with [^{3}H]lysine and [^{3}H]leucine. A sharp peak of radioactivity migrating with a molecular weight of 27,000 is observed. Fig. 1b is the pattern from an $HGPRT^-$ CRM^+ cell line (M345).

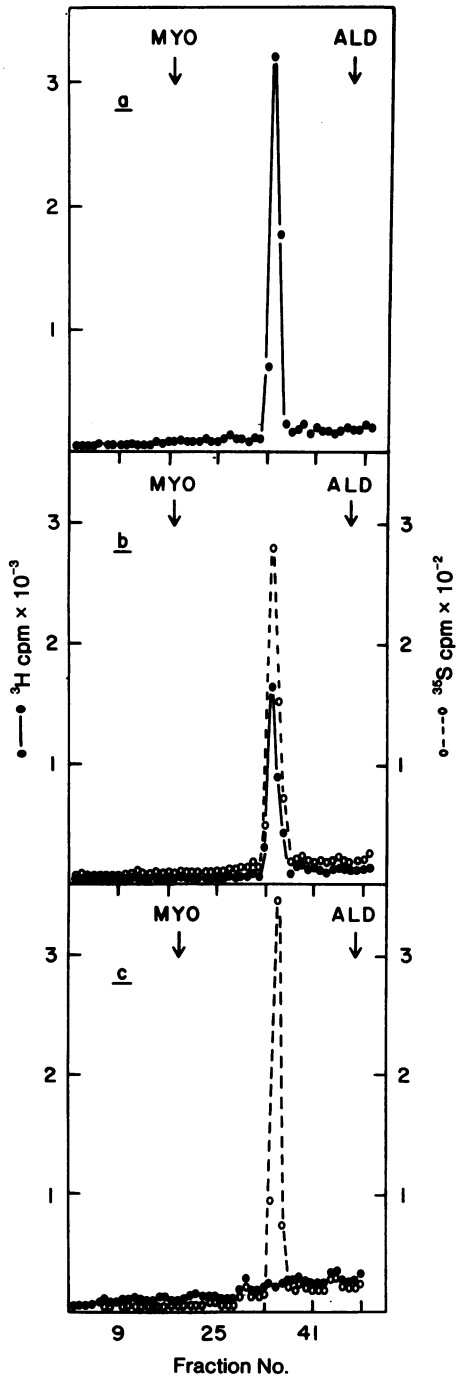

FIG. 1. Analysis of HGPRT isolated from L^+ and mutant ($HGPRT^-$) cell extracts by radioimmune precipitation. The L^+ and $HGPRT^-$ mutants were labeled with radioactive amino acids, the cell extracts were prepared, treated with antiserum against HGPRT and antibody against rabbit IgG prepared in goat, and the immunoprecipitate was processed and subjected to electrophoresis on $NaDodSO_4$–urea polyacrylamide gels as described in *Materials and Methods*. Each gel was internally standardized with fluoresceinated myoglobin (MYO), fluoresceinated aldolase (ALD), and bromphenol blue. The fluorescein-labeled proteins were shown to electrophorese with their unlabeled counterparts on $NaDodSO_4$–urea polyacrylamide gels. Panel a shows the pattern of radioactivity after electrophoresis of the immunoprecipitate isolated from L^+ extracts. Panel b shows the results of coprecipitation of [^{3}H]leucine- and [^{3}H]lysine-labeled $HGPRT^-$ CRM^+ cell extract (M345; ●) and [^{35}S]-methionine L^+ cell extract (○). The protein ratio of the extracts (^{35}S/^{3}H) was 1/20. The experiment in panel c is as in panel b, except that a [^{3}H]leucine- and [^{3}H]lysine-labeled $HGPRT^-$ CRM^- cell extract was used instead of an $HGPRT^-$ CRM^+ cell extract. The peak of radioactivity corresponds to a molecular weight of 27,000.

(*Legend continued*) →

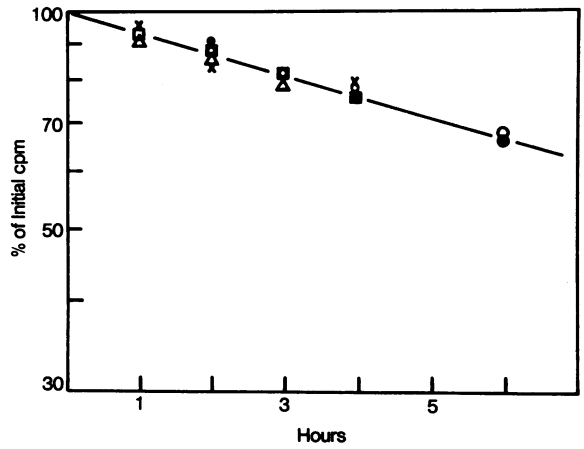

FIG. 2. Rate of total protein degradation in the L^+ and mutant cell lines M345, M343, M398, and M463. Plates (60 mm) of the cell lines L^+, M345, M343, M398, and M463 were grown to 75% confluency. The nonradioactive medium was then removed; plates were washed and radioactive medium was added as described in *Materials and Methods*. The cells were then incubated in the presence of radioactive amino acids for 3 hr at 37°. The radioactive medium was in turn removed, cells were washed, nonradioactive medium was added back, and incubation continued at 37°. At the designated times, cell extracts were prepared, and a 10-μl aliquot was removed to determine the total protein degradation rate in each cell line. The aliquot of cell extract was precipitated with 3 ml of 7% trichloroacetic acid in the presence of 0.5 mg of carrier bovine serum albumin. The precipitate was sedimented by centrifugation at 1000 × *g* for 15 min at 2°. The pellet was dissolved in 250 μl of 0.5 M NaOH to hydrolyze aminoacyl-tRNA ester bonds. The protein was reprecipitated with 3 ml of 7% trichloroacetic acid and collected by filtration on Whatman GFC fiber filters. The level of radioactive protein present in each sample was determined by liquid scintillation counting in 5 ml of Patterson–Greene scintillation fluid (18). ●, L^+; ○, M345; ×, M343; △, M398; and □, M463.

As an internal marker, [^{35}S]methionine-labeled L^+ immunoprecipitate was added. Both the ^{3}H and ^{35}S radioactivity were observed to coelectrophorese at 27,000 daltons. Fig. 1c is the pattern obtained with an $HGPRT^-$, CRM^- cell line. The tritium radioactivity peak is absent. These experiments confirm genetically that the 27,000-dalton peak is the HGPRT subunit.

Degradation of Wild-Type and Mutant HGPRT. For determination of the rates of degradation of wild-type and mutant HGPRT, the cell lines L^+, M345, M343, M398, and M463 were incubated with medium containing [^{3}H]lysine and [^{3}H]leucine followed by removal of the radioactive medium. The cells were thoroughly washed with buffer and placed in medium containing unlabeled leucine and lysine at concentrations 300 times higher than in the radioactive medium. After incubation for the indicated times at 37°, cell extracts were prepared and immunoprecipitated as described

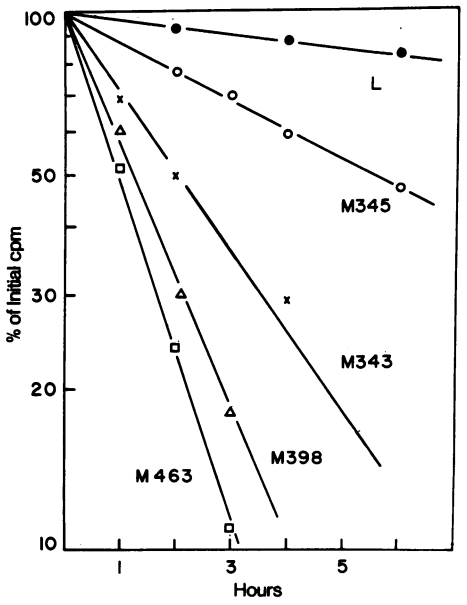

FIG. 3. Rate of degradation of L^+ and mutant HGPRT molecules. Plates (60 mm) of the cell lines L^+, M345, M343, M398, and M463 were grown. Cultures were labeled with radioactive amino acid, washed, and reincubated for the designated times in medium containing an excess of unlabeled amino acids. Cell extracts were prepared. An aliquot was removed, and total protein breakdown rate was measured (Fig. 2). The L^+ and mutant HGPRT molecules were then isolated from the extracts by immunoprecipitation with antiserum against HGPRT and antibody against rabbit IgG prepared in goat. The radioactively labeled immunoprecipitates were then processed and subjected to electrophoresis on calibrated $NaDodSO_4$–urea polyacrylamide gels. The level of HGPRT (L^+ and mutant) present in the extract was quantitated by integrating the radioactivity under the 27,000-dalton peak and normalized to the amount of radioactivity incorporated when the [^{3}H]aminoacids were removed. ●, L^+; ○, M345; ×, M343; △, M398; and □, M463.

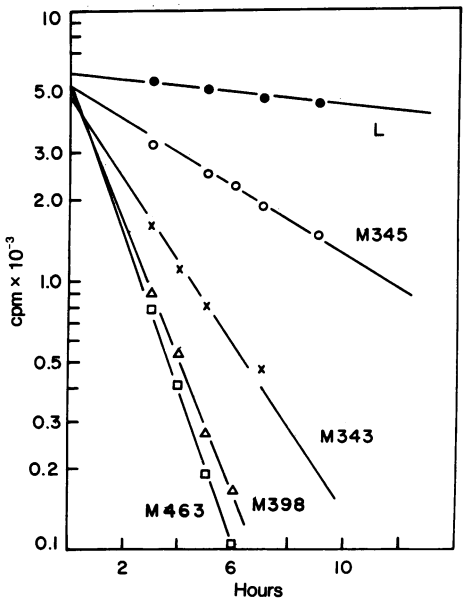

FIG. 4. Extrapolation of the L^+ and mutant HGPRT breakdown data back to zero time (i.e., the time of addition of radioactive amino acids). The breakdown data described in Fig. 3, after correction for the total amount of [^{3}H]aminoacid incorporation in the various cell lines, is extrapolated back to zero time. The 3-hr point is the time when the radioactive medium was replaced with nonradioactive medium. ●, L^+; ○, M345; ×, M343; △, M398; and □, M463.

in *Materials and Methods*. The radioactive immunoprecipitate was then examined on calibrated $NaDodSO_4$–urea polyacrylamide gels. The amount of wild-type or mutant HGPRT present in the cell extracts was quantitated by integrating the radioactivity under the 27,000-dalton peak.

In Fig. 2 we illustrate that the removal of radioactive amino acids from the cells was effective. We observe the expected logarithmic loss of base-stable, trichloroacetic acid-precipitable radioactivity as a function of time. Fig. 2 also shows that the rate of total protein degradation in the different cell lines is the same.

Fig. 3 demonstrates that the rates of degradation of L^+ HGPRT and the mutant forms of HGPRT are very different. The wild-type enzyme is observed to turn over more slowly than the average protein in the cell. Mutant HGPRT from M463 is degraded 17.5-fold faster than L^+ HGPRT. Fig. 3 also shows that the chase of radioactive amino acids was effective in these experiments. One would not expect straight logarithmic decay curves if reincorporation of radioactive amino acids were a problem.

The altered rates of degradation of the defective HGPRT molecules could be caused either by the structural alterations in the mutant HGPRTs, increasing their susceptibility to proteolysis, or the presence of an unselected second mutation that specifically effects HGPRT degradation without affecting total protein breakdown. We feel that this second mechanism is extremely unlikely since it would require the presence of a specific unselected mutation in each of the mutants that we have studied (i.e., 43 mutants).

Levels of L^+ and Mutant HGPRT Present in the Cells. After incubation in the presence of [^{3}H]aminoacids for 3 hr at 37°, the various mutant cell lines contain different levels of radioactively labeled defective HGPRT molecules, as determined by precipitation with an excess of antiserum against HGPRT and goat antibody against rabbit IgG serum. The breakdown data for HGPRT and the mutants is extrapolated back to zero time in Fig. 4. It can be seen that all of the curves extrapolate back, within experimental error, to the same point. This observation indicates that the rates of synthesis of mutant HGPRT in the various cell lines are equivalent to that of L^+. Since the overall level of proteolysis is the same in the various cell lines, this implies that the different concentrations of defective HGPRT molecules are due to the inherent differences in their susceptibility to breakdown.

DISCUSSION

We mentioned in the introduction that abnormal proteins could arise in cells by a number of different mechanisms. Proteins may age by denaturation or by chemical or enzymatic modification (4–6). Errors in transcription and translation would also produce defective proteins (7). Diploid cells can carry recessive mutations, causing the production of defective proteins by the mutated allele. Abnormal proteins could be deleterious to the cell as a result of their own cytotoxicity or by causing the production of other abnormal proteins. As examples of the latter, errors in the DNA replication, transcriptional, or protein synthetic machinery of the cell

could lead to ever increasing production of defective proteins. A simple model of cellular aging postulates just such an "error catastrophe" (21, 22). It would be advantageous for the cell to be able to selectively degrade abnormal proteins. The cell would then be able to maintain a low, stable level of errors, preventing premature death.

To test the hypothesis of selective degradation of abnormal proteins, we have examined the rates of proteolysis of HGPRT of several missense mutants. The experiments were done in a way that would not perturb the cells' physiology, since such perturbations could themselves cause increased rates of proteolysis. Under the growth conditions used, HGPRT is a nonessential enzyme. It represents only about 1/5000 of the total soluble protein pool. The loss of HGPRT enzymatic activity or a large change in its degradation rate should not affect the turnover of the total protein pool.

We have presented evidence in a previous communication that these HGPRT$^-$ CRM$^+$ mutants are of the missense type (17). They were derived by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis followed by selection for resistance of 8-azaguanine and 6-thioguanine (18). These mutants arose at a low frequency ($<10^{-8}$ spontaneous; about 10^{-6} with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis). The mutant proteins electrophorese with L$^+$ HGPRT on calibrated NaDodSO$_4$–urea polyacrylamide gels. Since the subunit molecular weights of the mutant proteins are the same as L$^+$ HGPRT, it is unlikely that they are nonsense or deletion mutants. Revertants from each of the HGPRT$^-$ CRM$^+$ cell lines have been isolated. This provides further evidence against the possibility of deletion mutants. Since *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as the mutagen, and the mutant proteins retain immunological crossreactivity, frameshift mutations are also unlikely. From this we conclude that the HGPRT$^-$ CRM$^+$ mutants used in this study are missense mutants.

The rates of degradation of a number of mutant forms of HGPRT (and of L$^+$ HGPRT) are shown in Fig. 3. The rates of total protein breakdown in the mutant and wild-type cell lines are the same (see Fig. 2). It can be seen that the defective HGPRT molecules are broken down 3- to 17.5-fold faster than wild-type enzyme. These studies show that even a small change in a protein, such as a missense mutant that still retains its antigenic determinants, is sufficient to cause selective degradation. This means that the cellular machinery for protein degradation is extremely efficient at detecting abnormal proteins. A simple model that could explain this selectivity is that most proteins have undergone a strong selective pressure to attain a configuration resistant to proteolytic breakdown (23). For these proteins, almost any change in configuration would result in increased sensitivity to proteolysis. There may, of course, exist classes of proteins for which a high rate of turnover is advantageous to the cell.

The HGPRT$^-$ CRM$^+$ mutants we have used in this study all have the same rate of total protein breakdown as the parental L$^+$ cell line. The rates of synthesis of the CRM are also the same as L$^+$. The levels of the mutant HGPRTs in the cells are, however, lower than L$^+$ HGPRT because of their increased rates of breakdown. This selective proteolysis appears to be the controlling factor in determining the cellular concentration of the abnormal protein. All 43 of the HGPRT$^-$ CRM$^+$ cell lines we have examined have lower levels of CRM than L$^+$ has HGPRT. The mutants selected for this study have relatively high levels of CRM to permit an accurate determination of their breakdown rates. This means, however, that most of the HGPRT$^-$ CRM$^+$ mutants that we have isolated probably contain forms of HGPRT that are broken down even more rapidly than those reported in these studies.

We have emphasized proteolytic degradation to account for the rapid loss of altered HGPRT molecules in the mutant cell lines. As an alternative mechanism, the mutant proteins could have inherently faster intracellular denaturation rates with the concomitant loss of antigenic reactivity. Our previous studies on the rates of heat denaturation *in vitro* of the altered HGPRT molecules in these mutants do not support this second explanation (17). M345 and M343 are much more resistant to heat denaturation than L$^+$ HGPRT (i.e., M345 and M343 have half-lives of 50 and 125 min at 80°, respectively, compared to 23 min for L$^+$ HGPRT). We have also observed no correlation between heat stability *in vitro* and degradation rates *in vivo* of the mutant HGPRTs. One would anticipate such a correlation if spontaneous denaturation were the explanation. We conclude, therefore, that mammalian cells contain a very sensitive mechanism for selectively degrading abnormal proteins.

This work was done while S.H.H. and G.M.W. were Predoctoral Fellows at the Department of Biological Chemistry, Harvard Medical School and M.R.C. had a Faculty Research Award from the American Cancer Society. This investigation was supported by a grant from the United States National Institutes of Health No. GM 16280.

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