Degradation of Proteins Microinjected into IMR-90 Human Diploid Fibroblasts

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ABSTRACT Erythrocyte ghosts loaded with ^{125}I-labeled proteins were fused with confluent monolayers of IMR-90 fibroblasts using polyethylene glycol. Erythrocyte-mediated microinjection of ^{125}I-proteins did not seriously perturb the metabolism of the recipient fibroblasts as assessed by measurements of rates of protein synthesis, rates of protein degradation, or rates of cellular growth after addition of fresh serum.

A mixture of cytosolic proteins was degraded after microinjection according to expected characteristics established for catabolism of endogenous cytosolic proteins. Furthermore, withdrawal of serum, insulin, fibroblast growth factor, and dexamethasone from the culture medium increased the degradative rates of microinjected cytosolic proteins, and catabolism of long-lived proteins was preferentially enhanced with little or no effect on degradation of short-lived proteins. Six specific polypeptides were degraded after microinjection with markedly different half-lives ranging from 20 to 320 h. Degradative rates of certain purified proteins (but not others) were also increased in the absence of serum, insulin, fibroblast growth factor, and dexamethasone.

The results suggest that erythrocyte-mediated microinjection is a valid approach for analysis of intracellular protein degradation. However, one potential limitation is that some microinjected proteins are structurally altered by the procedures required for labeling proteins to high specific radioactivities. Of the four purified proteins examined in this regard, only ribonuclease A consistently showed unaltered enzymatic activity and unaltered susceptibility to proteolytic attack in vitro after iodination.

Intracellular protein degradation is a fundamentally important process occurring in all organisms from bacteria to humans (5, 20, 23, 24, 53). The continued breakdown and replacement of proteins allows the cell to regulate concentrations of specific enzymes as well as to alter overall protein content in response to changing physiological demands. The major areas of current study in protein degradation can be divided into three broad topics: (*a*) the influence of polypeptide structure on protein half-lives (20, 23), (*b*) the physiological regulation of protein degradative rates (5, 20, 24), and (*c*) the mechanisms by which proteins are degraded within cells (5, 24, 53). Despite considerable recent progress in each of these areas, many of the major questions in this field of research remain unanswered.

Microinjection offers several advantages for analysis of protein degradation over more conventional approaches in which endogenous cellular proteins are radiolabeled and their degradative rates determined (11, 34, 51). Microinjection of mammalian cells in culture has been achieved using microneedles (6, 55) or by inducing fusion of the recipient cell with erythrocyte ghosts containing the protein to be microinjected (6, 34, 51). Most studies of protein degradation have used erythrocyte-mediated microinjection because sufficient numbers of cells can be microinjected for subsequent biochemical analysis. Several investigators have noted that microinjected hemoglobin (26), BSA (34, 66, 68), antibodies (62, 67, 68), and nonhistone chromosomal proteins (47, 66, 68) are catabolized after microinjection into cultured cells.

Although erythrocyte-mediated microinjection has great potential to answer many of the unresolved questions in the field of intracellular protein degradation, the few previous investigations using this technique have not fully evaluated its validity. Thus, it is not yet clear whether results from microinjection

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studies can be legitimately used to draw conclusions about normal, endogenous protein breakdown. We now report that erythrocyte-mediated microinjection of proteins into confluent cultures of IMR-90 human fibroblasts appears to be a valid approach for the study of intracellular protein degradation. However, certain proteins are damaged by radiolabeling procedures so that their catabolism after microinjection may reflect breakdown of abnormal rather than native proteins.

MATERIALS AND METHODS

Cell Culture

IMR-90 human diploid lung fibroblasts (40) were obtained at population doubling level 20 from the American Type Tissue Culture Collection, Rockville, Md. Cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (K-C Biologicals, Lenexa, Kan.), penicillin (100 μg/ml) and streptomycin (100 μg/ml) (both from Gibco Laboratories) in a humidified atmosphere of 90% air and 10% CO_2 at 37°C. Subcultivation of cell monolayers was done using 0.25% trypsin in calcium- and magnesium-free phosphate buffered saline (Gibco Laboratories) at a split ratio of 1:4 (5×10^5 cells/100 mm Corning tissue culture plate, Corning Glass Works, Corning, N. Y.). Cells were fed every 3–4 d with fresh complete medium. Cultures were routinely screened for mycoplasma contamination (Bioassay Systems Corp., Woburn, Mass.), and cells used for experiments were at population doubling levels between 20 and 40.

Preparation and Loading of Erythrocyte Ghosts

Erythrocyte ghosts were prepared and loaded with radioactive proteins according to a modification of the method of Rechsteiner (46). Fresh human blood, a gift from Dr. Fred Tauber, Department of Medicine, Harvard Medical School, was collected in citrate anticoagulant buffer (6% Dextran-70 in 0.9% NaCl diluted with an equal volume of 0.23 M citrate buffer, pH 5.2) at a ratio of 1 part whole blood to 3 parts buffer. The erythrocytes were allowed to settle out at room temperature, and the serum and white cells were removed by aspiration. The erythrocytes were washed three times in HBSS (Hanks' buffered salt solution, Gibco Laboratories) containing 100 μg/ml each of penicillin and streptomycin. The erythrocytes were collected by centrifugation at 630 *g* for 10 min and the final pellet was adjusted to 30% (v/v) with HBSS. One ml of the 30% erythrocyte solution was diluted with 9 ml of ⅓ strength HBSS and centrifuged at 630 *g* for 20 min at 4°C. The supernatant fluid was removed and, while the pelleted cells were vortexed, 0.3 ml of 5 mM sodium phosphate solution containing the protein to be loaded was added. The suspension was vortexed for 4 min at room temperature, then 0.03 ml of concentrated HBSS (10×) was added, and the ghosts were incubated with gentle shaking for 1 h at 37°C. After this period, the ghosts were washed three times in 10 ml of HBSS and collected by centrifugation at 320 *g* for 20 min. The final pellet was resuspended in 1 ml of HBSS, and the number of ghosts was determined with a hemocytometer. Loaded ghosts were stored at 4°C and used for fusion experiments within 24 h.

Fusion

A 10% (w/v) solution of PEG(polyethylene glycol)-1000 (J. T. Baker Chemical Co., Phillipsburg, N. J.) was freshly prepared for each fusion. One gram of PEG-1000 was autoclaved, 9 ml of DMEM were added, and the resulting solution was kept at 37°C. Confluent monolayers of fibroblasts in 100 mm plates ($\sim 2 \times 10^6$ cells/plate) were washed three times with HBSS. Approximately 3×10^8 loaded ghosts were added in a volume of 0.15 ml to each plate. The solution was gently swirled to distribute the ghosts over the entire surface, and immediately thereafter, 0.96 ml of 10% PEG-1000 was added and the solution swirled again. After exactly 1 min at room temperature, 4.5 ml of DMEM at 37°C were added to each plate with minimal disturbance of the PEG layer, and the cells were incubated for 30 min at 37°C. The medium was then removed, the monolayers were washed twice with 10 ml of HBSS containing 10% fetal calf serum (FCS), and 10 ml of DMEM containing 10% FCS were added to each plate. After incubation at 37°C for 7 h this medium was removed and replaced with 10 ml of fresh medium with or without added factors, and degradation measurements were begun at this time.

Autoradiography

Fibroblasts on cover slips were rinsed with PBS, pH 7.4, and fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After several rinses in the same solution, the cover slips were air-dried and mounted onto glass slides. The slides were coated in the dark with Kodak NTB-2 emulsion diluted 1:1 with distilled water. After a 2 wk exposure the slides were developed in D-19 (Kodak) and fixed (rapid fixer, Kodak).

Protein Fractionation

Proteins were separated according to subunit molecular weight by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or according to isoelectric point by isoelectric focusing as described previously (19) except that the isoelectric focusing was done in the presence of 6 M urea to reduce adventitious association of radioactive proteins with cellular constituents.

Degradative Rates of Cellular Proteins

Confluent cultures of fibroblasts were labeled for 3 d in DMEM containing 10% FCS and 1 μCi/ml [^{14}C]leucine (>300 Ci/mmol, New England Nuclear, Boston, Mass.). Medium was replaced with DMEM plus serum containing excess unlabeled leucine (2 mM) for 1 d to permit degradation of short-lived proteins containing [^{14}C]. The fibroblasts were then labeled for 1 h with 5 μCi/ml [^{3}H]-leucine (>110 Ci/mmol, New England Nuclear), and the medium was again replaced with medium containing excess unlabeled leucine (2 mM) for 1 h to permit secretion of exported proteins (7, 41). The cells were rinsed twice with 10 ml of HBSS and finally were incubated in nonradioactive medium containing excess unlabeled leucine (2 mM). Aliquots of the medium were removed at various times and TCA(trichloroacetic acid)-soluble radioactivity determined as a measure of degradation of short-lived (^{3}H) and long-lived (^{14}C) proteins. At the end of the experiment the total radioactivity in the cells was determined and the acid-soluble radioactivity expressed as a percentage of the initial radioactivity in the cells.

Degradation of Microinjected Proteins

Protein degradation was measured by assaying TCA-soluble radioactivity released into the medium after microinjection. Separate experiments indicated that >95% of the acid-soluble radioactivity produced after microinjection of ^{125}I-RNase A was iodotyrosine (64). This result indicates that little dehalogenation occurs in IMR-90 fibroblasts as has also been reported for other cell lines in culture (68). Aliquots (0.5 ml) was taken from 100-mm plates initially containing 10 ml of medium. To each aliquot, TCA was added to a final concentration of 10%, except that phosphotungstic acid (PTA) at a final concentration of 3.25% in 5% HCl was used to precipitate RNase A (13). Precipitated aliquots were centrifuged for 2 min in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and radioactivity in the supernatant fluid was determined. At the end of the experiment the cell monolayer was solubilized in 0.1 M NaOH-0.1% deoxycholate and the radioactivity remaining in the cells was determined. The radioactivity in the cells plus the cumulative radioactivity released into the medium was defined as total radioactivity initially microinjected into the cells, and results are expressed as the percentage of radioactivity remaining in the cells as a function of time.

Effects of Nutrients and Hormones on Degradation of Microinjected Proteins

Fibroblasts were grown in DMEM containing 10% FCS as described. The cells remained in DMEM containing 10% FCS until the beginning of the protein degradation measurements 7 h after microinjection. At this time the medium was changed to rich ("+") conditions consisting of medium supplemented with 10% FCS, insulin (50 ng/ml), fibroblast growth factor (FGF, 120 ng/ml), and dexamethasone (400 ng/ml) or to poor ("−") conditions consisting of medium without these supplements.

Counting of Radioactivity

In experiments involving ^{125}I only, the isotope was counted in a Packard Instruments gamma counter (Packard Instrument Co., Downers Grove, Ill.). In dual label experiments, ^{125}I and ^{131}I radioactivity or ^{3}H and ^{14}C radioactivity were determined by liquid scintillation counting in Soluscint A fluor (National Diagnostics, Inc., Somerville, N. J.) using a Packard Model 3255 liquid scintillation spectrometer (Packard Instrument Co., Inc.). Quenching was monitored with an external standard.

Radioiodination of Proteins

Bovine serum albumin (BSA) and ovalbumin (fraction V recrystallized to >99% purity) were obtained from Sigma Chemical Co., St. Louis, Mo. Lysozyme

and RNase A were purchased from Worthington Biochemicals Corp., Freehold, N. J., polyglutamate:tyrosine (1:1) was obtained from Miles Biochemicals, Miles Laboratories, Inc., Elkhardt, Ind., and ubiquitin was generously provided by Drs. A. L. Goldberg, Department of Physiology and Biophysics, Harvard Medical School, and G. Goldstein, Ortho Pharmaceutical Co., Raritan, N. J. Rat liver proteins, soluble after centrifugation at 100,000 *g* for 1 h (cytosolic proteins), were prepared as described previously (18).

Proteins were iodinated using lactoperoxidase and glucose oxidase (29) immobilized on Sepharose beads (Enzymobeads, Bio-Rad Laboratories, Richmond, Calif.). A typical 1-ml reaction contained 5 mg of protein in 100 mM sodium phosphate buffer, pH 7.4, 5 mCi $Na^{125}I$ (17 Ci/mg, New England Nuclear), 0.2% β-D-glucose, 0.2 ml rehydrated Enzymobeads, and various concentrations of NaI depending upon the desired I:protein ratio. The glucose was added last to initiate the reaction. Incubation was carried out with gentle shaking for 1 h at room temperature in a biochemical hood equipped for radioiodinations. The reaction mixture was then transferred to a previously calibrated 1 × 25 cm Sephadex G-25 column equilibrated with 5 mM sodium phosphate (pH 7.4). To reduce nonspecific adsorption of the radiolabeled protein, the column was prerun with the appropriate unlabeled protein at a concentration of 5 mg/ml. Fractions containing the radiolabeled protein were collected in 5 mM sodium phosphate, (pH 7.4) aliquoted into 0.1-ml samples, and frozen at −20°C. Protein was determined by assaying for fluorescamine-positive, TCA-precipitable material (59), except in the case of RNase A where PTA in HCl was used to precipitate the protein (13).

Assays for RNase A and Lysozyme Activity

Lysozyme was assayed by its ability to hydrolyze *Micrococcus lysodeikticus* cell walls (Sigma Chemical Co.) as described by Shugar (54). RNase A activity was determined by its ability to hydrolyze yeast RNA (Sigma Chemical Co.) according to the method of Kalnitsky, Hummel, and Dierks (30).

Measurement of In Vitro Susceptibility to Proteolysis

Proteolysis was measured in vitro essentially as described previously (19). A small amount (10–20 μg) of iodinated protein was mixed with an excess (10 mg) of the same uniodinated protein in 1 ml of 100 mM sodium phosphate buffer (pH 7.4). Trypsin, chymotrypsin (both from Worthington Biochemical Corp.), or Pronase (Sigma Chemical Co.) was then added, and the reaction was allowed to proceed at room temperature. Aliquots were withdrawn periodically and precipitated with 10% TCA or, in the case of RNase A, with 3.25% PTA in 5% HCl (13). Radioactivity and fluorescamine-positive material in the acid-soluble supernatant fluid were determined. The total radioactivity and total fluorescamine-positive material were defined as those amounts that appeared in the acid-soluble fraction after extensive hydrolysis (5 d).

A similar protocol was used to compare the susceptibility of ^{125}I- and ^{131}I-labeled cytosolic proteins to proteolytic attack (See Fig. 5). In these experiments the appearance of ^{125}I and ^{131}I in the acid-soluble fraction was compared to the acid-insoluble radioactivity before addition of the protease to calculate the percent of the protein hydrolyzed.

Statistical Calculations

To determine whether correlations existed between half-lives of microinjected proteins and their subunit molecular weight or isoelectric point, we calculated linear correlation coefficients between ^{131}I:^{125}I ratios of proteins and their migration distances in SDS-polyacrylamide gels or their isoelectric points, respectively. The significance of the correlation coefficient was obtained from statistical tables (43). The significance of changes in rates of synthesis and degradation of endogenously labeled proteins due to the microinjection procedure and the significance of altered half-lives of microinjected proteins in the presence and absence of serum, insulin, FGF, and dexamethasone were calculated using a two-tailed Student's *t* test.

RESULTS

Microinjection Procedure

LOADING AND STABILITY OF PROTEINS IN ERYTHROCYTE GHOSTS: The amount of protein loaded into ghosts varied between 20 and 30% of each of the proteins studied except for lysozyme and RNase A where the capture was somewhat lower (10–15%). Proteins loaded into erythrocyte ghosts appeared to be stable because ghosts loaded with ^{125}I-lysozyme, ^{125}I-BSA, ^{125}I-RNase A, or ^{125}I-cytosolic proteins from rat liver produced no acid-soluble radioactivity when incubated for 5 d at 4°C. Furthermore, all the acid-soluble radioactivity migrated in SDS-polyacrylamide gels at the expected positions for the native proteins (data not shown). However, a small amount of each protein (2–10%) was degraded to acid-soluble products when incubated for 1 d at 37°C. Because the loaded ghosts are exposed to 37°C for only 30 min during fusion, hydrolysis of the labeled protein before microinjection is not likely to be a serious problem.

FUSION OF LOADED GHOSTS WITH FIBROBLASTS: The fusion procedure we used in these studies is similar to that used by other laboratories (11, 31, 33, 52) except that the PEG concentration was lower in our experiments (9% vs. 40–50%).

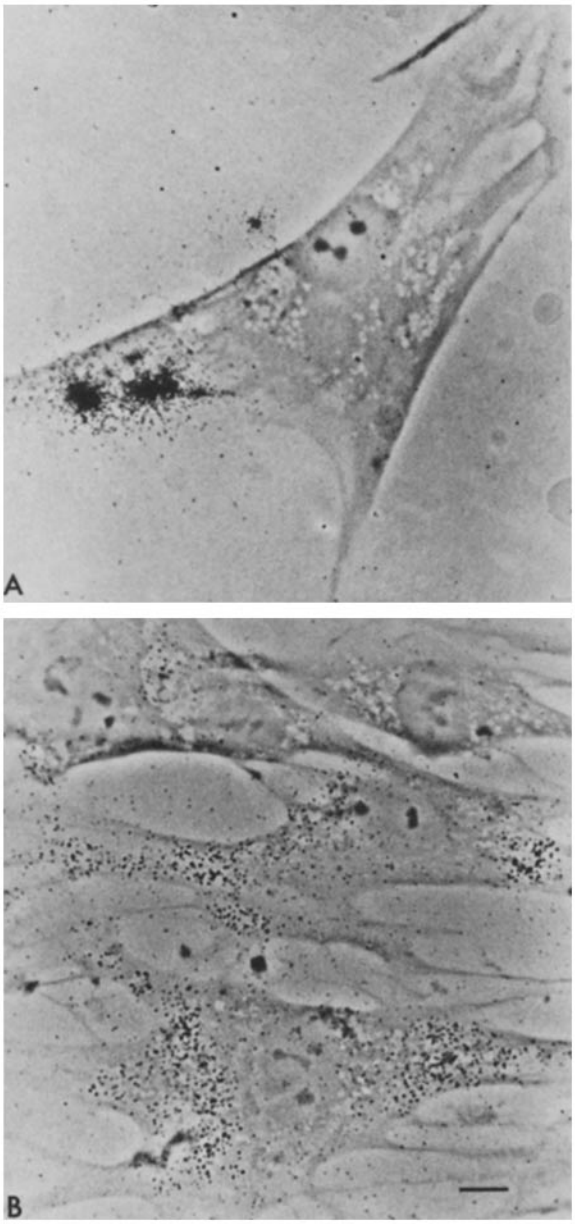

FIGURE 1 Autoradiograph of IMR-90 fibroblasts microinjected with ^{125}I-cytosolic proteins. Cytosolic proteins from rat liver were isolated, iodinated, and microinjected as described in Experimental Procedures. The cells were fixed one min (A) or 7 h (B) post fusion, and autoradiographic analysis was carried out as described in the text. Bar (*B*), 10 μm. × 960.

High concentrations of PEG (40–50%) actually worked less well in our hands than did lower concentrations, probably due to the reduced contact between the overlying ghosts and the fibroblast monolayer with the more viscous, high concentrations of PEG.

Our initial studies showed that an average of two to five ghosts fused with one fibroblast with an initial incubation ratio of 150:1 (ghosts:fibroblast). However, for unknown reasons fusion multiplicities were lower with ghosts loaded with lysozyme or RNase A (0.5–1.0 ghosts:fibroblast). We calculated the fusion multiplicities by determining the average radioactivity loaded per ghost and the amount of radioactivity microinjected into cells at 7 h post fusion.

Autoradiographic examination of fibroblasts microinjected with ^{125}I-labeled cytosolic proteins from rat liver showed that the microinjected proteins were distributed randomly throughout the cytoplasm by 7 h post fusion (Fig. 1 *B*). In contrast, cultures that were fixed 1 min after addition of PEG contained highly localized radioactivity that was presumably still contained in erythrocyte ghosts or was localized in the fibroblasts' cytoplasm at the site of injection (Fig. 1 *A*).

Microinjection appeared to be complete by 30 min after addition of PEG because the percentage of cells with fluorescent cytoplasm after fusion with ghosts loaded with fluoresceinated BSA or fluoresceinated RNase A was constant between 30 min and 7 h after fusion (50–60% of the cells, data not shown). However, at 30 min some unfused, fluorescent ghosts remained associated with the fibroblasts. By 7 h after fusion all such ghosts had detached from the monolayer and could be recovered from the culture medium.

Our ability to fuse ghosts with fibroblasts using low PEG concentrations depended on several factors. Fresh erythrocytes were required because storage of erythrocytes or loaded ghosts for >1 d at 4°C resulted in a dramatic decrease in their fusibility with fibroblasts. Furthermore, addition of only a small volume of PEG appeared to be important to increase the frequency of interactions between ghosts and the fibroblast monolayer. Other considerations that may be important in our procedure include the composition of the anticoagulant buffer in which the erythrocytes are collected, the commercial source and average mol wt of the PEG, and the specific properties of confluent cultures of IMR-90 fibroblasts.

Little or no fibroblast to fibroblast fusion occurred with our microinjection procedure because we could detect no multinucleated fibroblasts 30 min or 7 h after PEG treatment.

Effects of Microinjection on Cellular Protein Metabolism

The recipient fibroblast could theoretically be affected by several factors in the microinjection procedure including exposure to the PEG, integration of the erythrocyte membrane into the plasma membrane, and introduction of the microinjected protein into the cytoplasm. We did the following experiments to analyze the effects of microinjection on the recipient cells.

RATES OF PROTEIN SYNTHESIS AFTER MICROINJECTION: We loaded ghosts with nonradioactive RNase A and microinjected this protein into fibroblasts. As various times after fusion, we determined relative rates of cellular protein synthesis by pulse-labeling fibroblasts with [^{3}H]leucine for 1 h in the presence of high concentrations of unlabeled leucine (2 mM). This protocol minimizes possible differences in endogenous amino acid pool sizes and rates of amino acid transport

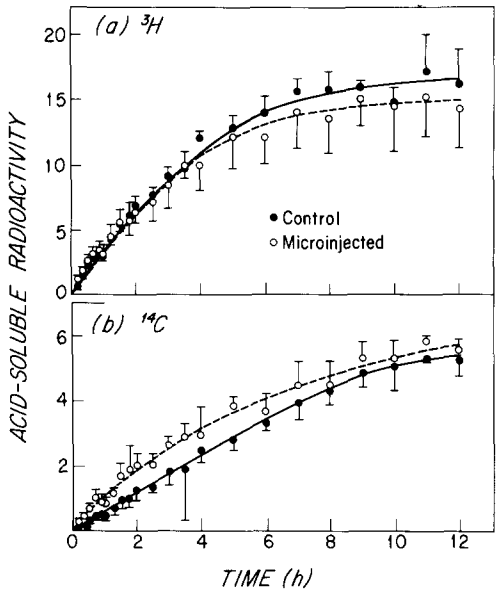

FIGURE 2 The effects of microinjection on the degradation of endogenously labeled proteins. Short-lived (^{3}H) and long-lived (^{14}C) proteins were selectively labeled in the same cell cultures. Cells were labeled for 3 d with [^{14}C] leucine followed by a 1-d chase. The same cultures were then labeled with [^{3}H] leucine for 1 h followed by a 1-h chase. One group of cultures was then microinjected with ghosts containing nonradioactive BSA (○) while another group served as a control (●). Immediately after the PEG was removed from the microinjected cells, rates of protein degradation were followed in DMEM containing 10% fetal calf serum. Each point represents the average ± one standard deviation for five cultures. No points in (*a*) are significantly different from controls. Most (7/10) points in (*b*) are significantly different ($p < 0.05$) from controls for the first 2 h, but most (10/12) are not in the subsequent 10 h.

and has been shown to be a valid measure of protein synthesis in cultured fibroblasts (28, 38). Relative rates of protein synthesis were essentially the same at 2, 6, and 24 h after fusion of erythrocyte ghosts with fibroblasts (data not shown). Furthermore, the stimulation of cell growth after addition of fresh serum, as assayed by net protein accumulation (35), was indistinguishable between untreated and microinjected cultures (data not shown).

RATES OF PROTEIN DEGRADATION AFTER MICROINJECTION: We also analyzed the effect of microinjection on degradative rates of endogenously labeled cellular proteins. We studied breakdown of both short-lived and long-lived proteins in the same cultures by differential labeling with [^{14}C]leucine and [^{3}H]leucine (9, 23). We then microinjected cells with nonradioactive BSA and followed the catabolism of the two classes of cellular proteins (Fig. 2). There was an initial 40% stimulation of breakdown of long-lived proteins in microinjected cells, but their degradative rates returned to normal well within the 7-h period before we began to measure degradation of microinjected proteins. Microinjection did not significantly affect degradation of short-lived proteins at any time-point examined. These results were obtained in four separate experiments.

DEGRADATION OF MICROINJECTED PROTEINS WITH INCREASING GHOST:FIBROBLAST FUSION RATIOS: To determine whether the amount of erythrocyte membrane introduced into the fibroblast plasma membrane or the amount of protein microinjected into the cytoplasm influenced rates of proteolysis, we microinjected fibroblasts with increasing num-

bers of ghosts loaded with ^{125}I-cytosolic proteins from rat liver. We varied the initial ratio of ghosts added to fibroblasts to obtain a range of fusion multiplicities and calculated the number of ghosts fused per fibroblast by measuring the average amount of radioactive protein captured per ghost and the radioactivity introduced per fibroblast 7 h after PEG treatment. The microinjected cytosolic proteins were degraded at the same average rate ($T_{1/2} = 45 \pm 6$ h, with fusion multiplicities ranging from 0.8 to 15 (data not shown). Furthermore, similar experiments in which BSA and RNase A were microinjected at increasing fusion multiplicities indicated that half-lives of these proteins were also independent of the number of ghosts fused per fibroblast.

Influence of Polypeptide Structure on Half-Lives of Microinjected Proteins

Many studies of the degradation of endogenously labeled cytosolic proteins have indicated that the degradative rates of proteins, like their catalytic and regulatory properties, are determined primarily by differences in protein structure (reviewed in 20, 23, and 24). For example, the catabolic rates of normal polypeptides in all eucaryotic cells examined, including IMR-90 fibroblasts, are related to protein size, isoelectric point, and susceptibility to proteolytic attack such that rapidly degraded proteins tend to be large, acidic, and/or especially protease sensitive (see Discussion).

Dual-isotope labeling has been the major experimental approach used to establish the various correlations between the structure of cellular proteins and their half-lives (3, 23), and we have used a similar approach to determine whether these general correlations also apply to microinjected proteins. Cytosolic proteins from rat liver contain polypeptides of widely varying mol wt, isoelectric points, and protease sensitivities, and the various correlations between protein structure and half-life have been best studied in rat liver cytosol. We therefore prepared rat liver cytosolic proteins (18) and divided them into two identical aliquots. We labeled one aliquot with ^{125}I and the other with ^{131}I. We then loaded these proteins into separate groups of erythrocyte ghosts and microinjected the ^{125}I-labeled proteins into fibroblasts where they were degraded for various times within the cells.

Our initial experiments showed that the ^{125}I- and ^{131}I-labeled proteins loaded into the two separate ghost preparations were indistinguishable based on SDS-PAGE, isoelectric focusing, or sensitivity to digestion by Pronase. Furthermore, the proteins initially microinjected into cells were a random representation of the proteins loaded into ghosts. Therefore, in certain experiments we microinjected the ^{131}I-labeled proteins but did not allow them to degrade (harvested at 7 h post fusion) whereas in other experiments we simply added ghosts loaded with ^{131}I-labeled proteins to the cell cultures (3 ghosts:fibroblast) immediately before harvesting. With either protocol, the ^{131}I in any protein fraction indicates the relative amount of those proteins initially microinjected whereas the ^{125}I reflects the amount remaining after the period of degradation within the cell. Thus, fractions with high ^{131}I:^{125}I ratios represent rapidly degraded proteins, whereas those with low ^{131}I:^{125}I ratios represent slowly degraded proteins.

MOLECULAR SIZE: The average half-life of the ^{125}I-labeled cytosolic proteins microinjected into fibroblasts was 43 h in this experiment, but degradative rates of polypeptides of different sizes were strikingly heterogeneous. Fig. 3 shows ^{131}I:^{125}I ratios for protein fractions after separation by SDS-PAGE. Proteins with subunit mol wt greater than 60,000 did not load into ghosts as efficiently as smaller proteins, so there was not enough radioactivity in these larger fractions for accurate determination of isotopic ratios. Nevertheless, it is clear for the range of proteins analyzed that proteins with larger subunit mol wt tend to be degraded more rapidly than proteins with smaller subunit mol wt at all periods of degradation examined (7 hr, $\bar{r} = -0.77$; 29 h, $\bar{r} = -0.89$; 39 h, $\bar{r} = -0.90$; 55 h, $\bar{r} = -0.93$; all values are $p < 0.005$).

ISOELECTRIC POINT: In less extensive experiments of identical design, we fractionated cytosolic proteins by isoelectric focusing after 39 h of degradation. Of the iodinated protein analyzed, 70% was acidic (pI 4–7) and 30% was basic (pI 7–12) as determined by the radioactive profile of the isoelectric focusing column. There were 16 identifiable radioactive peaks in the acidic region (pI 4–7) and 14 in the basic region (pI 7–12). As shown in Fig. 4, in general, acidic proteins were broken down more rapidly than basic proteins ($\bar{r} = -0.72$, $p < 0.005$). We obtained this result in two separate experiments.

SENSITIVITY TO PROTEASES *IN VITRO*: When we microinjected both ^{125}I-labeled and ^{131}I-labeled cytosolic proteins loaded into different ghost preparations into fibroblasts, they were equally susceptible to Pronase digestion in vitro 7 h post fusion (Fig. 5*a*). However, when we microinjected the ^{125}I-labeled proteins into fibroblasts and allowed them to be degraded for 32 h, the proteins remaining were much less sensitive to Pronase (Fig. 5*b*). Similar results were obtained using trypsin or chymotrypsin. These results suggest that the microinjected proteins that are more rapidly degraded by fibroblasts tend also to be more susceptible to proteolytic attack in vitro.

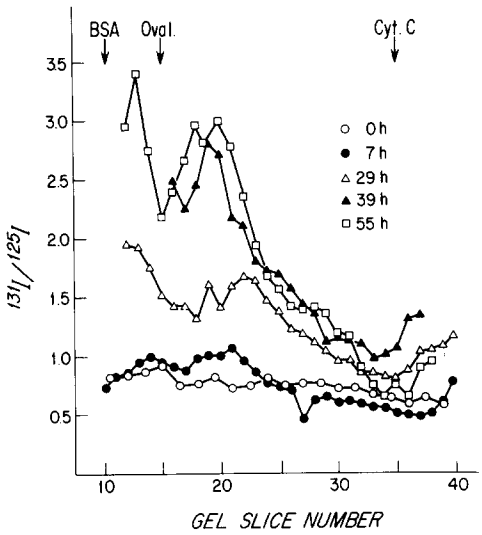

FIGURE 3 The relationship between subunit size and degradative rate of microinjected cytosolic proteins. ^{125}I-labeled cytosolic proteins were microinjected into confluent cultures of fibroblasts and were allowed to be degraded intracellularly for various times before harvesting (○, 0 h; ●, 7 h; △, 29 h; ▲, 39 h; and □, 55 h). An aliquot of ghosts loaded with ^{131}I-labeled proteins was added to the cells before harvesting, and proteins were then separated according to subunit molecular weight by SDS-polyacrylamide gel electrophoresis. Protein fractions with high ^{131}I:^{125}I ratios reflect more rapid degradation after microinjection. Arrows indicate the positions of protein standards of known molecular weight run simultaneously with the other gels. *BSA* = bovine serum albumin, 67,000; *Oval.* = ovalbumin, 45,000; Cyt. C = cytochrome C, 13,000.

However, we have not yet ruled out an alternative interpretation of our findings which would propose that some proteins are preferentially adsorbed to intracellular membranes between 7 and 32 h after microinjection and thereby become less susceptible to proteolytic attack.

Effects of Medium Composition on Degradation of Microinjected Cytosolic Proteins

Average rates of degradation of endogenously labeled proteins in cultured cells are enhanced by withdrawal of certain hormones and nutrients from the medium (4, 24). This enhanced degradation applies preferentially to long-lived proteins with little or no effect on catabolism of short-lived proteins (see Discussion). Furthermore, we have recently shown that withdrawal of serum, insulin, fibroblast growth factor (FGF), and dexamethasone from IMR-90 fibroblasts increases the degradative rates of endogenously labeled long-lived proteins twofold with no effect on the half-lives of short-lived proteins (J. F. Dice, L. Bourret, and V. Bochaki. Manuscript in preparation).

Catabolism of microinjected cytosolic proteins is also enhanced in medium lacking serum, insulin, FGF, and dexamethasone (Fig. 6).The kinetics of degradation suggest a preferential effect on catabolism of long-lived proteins because the most rapidly degraded fraction of protein is not affected by deprivation conditions ($t_{1/2}$ = 24 h in both supplemented and deprived cultures) whereas the degradation of more slowly degraded proteins is enhanced ~twofold ($t_{1/2}$ = 58 h in supplemented cultures and $t_{1/2}$ = 24 h in deprived cultures). The alternative explanation that deprivation requires ~10 h to achieve the increased degradative rates appears to be incorrect

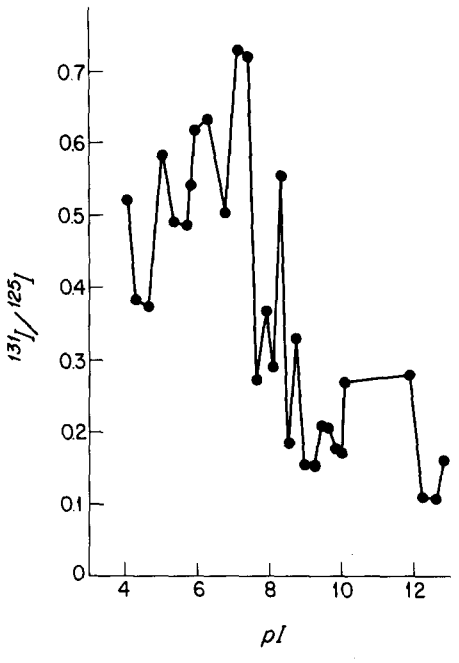

FIGURE 4 The relationship between isoelectric point and degradative rate of microinjected cytosolic proteins. ^{125}I-labeled proteins were microinjected and allowed to degrade in fibroblasts for 39 h. Ghosts loaded with ^{131}I-labeled proteins were added, and the proteins were separated by isoelectric focusing in 6 M urea. Radioactivity was determined for TCA-insoluble proteins in each fraction (19).

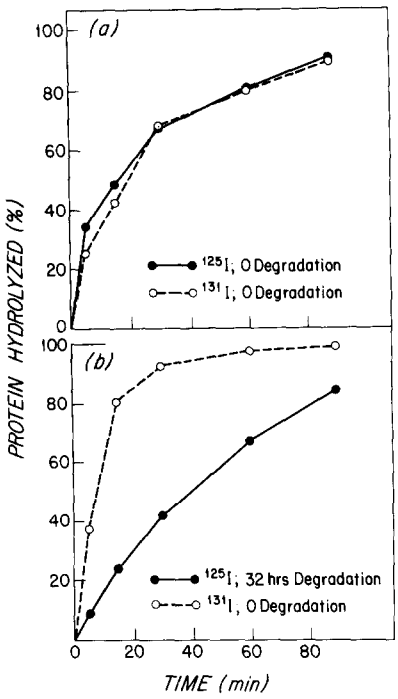

FIGURE 5 The relationship between in vitro susceptibility to Pronase and intracellular degradative rates of microinjected cytosolic proteins. ^{125}I- and ^{131}I-labeled proteins were separately loaded into ghosts. In (*a*) both ^{125}I- and ^{131}I-labeled proteins were microinjected into fibroblasts, and the cells were harvested and sonicated 7 h post fusion ("0 degradation"). Pronase was added to a final concentration of 200 μg/ml, and the appearance of TCA-soluble ^{125}I and ^{131}I followed. The proteins were initially equivalently susceptible to proteolytic attack. In (*b*) the ^{125}I-labeled proteins were microinjected into fibroblasts and allowed to degrade for 32 h. The remaining ^{125}I-labeled proteins were much more resistant to digestion by Pronase (250 μg/ml in this case) than the ^{131}I-labeled protein microinjected into separate cultures which were harvested at 7 h postfusion.

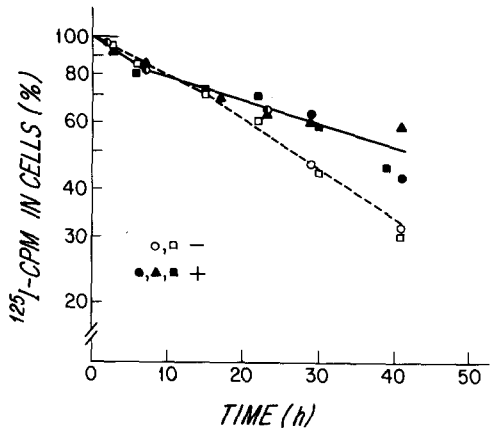

FIGURE 6 Effect of serum, insulin, FGF, and dexamethasone on degradation of microinjected cytosolic proteins. ^{125}I-labeled cytosolic proteins from rat liver were microinjected into confluent cultures of fibroblasts, and degradation of the microinjected proteins was followed. The data presented are from three separate experiments in the presence of 10% fetal calf serum, insulin (50 ng/ml), FGF (120 ng/ml), and dexamethasone (400 ng/ml) (●, ▲, ■) and two separate experiments in the absence of these medium supplements (O, □). Each point represents the average for five different cultures.

because degradation of specific microinjected proteins and of endogenously labeled cellular proteins is enhanced within 1 h under these conditions (data not shown).

The preferential effect of serum and hormone withdrawal on degradation of microinjected proteins that are long-lived is further supported by double-label experiments in which degradation of two classes of long-lived proteins (e.g. those that are small and those that are basic) appear to be selectively enhanced. We carried out double-isotope experiments similar to those described above except that the period of degradation of microinjected ^{125}I-proteins was either in the presence of or in the absence of serum and hormones. Degradation of small proteins is selectively enhanced in deprived cells with little or no effect on the breakdown of large proteins (data not shown). Also, degradation of basic proteins is preferentially increased with little effect on catabolism of acidic proteins (data not shown).

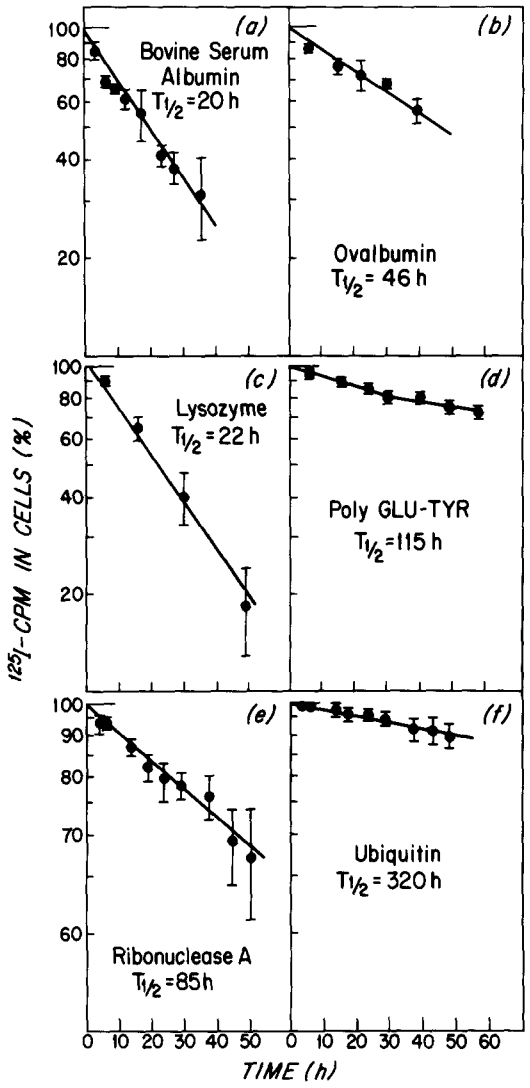

FIGURE 7 Degradation of specific polypeptides after microinjection into IMR-90 fibroblasts.

Proteins were iodinated, loaded into erythrocytes ghosts, microinjected into confluent monolayers of fibroblasts, and rates of degradation determined as described in Materials and Methods. The degradative curve for BSA (*a*) contains data points from three separate experiments, whereas other degradative curves are from single experiments representative of at least three trials yielding indistinguishable results. Each point shows the average ± one standard deviation for five cultures.

Degradation of Specific Proteins after Microinjection

Six different proteins microinjected into separate cultures of confluent fibroblasts were degraded at markedly heterogeneous rates in the presence of serum, insulin, FGF, and dexamethasone (Fig. 7). Half-lives ranged from 20 h for BSA to 320 h for ubiquitin. Degradation of each microinjected protein followed the expected exponential decay kinetics for intracellular protein degradation with the exception of the synthetic polypeptide, polyglutamate:tyrosine, 1:1 (Fig. 7*d*). This copolymer consists of a mixture of molecules with heterogeneous sizes (mol wt = 10,000–50,000), and within this mixture, larger mol wt copolymers tended to be degraded more rapidly than smaller ones after microinjection (data not shown). Therefore, the complex kinetics of breakdown of polyglutamate:tyrosine are not surprising.

Repeated microinjection experiments with the same preparation of ^{125}I-labeled proteins yielded very reproducible half-lives. For example, the degradative curve for BSA (Fig. 7*a*) contains data points from three separate experiments. However, different preparations of ^{125}I-BSA yielded different half-lives (10–40 h) as has also been reported by Zavortink, Thacher, and Rechsteiner (68). Because the conformation of BSA is altered by iodination (see below), variable extents of disruption of the protein conformation may explain the variability of half-lives obtained. For reasons that have not yet been clarified, the half-life of RNase A varied substantially even in multiple experiments using the same protein preparation ($t_{1/2}$ = 40–95 h). This variability is not related to the preparation of ^{125}I-RNase A used and is independent of the amount of RNase A injected.

Degradative rates of specific microinjected proteins were either decreased or unaffected by withdrawal of serum, insulin, FGF, and dexamethasone (Table I). The catabolic rates of BSA, ovalbumin, and lysozyme were not significantly altered by the medium composition, but those of RNase A, ubiquitin, and polyglutamate:tyrosine were increased. These results indicate that factors that regulate average rates of protein degradation have heterogeneous effects on the breakdown of individual microinjected proteins.

Effects of Iodination on Protein Conformation

Proteins of high specific radioactivities are required for microinjection studies so that enough radioactivity is injected for accurate determinations of protein half-lives. The lactoperoxidase/glucose oxidase method for protein iodination (29) results in covalent coupling of iodine to tyrosine residues (36), and 5 mg of protein can be labeled to $\sim 1 \times 10^6$ dpm/μg starting with 5 mCi of Na^{125}I. However, we have found that this procedure disrupts the structure of certain proteins. We calculated the molar ratio of iodines incorporated per enzyme molecule from the initial specific radioactivity of the iodine in the reaction mixture and the resulting specific radioactivity of the iodinated protein. Iodination of lysozyme reduced its enzymatic activity and was dependent upon the average number of iodines per enzyme molecule (Fig. 8). We also subjected lysozyme to identical conditions except that no iodine was added to the reaction mixture. In this case lysozyme retained full enzymatic activity, indicating that iodination itself rather than the reaction conditions caused the inactivation of this

TABLE I

Effects of Serum, Insulin, FGF, and Dexamethasone on Degradation of Microinjected Proteins

Protein	Experiment	N	$t_{1/2}(+)$	$t_{1/2}(-)$	Change	Significance
					%	
BSA	1	5	20 ± 6	25 ± 3	−20	N.S.
	2	3	27 ± 3	24 ± 3	+12	N.S.
	3	5	22 ± 6	30 ± 5	−27	$p < .05$
Lysozyme	1	4	22 ± 4	16 ± 6	+38	N.S.
Ovalbumin	1	3	55 ± 5	49 ± 6	+12	N.S.
RNase A	1	4	58 ± 8	32 ± 6	+81	$p < .005$
	2	5	95 ± 8	42 ± 3	+126	$p < .005$
	3	6	59 ± 6	27 ± 7	+199	$p < .005$
	4	4	69 ± 6	48 ± 3	+60	$p < .005$
	5	5	40 ± 4	22 ± 2	+82	$p < .005$
Ubiquitin	1	4	320 ± 10	238 ± 20	+34	$p < .005$
Polyglutamate:tyrosine	1	5	126 ± 16	66 ± 14	+91	$p < .005$

Proteins were iodinated, microinjected, and degradative rates determined in the presence or absence of added factors as described in the text. N refers to the number of cultures microinjected, and half-life values are averages ±1 standard deviation for cultures in the presence of serum, insulin, FGF, and dexamethasone ($t_{1/2}+$) or in the absence of these factors ($t_{1/2}-$). The number of iodines:protein molecule was 0.7 to 1.0 for all proteins except BSA where the ratio was 2.3. Subunit molecular weights and isoelectric points, respectively, for the proteins are as follows: BSA, 67,000 and 4.8; Lysozyme, 14,400 and 11.0; Ovalbumin, 45,000 and 4.0; RNase A, 13,700 and 9.4; Ubiquitin, 8,600 and 6.7; polyglutamate:tyrosine, 30,000 and 4.3. These values were obtained from Worthington Biochemicals except for ubiquitin (63) and our direct determinations for the polyglutamate:tyrosine.

enzyme. In contrast, RNase A was unaffected by iodination. We attached up to three iodines per molecule of RNase A with full retention of enzymatic activity (Fig. 8).

We also monitored the effects of iodination on protein structure by comparing the susceptibilities of iodinated and native proteins to proteolytic attack in vitro. The sensitivity of proteins to in vitro digestion by proteases has been widely used as a probe of protein conformation because even slight changes in structure may result in large alterations in susceptibility to proteolytic attack (2, 8, 23, 49, 50). Fig. 9 compares the rates of digestion of four different iodinated proteins with their native counterparts in the same reaction mixture. In each case, the labeled protein contained an average of ~0.5 iodines per protein molecule so that most radioactive molecules were monoiodinated. Fig. 9*a* indicates that the initial sensitivity of ^{125}I-lysozyme to digestion by Pronase was enhanced fourfold over that for native lysozyme. In contrast, the susceptibility of ^{125}I-RNase A was identical to that of native RNase A (Fig. 9*b*). These results are in accord with the enzymatic activity of lysozyme being reduced by iodination whereas that of RNase A was unaffected. The initial protease sensitivity of BSA was three times higher after iodination (Fig. 9*c*), whereas we unexpectedly found that ovalbumin was four times less susceptible after iodination (Fig. 9*d*). We obtained qualitatively similar results for each of the proteins tested when trypsin or chymotrypsin rather than Pronase was used and when the digestion was carried out at 37°C instead of 22°C. Similar studies with a mixture of cytosolic proteins from rat liver showed that iodination had no effect on the average sensitivity to proteases in vitro if the number of iodines incorporated per protein was low (0.1–0.5; data not shown). These studies indicate that the effects of iodination on protein conformation are highly variable.

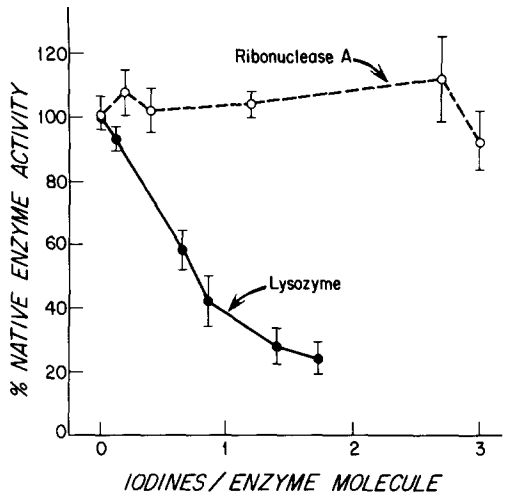

FIGURE 8 Effect of iodination on RNase A and lysozyme enzymatic activities. The enzymes were iodinated to different average iodine: protein ratios by varying the amount of unlabeled iodine added to the reaction mixture. The enzyme assays were carried out as described in Materials and Methods. Each point represents the average ± one standard deviation for five determinations.

DISCUSSION

Our results indicate that the microinjection procedure described here does not greatly perturb protein metabolism in confluent cultures of IMR-90 fibroblasts, so erythrocyte-mediated microinjection promises to be a very useful approach to the study of protein degradation in these cells. Relative rates of protein synthesis, rates of growth after addition of fresh serum, and degradative rates of short-lived proteins were indistinguishable in microinjected and control cultures (Fig. 2). Furthermore, degradation of long-lived proteins, although initially enhanced by ~40% because of microinjection, returned to normal within 2–4 h (Fig. 2).

We believe that microinjected proteins are catabolized by the same mechanisms responsible for breakdown of endogenous cytosolic proteins because microinjected cytosolic proteins were degraded according to several of the known characteristics of intracellular protein degradation.

(*a*) Large microinjected proteins were degraded more rapidly than small ones (Fig. 3). This result applied to all time-points of degradation examined from 7 to 55 h. This "size correlation" is a general feature of the catabolism of endogenous cytosolic proteins in eucaryotic cells (1, 12, 14, 15, 16, 25) including IMR-90 fibroblasts (L. Bourret and J. F. Dice. Manuscript in preparation).

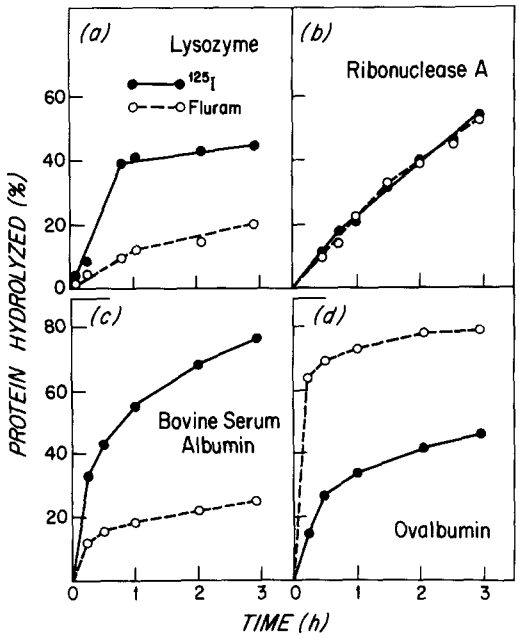

FIGURE 9 Susceptibility of iodinated and uniodinated proteins to Pronase digestion. Proteins were iodinated at an iodine:protein ratio of ~0.5. A trace of labeled protein (10–20 μg) was added to an excess of unlabeled protein (10 mg) and their rates of digestion by Pronase (200 μg/ml) were compared in the same reaction mixture as described in Materials and Methods. Each point is the average of three determinations. *Fluram* refers to fluorescamine-positive material as assayed according to the procedure of Udenfriend *et al.* (59).

(*b*) Microinjected proteins with acidic isoelectric points were degraded more rapidly than those with basic isoelectric points (Fig. 4). This "charge correlation" has also been observed for the degradation of cytosolic proteins from a wide variety of eucaryotic cells (12, 17, 19).

(*c*) Microinjected proteins that are rapidly degraded within cells appeared to be especially susceptible to proteolytic attack in vitro (Fig. 5) although other explanations for our results are also possible. The relationship between in vivo half-lives and sensitivity to proteases in vitro is a general characteristic of degradation of endogenous proteins in every cell type examined (reviewed in reference 23). Some aspect of the conformation of the proteins appears to determine their relative proteolytic susceptibility because the correlation is obtained with proteases of widely differing physical properties and specificities, and the correlation is abolished if the proteins are denatured before they are digested (57).

(*d*) Overall rates of catabolism of microinjected cytosolic proteins were increased under conditions of hormone and nutrient deprivation (Fig. 6). Serum, insulin, FGF, and dexamethasone are also important regulators of overall rates of endogenous protein degradation in rat fibroblasts (25, 61) and in human fibroblasts (4, 10; J. F. Dice, L. Bourret, and V. Bochaki. Manuscript in preparation). Degradative rates of certain purified proteins that were microinjected were also increased by withdrawal of serum, insulin, FGF, and dexamethasone (Table I).

(*e*) Degradative rates of microinjected cytosolic proteins that are long-lived were preferentially enhanced during deprivation conditions (Fig. 6). Degradative rates of endogenous long-lived proteins are also selectively increased in several cell types deprived of serum and hormones (21, 32, 39, 61). We have recently confirmed these findings in IMR-90 fibroblasts deprived of serum, insulin, FGF, and dexamethasone (J. F. Dice, L. Bourret, and V. Bochaki. Manuscript in preparation). Furthermore, two specific classes of microinjected proteins that are long-lived were degraded more rapidly in the absence of serum and hormones. That is, degradative rates of small proteins and basic proteins were selectively increased during deprivation thereby reducing the normal correlations between protein size and half-life and between protein net charge and half-life (data not shown). These reduced correlations also characterize the increased protein degradation in rat liver and muscle during diabetes and starvation (18) and in IMR-90 fibroblasts deprived of serum, insulin, FGF, and dexamethasone (L. Bourret and J. F. Dice. Manuscript in preparation).

These similarities in degradation of microinjected cytosolic proteins and endogenous cytosolic proteins strongly suggest that normal degradative systems are operative in IMR-90 fibroblasts after microinjection. Whether these conclusions apply to other cell lines after PEG-mediated microinjection remains to be established. Furthermore, the use of higher concentrations of PEG (11, 31, 33, 52) and lectins (52) in the microinjection procedure must be evaluated to determine whether these different microinjection conditions might perturb intracellular protein degradation.

Less extensive studies suggest that the use of inactivated Sendai virus as the fusogen for erythrocyte-mediated microinjection is also a valid procedure for the study of intracellular protein degradation. For example, proteins microinjected by virus-induced fusion are degraded at heterogeneous rates and with the expected exponential kinetics in a variety of cell lines (68). Furthermore, rates of degradation of endogenously labeled proteins in baby-hamster kidney (BHK) cells are normal within 2 h after the microinjection (26), and degradative rates of endogenous proteins as well as microinjected hemoglobin are increased in response to serum withdrawal (26).

The most serious potential problem in studying the degradation of microinjected proteins as a model for normal intracellular protein degradation concerns the effects of iodination on the conformation of the proteins to be microinjected. A variety of abnormal proteins are degraded especially rapidly by cells in culture (reviewed in references 23 and 24), and such proteins may be degraded by pathways distinct from those responsible for catabolism of normal proteins (32, 39, 55, 68). Although a complex mixture of proteins from rat liver cytosol showed no average alteration in sensitivity to proteolytic attack after iodination with low iodine:protein ratios, certain purified proteins were greatly affected by iodination (Figs. 8 and 9). The decreased enzymatic activity of lysozyme and the increased susceptibility to proteolytic attack of lysozyme and BSA after iodination are likely to reflect denaturation or unfolding of the polypeptide chain (50), whereas the decreased susceptibility of iodinated ovalbumin to proteolytic attack may reflect shielding of a particularly sensitive peptide bond by the iodine molecule, or, more likely, the aggregation of ovalbumin subsequent to its denaturation. These results agree with a growing number of reports that show that iodination of certain enzymes (22), hormones (27, 45, 65), and other proteins (42) results in reduced function, complete inactivation, or enhanced rates of endocytosis of the polypeptide. Proteins that are disrupted by iodination can possibly be labeled by reductive methylation with less drastic disruption of protein structure (42, 48, 56), and we are pursuing this approach for labeling lysozyme before microinjection.

These variable effects of iodination on protein conformation may explain why there is no significant relationship between half-lives and subunit size or net charge among the six specific proteins microinjected. Notice, for example, that RNase A is degraded 3–4 times more slowly than lysozyme (Fig. 7) even though both are small, basic proteins. Also, degradation of RNase A is enhanced when serum and hormones are withdrawn from the medium, but catabolism of lysozyme is unaltered in these conditions (Table I). These findings may be explained by the consequences of iodination of lysozyme compared to RNase A. However, it should also be pointed out that the relationships between protein structure and half-life are general trends only, and many exceptions to these correlations are known (16). Therefore, general correlations between protein structure and half-life are not necessarily expected when only six proteins are examined.

We are currently studying in detail the degradation of microinjected RNase A, because our results (Figs. 8 and 9), as well as those of other laboratories (reviewed in reference 49), indicate that up to three iodines can be added per protein molecule with no detectable effect on RNase A structure or enzymatic activity. We intend to probe the structural features of RNase A that influence its half-life by specifically modifying the structure of this enzyme before microinjection. In addition, we are determining whether specific alterations in RNase A structure affect the cells' ability to regulate the half-life of microinjected RNase A in response to hormonal or nutritional signals. Finally, we are analyzing the role of lysosomes in the catabolism of microinjected RNase A by covalently coupling radioactive raffinose to the protein before microinjection. The raffinose is retained in lysosomes after the polypeptide has been degraded and so serves as a marker for protein that has been degraded by lysosomal pathways (44, 60). Such studies promise to yield much new information concerning the mechanisms and regulation of intracellular protein breakdown.

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