Protein Degradation in the Mouse Blastocyst*

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The degradation characteristics of 56 individual newly synthesized proteins of the Day 4 mouse blastocyst have been examined employing double isotope labeling of proteins for half-life measurement and two-dimensional electrophoresis for separation of proteins. The half-lives ranged from 1 to approximately 30 h with a mean of 12.4 h. Several proteins appeared to have half-lives greater than 30 h but decay times were insufficient to provide precise information for these proteins. The results suggest there is a tendency for proteins with acidic isoelectric points to be degraded more rapidly than basic proteins, and for high molecular weight proteins to be degraded more rapidly than low molecular weight proteins. Although the regressions of these two parameters on half-life were not significant, the direction and magnitude of the trends were similar to those previously described for liver proteins. Two specific proteins, tubulin and actin, were tentatively identified, and their half-lives determined. Tubulin had a half-life of 9.0 h. The half-lives of the provisionally identified γ, β, and α forms of actin were 2.2, 8.7, and 5.4 h respectively.

During the past 10 years, a considerable volume of work has appeared describing protein synthesis in the early mammalian embryo (for example, Refs. 1 to 4) with the most detailed studies on the mouse. The evidence indicates that there is no quantitative change in synthetic rate at the time of fertilization and that the major increase (6- to 8-fold) in rate occurs between the 8-cell and blastocyst stage (3, 5). Qualitative changes in synthesis of individual proteins have been demonstrated throughout the preimplantation period of the mouse and a considerable number of these occur before the rate increase (for example, Refs. 6 to 8).

However, little work has been performed in the area of protein degradation, a function which is of equal importance to synthesis in regulating protein content of the embryo. Epstein and Smith (6) labeled 8-cell mouse embryos with amino acids and noted that recovery of radioactivity decreased about 50% during a 24-h "chase" period, indicating that turnover did occur. They found no evidence of a difference in turnover among the proteins separated by polyacrylamide disc gel electrophoresis. Brinster, et al. (5) examined degradation at the 2-cell and blastocyst stage of mouse development. They found the average half-life of newly synthesized proteins was 18.3 h for the two-cell stage and 11.2 h in the blastocyst. Thus, there exists considerable difference between these two early stages in breakdown of protein. However, the interesting question of the variation in stability of individual proteins has not been determined at any stage of embryonic development. In fact, such studies have not been made for any cell type. We report here the stability of 56 individual proteins synthesized by the Day 4 mouse blastocyst.

MATERIALS AND METHODS

Blastocyst Collection and Labeling—Blastocysts were obtained from superovulated Swiss mice on the morning of the 4th day after mating (day of the vaginal plug was Day 1) and washed free of reproductive tract fluid (9). The basic medium for collection, manipulation, and culture was a modified Krebs-Ringer bicarbonate medium (9, 10), and the embryos were cultured in small drops (25 to 50 μl of medium under silicone oil in a plastic tissue culture dish (11).

To measure half-life of individual newly synthesized proteins, the double isotope labeling method developed by Arias et al. (12) was used with minor modifications. After the blastocysts (100 to 150) were collected and pooled, they were randomly divided into two groups. The first group was incubated at 37°C for 1 h in a medium containing [3H]methionine at 20 × 10^-6 m (80 Ci/mmol) and the second group was incubated for 4 h in medium without isotope. At the end of 1 h, the 3H-labeled blastocysts were placed in medium containing cold nonradioactive methionine (5 × 10^-2 m) for 4 h. The concentration of nonradioactive methionine was more than 150 times the concentration of the labeled methionine. This fact combined with the rapid uptake of amino acid by the mouse blastocyst (3-5) would greatly limit redistribution of labeled methionine, probably to less than 1% of incorporation. The second group of blastocysts was removed from medium without isotope after the initial 1 h of incubation and transferred into medium containing [35S]methionine at 20 × 10^-6 m (approximately 80 Ci/mmol) and incubated for 1 h. The 35S incubation occurred during the last hour of the 4-h cold methionine chase of the 3H-labeled blastocysts. At the end of the 35S incubation, these blastocysts were washed in nonradioactive methionine. The [3H]methionine-labeled and [35S]methionine-labeled blastocysts were then pooled in a tube (6 × 60 mm) and stored at -70°C.

To measure overall or mean protein half-life, blastocysts were incubated for 1 h in [3H]methionine (80 Ci/mmol) and then placed in nonradioactive methionine (5 × 10^-2 m) medium. Groups of blastocysts were removed at the end of the 3H pulse and after 2 h and 4 h in the nonradioactive methionine. The groups of blastocysts were placed in 3-ml centrifuge tubes and stored at -70°C. The 3H disintegrations present in the trichloroacetic acid-precipitable protein were determined (3) for each time and the mean protein half-life was calculated.

Identification of Proteins—The tubes containing the mixture of 3H- and 35S-labeled blastocysts were freeze-dried in a Speed-Vac Concentrator (Savant Instruments, Hicksville, N. Y.) and then dissolved in 5 μl of Laemmli sample buffer containing 20% sodium dodecyl sulfate; 10% glycerol; 5% β-mercaptoethanol; 62.5 mM Tris-HCl, pH 6.8 (13). Each tube contained approximately 1.5 × 10^2 dpm of 3H and similar 35S activity in trichloroacetic acid-precipitable protein. These samples were then fractionated by the high resolution two-dimensional electrophoresis system of O'Farrell (14), with minor modification. Early studies indicated that it was not necessary to treat the sample with DNase or RNase, perhaps because of the small amount of protein (2 to 3 μg). To each sample dissolved in Laemmli buffer, 20 μl of O'Farrell's lysozyme buffer was added and isoelectric focusing was carried out in tubes (2.5 × 130 mm) as described by
O'Farrell (14). This procedure separates the proteins by isoelectric point along a gradient from approximately pH 7.5 to 4.5. After isoelectric focusing, the cylindrical gel was equilibrated for 1 h in 5 ml of O'Farrell's SDS sample buffer and then laid along the top of a gradient (linear 9 to 15%) polyacrylamide-SDS slab gel for electrophoretic separation of the proteins according to molecular weight. This technique can resolve proteins differing by a single charge (14).

Following electrophoresis, the gels were prepared for fluorography according to the method of Bonner and Laskey (15). After drying, the corners of the gel were marked with black radioactive ink for identification and wrapped against Kodak SP-5 x-ray film. Following a 1 to 7-day exposure at -70°C, the film was developed. The desired spots on the film were marked on tracing paper as well as the identification marks. Using the identification marks, the tracing paper was positioned precisely on the dried gel, and the selected spots removed with a fine scalpel. The dried gel spots were placed in 0.4 ml of NCS (Amersham/Searle) solution (9 parts NCS:1 part water) in an 8-ml scintillation vial and incubated overnight at 55°C during which time the gel swelled. After cooling, 4 ml of scintillation mixture were added and the radioactivity was determined by counting in a scintillation counter programmed for separation of H and 35S disintegrations.

Data Interpretation—The experiments provide 35S/methionine and [3H]methionine disintegrations in individual proteins of the blastocysts. The 35S disintegrations per min represent zero time protein and the [3H] disintegrations per min represent protein remaining after the 4-h chase period. The logarithm of the 35S/[3H] ratio is a function of the first order decay of the protein (12-16). This ratio alone provides a relative indication of the rate constant of degradation or the half-life (t1/2) of the protein. Proteins with high 35S/[3H] ratios are being degraded faster than those with low 35S/[3H] ratios. To calculate the actual half-life of an individual protein, the 35S/[3H] ratio of the protein is related to the 35S/[3H] ratio of the total blastocyst protein present in the lysis buffer before isoelectric focusing. This is determined by removing a small aliquot (0.5 to 1.0 ml) of the mixture and measuring the 35S and [3H] disintegrations per min in a trichloroacetic acid precipitate of the aliquot. Since the mean half-life of total blastocyst protein is known from measurement of 35S disintegrations in trichloroacetic acid-precipitable protein from small groups of blastocysts removed during protein decay, the half-life of individual proteins can be calculated using the formula

\[ t_{1/2} = \frac{t \cdot \ln 2}{\ln R_0 - \ln R} \]

where \( t_{1/2} \) is the half-life, \( t \) is the chase period, \( R_0 \) is the reference ratio value calculated from the ratio for the mean protein half-life, and \( R \) is the experimental individual protein ratio value (17).

RESULTS

Preliminary experiments indicated that several hundred individual radioactive spots could be identified on autoradiographs of two-dimensional electrophoretic protein separations of Day 4 mouse blastocysts. The pattern of the protein spots was essentially identical between experiments. On the basis of these findings, the experimental protocol described above was established. An important criterion is that sufficient labeled methionine be incorporated to allow scintillation counting and quantitation of the radioactivity contained in the protein spots. This criterion is met by employing 50 or more blastocysts in each labeled group and an incubation time of 1 h. This provides greater than 108 dpm of [3H] and 35S in trichloroacetic acid-precipitable protein. Under these conditions, more than 50 spots were found to contain sufficient counts for quantitation and dual label separation.

Three separate experiments were performed on Day 4 mouse blastocysts. A representative autoradiograph is shown in Fig. 1. Many more spots could be seen on the original autoradiograph than appear in the photograph. However, the most intense spots contain the highest counts and are therefore of most interest for quantitation. The 75 most intense spots were removed from each of the three experimental gels for counting. The half-life was calculated for each protein spot on each of the three gels and the results from the three were combined. In most cases, three estimates of half-life for each protein could be made. However in some instances the counts from a spot would be low (less than twice background), and the spot would be excluded. The overall or mean protein half-life for newly synthesized protein in the blastocysts was determined in three separate experiments. In each experiment, duplicate groups of blastocysts were assayed at each time. The mean half-life calculated from the data was 12.4 h.

Table I shows the location and half-life of 56 radioactive protein spots removed from the gels. An attempt was made to use the most intense spots but also to select proteins from various locations on the gel. Such a procedure would maximize the possibility of detecting difference in half-life resulting from protein isoelectric point or molecular weight. The distribution of the proteins analyzed is shown in Fig. 2. The half-lives of the proteins range from 1 to approximately 30 h. Several proteins did show half-lives greater than 30 h, but the variation between the estimates from different gels was large and limits the accuracy of these half-lives. One reason for this is that the inaccuracy associated with half-life calculation increases as the interval between the decay time (chase period) and half-life increases due to the exponential nature of the decay function. The four proteins with long half-lives are designated 30+ only to indicate they appear to be more stable than the others listed. Their true half-life cannot be accurately estimated with a 4-h decay period. Our studies concentrated on proteins with short half-lives between 1 and 30 h.

The distribution of half-lives on the gel is shown in Fig. 3. It is not possible to discern an effect of position (isolectric point or molecular weight) on protein half-life from the figure.
TABLE I

Half-lives of individual proteins from the Day 4 mouse blastocyst

Spot number identifies the location of the protein on the two-dimensional electrophoretic separation shown in Figs. 1 and 2. Half-life is the mean ± S.E. for three determinations. An asterisk indicates only two determinations, and in these cases the half-life is the mean ± one-half the range. Several protein spots are designated 30.0+ which indicates that the half-life is greater than 30 h. An accurate estimate could not be made in these cases because the experimental decay period was too short.

| SPOT NUMBER | HALF-LIFE (HRS) | SPOT NUMBER | HALF-LIFE (HRS) | SPOT NUMBER | HALF-LIFE (HRS) |
|-------------|-----------------|-------------|-----------------|-------------|-----------------|
| 0.2 x 13.6  | 30.0+           | 4.8 x 6.8   | 16.3 ± 3.8      | 9.2 x 5.3   | 7.6 ± 1.5*      |
| 0.3 x 15.1  | 15.7 ± 0.6*     | 5.0 x 12.1  | 7.5 ± 2.6*      | 9.3 x 6.9   | 8.7 ± 1.0       |
| 0.8 x 12.6  | 30.0+           | 5.5 x 6.4   | 12.0 ± 2.8      | 9.4 x 4.3   | 3.7 ± 0.8       |
| 1.4 x 14.0  | 10.0 ± 1.0      | 5.6 x 9.3   | 6.0 ± 2.0*      | 9.8 x 6.9   | 5.4 ± 0.3       |
| 1.5 x 3.4   | 9.2 ± 2.1       | 6.3 x 8.3   | 7.5 ± 1.3       | 10.3 x 3.6  | 14.7 ± 3.6      |
| 1.5 x 7.5   | 30.0+           | 6.9 x 14.2  | 3.8 ± 0.5       | 10.8 x 6.1  | 10.4 ± 2.2      |
| 1.8 x 5.3   | 7.0 ± 1.2*      | 7.0 x 5.3   | 9.0 ± 1.6       | 10.9 x 13.0 | 30.0+           |
| 2.2 x 13.6  | 22.5 ± 2.6      | 7.7 x 5.3   | 7.3 ± 1.4       | 11.1 x 5.8  | 9.0 ± 1.3       |
| 2.1 x 10.9  | 6.2 ± 1.1       | 7.9 x 7.1   | 14.8 ± 6.9      | 11.1 x 14.3 | 3.5 ± 0.1*      |
| 2.7 x 6.6   | 9.9 ± 0.4       | 8.0 x 4.9   | 28.3 ± 11.3     | 11.3 x 13.0 | 2.9 ± 0.4       |
| 2.7 x 7.4   | 7.2 ± 0.9       | 8.2 x 6.6   | 5.8 ± 0.5       | 11.8 x 4.8  | 10.4 ± 0.9      |
| 2.9 x 3.7   | 9.6 ± 1.8       | 8.3 x 6.9   | 6.7 ± 0.9       | 12.2 x 9.8  | 11.0 ± 1.2      |
| 3.5 x 9.2   | 2.2 ± 0.2       | 8.4 x 4.6   | 8.9 ± 1.6       | 12.5 x 2.9  | 1.0 ± 0.3       |
| 3.7 x 6.7   | 15.7 ± 3.2      | 8.5 x 8.5   | 15.2 ± 2.3      | 12.5 x 5.9  | 8.5 ± 0.5       |
| 4.0 x 14.0  | 2.3 ± 0.2       | 8.5 x 9.7   | 5.9 ± 0.5       | 12.5 x 8.6  | 10.8 ± 3.8*     |
| 4.1 x 5.0   | 7.5 ± 1.0       | 8.7 x 4.6   | 21.2 ± 0.7      | 12.5 x 10.9 | 22.6 ± 6.2      |
| 4.3 x 10.5  | 9.1 ± 1.0*      | 8.9 x 3.2   | 12.3 ± 4.1      | 12.7 x 9.3  | 8.9 ± 1.8       |
| 4.4 x 11.0  | 10.7 ± 4.0      | 8.9 x 6.9   | 2.2 ± 0.4       | 13.7 x 13.7 | 6.0 ± 0.9       |
| 4.5 x 2.7   | 8.4 ± 2.4       | 9.0 x 6.6   | 9.9 ± 2.5       |             |                 |

Fig. 2. Distribution of proteins analyzed for half-life. The gel shown in Fig. 1 was used as the model. Protein spots are identified by their location on the gel. The first number of the spot identification is the distance in centimeters from the left margin of the gel. The second number is the distance in centimeters from the top of the gel.

Fig. 3. Distribution of half-lives of protein from Day 4 mouse blastocysts. The gel shown in Fig. 1 was used as the model. Locations are shown in Fig. 2. Each value is the mean of two or three determinations. Standard errors of the means are shown in Table I. The spots marked γ, β, and α have been tentatively identified as the three forms of actin. Several protein spots are designated 30.0+ which indicates that the half-life is greater than 30 h. An accurate estimate could not be made in these cases because the experimental decay period was too short.

To resolve this question, a regression analysis was performed between horizontal position (protein isoelectric point) and half-life, as well as between molecular weight and half-life. There was a tendency for acidic proteins (high first number in location identification) to be degraded more rapidly (correlation coefficient, $r = -0.23$). However, the regression was not significant. Likewise, high molecular weight proteins showed a tendency to be degraded faster than low molecular weight proteins ($r = -0.14$), but the regression again was not significant.
At the present time, two specific proteins can be tentatively identified in these gel separations. On the basis of cold marker migration and previous studies (18), spot 11.1 × 5.8 is thought to be tubulin and spots 8.9 × 6.9, 9.3 × 6.9 and 9.8 × 6.9 are thought to be the γ, β, and α forms of actin (19, 20). The position of these proteins relative to the molecular weight marker indicates they have not migrated as far as might be expected from their molecular weights (tubulin = 55,000; actin = 43,000). However, we have found that the molecular weight markers migrate slightly farther than identical proteins first separated by isoelectric points, because under the conditions of these experiments the molecular weight markers start from an adjacent well rather than from within the IEF gel. For accurate identification, specific protein markers in the IEF gel allow greater reliability than the mengo virus molecular weight markers. The separation of the three provisionally identified forms of actin provides important information about the technique. As shown in Fig. 3, the three protein spots lie adjacent to one another but have significantly different half-lives. The technique clearly discriminated these three very similar proteins. Another excellent example of the resolving ability of the system is seen below actin in Fig. 3. In this case, the adjacent protein spots 10.9 × 13.0 and 11.3 × 13.0 have vastly different half-lives; the first is 30.0+ and the second is 2.9 h. The large difference in half-lives makes it unlikely that either protein influenced very much the estimate of the half-life for the other protein. Thus, it is possible to distinguish proteins differing widely in half-life even though they possess very similar electrophoretic mobilities.

**DISCUSSION**

The overall or mean protein half-life of 12.4 h found in these experiments is very similar to the value (11.2 h) found previously (5) for blastocysts using a slightly different but basically similar technique. More importantly, however, the present studies demonstrate that the various proteins synthesized by the developing embryo have a wide range of stabilities. Only a few of the large array of newly synthesized proteins were examined but the variability is clear. Furthermore, it is probable that the extremes of the range would be accentuated if shorter and longer decay times were used. Our study concentrated on proteins with relatively short half-lives, between 1 and 30 h.

The correlation of isoelectric point and molecular weight with half-life was not significant in our experiments. However, the direction of the trend was similar to previous reports (21, 22). The low correlation coefficients found for the blastocyst proteins probably result from the short half-lives used to calculate the regression. Dice and Goldberg (22) found the correlation was significant for the effect of molecular weight on half-life (r = 0.65) when they used 30 rat liver proteins with half-lives from 0.2 to 150 h for regression analysis. However, when only the proteins with half-lives shorter than 36 h were analyzed by Dice and Goldberg, the correlation was not significant (−0.15), and the coefficient was remarkably similar to that found for the blastocyst proteins (−0.14) which had half-lives of 1 to 28 h. Dice and Goldberg (21) found the effect of isoelectric point on half-life to have an even higher (−0.82) correlation coefficient than that found for molecular weight. Our studies also suggest that isoelectric point may have a greater tendency than molecular weight to influence protein half-life in the mouse blastocyst. Although the Dice and Goldberg (21) studies on isoelectric point effect do not have a separate regression for proteins with short half-lives, the graphical presentation of their data suggests the regression for proteins with half-lives shorter than 30 h is minimal and that most of the regression arises from proteins with half-lives between 30 and 160 h. This is a situation similar to that found for molecular weight. Thus, the data from the mouse blastocyst conforms to and supports the pattern seen in rat liver proteins regarding the effect of isoelectric point and molecular weight on half-life.

Apart from the relationship the studies on the mouse blastocyst bear to previous investigations, several new insights are provided regarding development. For instance, it is clear that spots on the autoradiograph which have equal intensity or 32S disintegrations represent proteins which are being synthesized at equal rates. However, the introduction of the information on protein degradation allows a more accurate appraisal of the accumulation of the half-lives. It has been established that the half-life of a protein strongly influences its steady state level in a cell. If two proteins, "a" and "b," are synthesized at equal rates and protein "a" has a half-life 10 times that of "b," then the steady state level of "a" will be 10 times that of "b" (23, 24). Clearly, this profoundly influences the interpretation of the radioactivity intensities or counts in the protein spots on gel separations. Thus, in Fig. 1, spot 12.5 × 2.9 and 8.5 × 8.5 have approximately equal 32S disintegrations and therefore synthetic rates. However, because the half-life of the former is 1.0 h and the latter is 15.2 h (Table I, Fig. 3), the blastocyst will accumulate protein 8.5 × 8.5 to a level 15 times that of 12.5 × 2.9 under steady state conditions. Of course, the embryo is dynamic and may change synthetic or degradation rate, but the importance of protein stability in determining protein levels and in interpreting autoradiographs of two-dimensional gels is clear.

Although the short half-life of protein 12.5 × 2.9 brings about a lower steady state level than for 8.5 × 8.5, the short half-life could possibly be advantageous. The possible advantage is that the levels of 12.5 × 2.9 could be altered more rapidly than 8.5 × 8.5. In fact, 12.5 × 2.9 could reach a new steady state level 15 times faster than 8.5 × 8.5 (23, 24). This would be a distinct advantage for certain protein functions such as regulatory roles. It has been found that the 12 liver proteins with the shortest reported half-lives catalyze either the first or the rate-limiting step in metabolic pathways (25). It is tempting to speculate that protein 12.5 × 2.9 may perform a critical regulatory function in the embryo. However, two other alternatives which could cause short half-lives must be considered. First, there is the possibility that some of the rapidly degraded proteins are abnormal since several studies have shown that abnormal proteins are very labile (25, 26). Second, the possibility exists that secretion of 3H-labeled proteins from the blastocyst could occur during the cold chase and thereby raise the ratio of 32S to 3H. The increased ratio would give the secreted protein a lower apparent half-life than the true value. This problem may be small in these studies since trichloroacetic acid precipitable counts in the cold chase medium, which might indicate secreted protein, were at background levels.

An additional important observation in these studies relates to the synthesis and degradation of the specific proteins tubulin and actin. Previous studies (18) have demonstrated that tubulin and actin represent approximately 2% and 5.7% of protein synthesis by the mouse blastocyst. Furthermore, the increase in synthetic rate from the 1-cell stage to the blastocyst is twice as great for tubulin and 14 times as great for actin as the increase in general protein synthesis. Clearly, they must have an increasing role in embryo development, probably related to cytokinesis, blastomere movement, and blastocyst formation. The present study indicates that both the tentatively identified actin and tubulin have faster degradation rates than the average for blastocyst protein. Tubulin and the putative β form of actin (18–20) have half-lives of 9.0...
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...and 6.7, respectively, which are not too different than the mean protein half-life of 12.4. However, the γ and α forms have short half-lives of 2.2 and 5.4 h, respectively. If the synthetic rates of the three tentatively identified forms were similar, the β form would accumulate to almost 4 times the level of the γ form. But the difference in accumulation is magnified because the 35S incorporation indicates that γ, β, and α forms are synthesized in the approximate proportion 3:15:2. Based on this data, under steady state conditions, the newly synthesized actin would accumulate to steady state levels in the proportion of 7:130:1 for γ, β, and α forms. The β form appears to dominate the actin of the blastocyst, but the γ form with its short half-life may have a dynamic function which requires rapid changes in its level. The large increase in synthesis of actin from the 1-cell to the blastocyst stage, the high rate of synthesis in the blastocyst, and the marked differences in degradation rates of the three forms suggest that actin metabolism plays an important role in embryo development.

The combination of double isotope labeling of proteins for half-life measurement and two dimensional electrophoresis for separation of proteins offers an excellent technique for determining the degradation characteristics of specific individual proteins in the embryo as well as in other tissues.

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