Actin in Young and Senescent Fibroblasts

By PETER J. ANDERSON

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N6N5, Canada

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Double labelling and the isolation of peptides specific to muscle actin were used to determine the amount of actin in young and senescent fibroblasts from chicken embryo. A 20-residue peptide produced from the C-terminus of muscle actin was found in amounts that indicated 6.9% of the protein of young fibroblasts and 11.5% of the protein of senescent fibroblasts to be actin. Two other peptides of muscle actin were present in very small amounts. This indicates that the actin of both young and senescent fibroblasts is homologous to, but not identical with, muscle actin. Increased actin content of senescent fibroblasts may be related to their loss of proliferative capacity and low cell density at confluence.

Decreases in the amount of actin (Wickus *et al.*, 1975; Fine & Taylor, 1976) and in the number of microfilaments formed from this protein (Pollack *et al.*, 1975) have been shown to accompany viral transformation of a variety of cell types. Two manifestations of viral transformation of cells are an infinite replicative capacity and high cell densities in culture. It is thought that alterations in both the amount (Fine & Taylor, 1976) and state of assembly (Edelman & Yahara, 1976) of actin may be related to these properties of transformed cells.

Diploid fibroblasts from human skin (Schneider & Mitsui, 1976), human fetuses (Hayflick, 1965; Nichols *et al.*, 1977) and chicken embryos (Lima & Macieira-Coelho, 1972) are capable of limited numbers of divisions *in vitro*. The decreasing ability of diploid cells to divide in culture as a function of division number has been used extensively in studies of biological aging (Cristofalo, 1972). Such studies indicate that certain features such as the correlation between cell size and division number and the percentage of cells capable of DNA synthesis as a function of age are variable for diploid cells from different sources. However, a common feature of cells that have exhausted their replicative capacity has been found to be decreased cell density at confluence. It might be expected that, if decreased cellular actin and microfilament content have a role in increasing cell-division capacity and cell density in virus-transformed cells, increased amounts of actin and microfilaments would be found in cells that have lost their capacity to divide and have low cell densities at confluence.

Fibroblasts from chicken embryos exhibit cellular senescence (Lima & Macieira-Coelho, 1972), and the amount of actin contained in early-passage chicken-embryo fibroblasts has been determined (Bray & Thomas, 1975; Anderson, 1976b). It was therefore decided to determine if changes in actin content accompanied senescence in these cells.

Experimental

Actin was prepared from chicken muscle and carboxymethylated with iodo[2-¹⁴C]acetic acid as previously described (Anderson, 1976a), except that iodo[2-¹⁴C]acetic acid was obtained from NEN Canada Ltd., Lachine, Que., Canada, and was diluted to a specific radioactivity of 0.5 mCi/mmol before use.

Chicken-embryo fibroblasts were prepared from embryos by trypsin treatment. They were cultured in 75 cm² Falcon flasks in Medium 199 supplemented with 10% (v/v) foetal calf serum and 0.5% chicken-embryo extract obtained from Flow Laboratories, Rockville, MD, U.S.A. The medium also contained penicillin (100 i.u./ml) and streptomycin (100 µg/ml) obtained from Gibco Canada, Burlington, Ont., Canada. Flasks containing medium and cells were gassed with CO₂/air (1:19), and incubated at 37°C. Confluent cultures were harvested by treatment with trypsin and subcultivated at split ratios of 1:4. When declining proliferative rates were observed, the split ratios were changed to 1:2. Trypsin-treated cells were counted by using a haemocytometer. Cells to be used for actin determination were harvested by scraping with a rubber policeman. They were then washed three times in 10 vol. of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (Dulbecco & Vogt, 1954) and frozen at -20°C before preparation of acetone-dried powders as previously described (Anderson, 1976a).

The acetone-dried powders were dissolved in 6M-guanidine hydrochloride containing 100 mM-Tris/HCl, pH 8.0, and 2 mM-dithiothreitol, and carboxymethylated with iodo[2-³H]acetic acid from NEN Canada Ltd. (diluted with carrier to a specific radioactivity of 100 mCi/mmol before use). Peptides were generated from mixtures of carboxymethylated acetone-dried powders of fibroblasts by using CNBr

and trypsin and were subsequently purified and analysed as previously described (Anderson, 1976a). From the $^3\text{H}/^{14}\text{C}$ ratios of peptides so obtained, the amount of actin present in the acetone-dried powders can be calculated by assuming a molecular weight for actin of 42000 (Anderson, 1976a).

Results

Fig. 1 shows a typical growth curve for a fibroblast culture from an 11-day embryo. Similar curves were obtained from 9-, 10- and 12-day embryos. Some variation was noted between individual cultures, but this did not seem to be related to the age of the embryo. In some cases growth was slow from the start and cell division ceased before the 20th population doubling was reached. No cultures underwent more than 35 population doublings. Generally, the loss of proliferative capacity occurred abruptly. However, in some cases, particularly with cells that were slow to divide even at early population doublings, a more gradual decrease in proliferative rate was observed.

Table 1 shows the number of cells present at confluence after various numbers of population doublings. In general, up to ten population doublings, approx. 20×10^6 cells/flask, were present at confluence. There was then a gradual decline in cell density at confluence until just before the cells lost their ability to divide. At this stage, cell numbers at confluence decreased to about 5×10^6 . From these observations, cells that had gone through less than ten population doublings and that were present in concentrations of 20×10^6 cells/flask were used as young cells for actin measurement. Old cells used for actin measurement were cells that had gone through at least 25 population doublings, had a very slow proliferative rate, and would not grow to densities of

Fig. 1. Population doublings as a function of time in culture. Chicken-embryo fibroblasts were allowed to grow until confluent, then harvested with trypsin and subcultivated at split ratios of 1:4. Harvested cells were counted by using a haemocytometer.

more than 5×10^6 cells/flask. To avoid individual variations, actin was determined in young and old cells derived from single embryos.

Table 2 gives the amounts of three peptides generated from acetone-dried powders of chicken muscle and of young and old fibroblasts from chicken embryos after carboxymethylation. Peptide X1-T-A (AcAsp - Glu - Thr - Glu - Asp - Thr - Ala - Leu - Val - CmCys-Asp-Asp-Gly-Ser-Gly-Leu-Val-Lys, where AcAsp is *N*-acetylaspartic acid and CmCys is carboxymethylcysteine) is produced from the *N*-terminal region of carboxymethylated muscle actin by treatment with trypsin. The internal peptide X2-A (Lys-CmCys - Asp - Ile - Asp - Ile - Arg - Lys - Leu - Tyr - Ala-Asn-Asn-Val-Hse) and the *C*-terminal peptide X2-N (Trp-Ile-Thr-Lys-Gln-Glu-Tyr-Asp-Glu-Ala-Gly-Pro-Ser-Ile-Val-His-Arg-Lys-CmCys-Phe) are produced from carboxymethylated muscle actin by treatment with CNBr. From the amounts of these peptides, the amount of actin in muscle and fibroblasts given in Table 2 was calculated by assuming a molecular weight of 42000 for actin. All three peptides can be generated in large amount from acetone-dried powders of muscle. From the amounts of these peptides present, approx. 18% of the protein of muscle is actin. In fibroblasts, however, only peptide X2-N is present in large amount. From the amount of this peptide present, 6.9% of the protein of young fibroblasts is actin, whereas 11.5% of the protein of senescent fibroblasts is actin. On the basis of the amounts of peptides X1-T-A and X2-A produced, less than 1% of the protein of both young and old fibroblasts is actin. Since the presence of carboxymethylcysteine-containing peptides co-purifying with the above peptides would lead to high estimates of the amount present, smaller peptides were generated from the isolated peptides and repurified. Treatment of peptide X1-T-A with chymotrypsin produced the peptide CmCys-Asp-Asp-Gly-Ser-Gly-Leu-Val. Calculation of the amount of actin present from the amount of this peptide indicated that 18%

Table 1. Number of cells present at confluence as a function of number of population doublings

Each value is the average of three counts, with a haemocytometer, of trypsin-treated cells at confluence in 75 cm² flasks. The values given are for three cultures derived from different embryos.

No. of population doublings	$10^{-4} \times$ No. of cells/cm ² at confluence
3	39, 32, 25
7	29, 24, 25
10	24, 27, 24
15	19, 23, 21
20	16, 16, 12
25	8, 5, 9

Table 2. *Actin content of young and old fibroblasts and of muscle as determined by double labelling and peptide isolation*
 Proteins were carboxymethylated with iodo[³H]acetic acid, and peptides were generated and isolated after mixing with iodo[¹⁴C]acetic acid-treated purified muscle actin as previously described (Anderson, 1976a,b). ³H/¹⁴C ratios of purified peptides were determined by liquid-scintillation counting, and from these values and values obtained when known amounts of purified actin were added, the quantities of peptides in the fibroblasts and muscle were calculated. The actin content was then calculated from the amount of peptide, assuming a molecular weight for actin of 42000.

Experiment	Peptide	Amount (nmol of peptide/mg of protein)			Actin content (%, w/w)		
		Young	Old	Muscle	Young	Old	Muscle
1	X1-T-A	0.32	0.23	4.55	1.3	1.0	19.1
	X2-A	0.10	0.14	4.23	0.4	0.6	18.0
	X2-N	1.59	3.00	4.67	6.5	12.6	19.6
2	X1-T-A	0.09	0.06	4.64	0.4	0.3	19.5
	X2-A	0.12	0.15	4.15	0.5	0.6	17.4
	X2-N	1.76	2.64	4.29	7.4	11.1	18.0
3	X1-T-A	0.17	0.10	4.33	0.7	0.4	18.2
	X2-A	0.11	0.18	4.17	0.5	0.7	17.5
	X2-N	1.60	2.57	4.02	6.7	10.8	16.9

of the protein of muscle was actin. Less than 0.5% of the protein of fibroblasts was actin, on the basis of the amount of this peptide, and there was no detectable difference between the amount produced from young and senescent fibroblasts. Treatment of peptide X2-A with trypsin produced the peptide CmCys-Asp-Ile-Asp-Ile-Arg. On the basis of the amount of this peptide present, 17% of the protein of muscle was calculated to be actin. The amount of this peptide produced from young and senescent fibroblasts indicated that less than 0.25% of the protein of fibroblasts was actin, and no difference between the amount produced by young and senescent fibroblasts was seen. Treatment of peptide X2-N with chymotrypsin gave rise to the peptide Arg-Lys-CmCys-Phe. Calculation of the amount of actin present on the basis of this peptide gave very similar values to those obtained when peptide X2-N was used. As a further indication of peptide purity, after isolation of the peptide Arg-Lys-CmCys-Phe, the peptide CmCys-Phe was generated by treatment with trypsin and isolated. Again values very similar to those calculated from the amount of peptide X2-N were calculated for the actin content of muscle of young and senescent fibroblasts.

Discussion

Fibroblasts from chicken embryos have been reported to have a limited lifespan *in vitro* (Hay & Strehler, 1967; Lima & Macieira-Coelho, 1972). Although different culture conditions were used, the results obtained in the present study are in very good agreement with a previous study of the properties of these cells in culture (Lima & Macieira-Coelho, 1972). In both cases variations between individual

cultures were observed, but typically early-passage cells became confluent at densities of about 3×10^5 cells/cm², whereas late-passage cells would not grow to densities greater than about 5×10^4 cells/cm². Also in both studies, the fibroblasts tended to grow at a constant rate, with a very gradually declining density at confluence until about the 25th population doubling. At about this number of doublings, the division time became much longer and the number of cells present at confluence became markedly less. Therefore cellular senescence of chicken embryos as indicated by prolonged division time and low cell densities is reproducible under at least two different culture conditions.

As found in previous studies (Anderson, 1976a,b), only one of three carboxymethylcysteine-containing peptides that can be produced from carboxymethylated muscle actin is produced in large amount from the carboxymethylated proteins of chicken-embryo fibroblasts. This indicates that a protein that is homologous to, but not identical with, muscle actin is present in fibroblasts. Studies using two-dimensional electrophoresis on polyacrylamide gels indicate that there are at least three species of actin with the same molecular weight, but different isoelectric points, in a variety of non-muscle cells (Whalen *et al.*, 1976; Rubenstein & Spudich, 1977). Other studies have shown that a protein is found in brain (Storti & Rich, 1976) and platelets (Elzinga *et al.*, 1976) with a structure similar to, but not identical with, that of muscle actin, and that the structural changes are due to alterations in structural genes. It is clear in the present study that only a very small amount of protein identical with muscle actin in the three regions of the amino acid sequence examined can be present. The amount of peptide X2-A compared with the amount

of peptide X2-N produced from fibroblasts indicates that, at most, 4% of the actin found in fibroblasts can be the muscle type. The amount of peptide X2-N present may not be an indication of the total amount of protein homologous to actin present in fibroblasts. An actin species differing from muscle actin in this region of the amino acid sequence would not be detected. Whether such an actin species exists in fibroblasts awaits further amino acid-sequence studies. Despite this uncertainty, a clear difference between the amount of peptide X2-N that can be generated from the proteins of young and senescent fibroblasts of chicken embryo was obtained. The amount of protein that can give rise to a peptide homologous to a peptide that can be generated from the C-terminal region of muscle actin is clearly much greater in senescent fibroblasts. From probability considerations it is highly unlikely that any protein other than a protein genetically related to muscle actin would give rise to such a peptide. It therefore may be concluded that the content of at least one species of protein homologous to muscle actin is present in increased amounts in senescent fibroblasts from chicken embryo as compared with young fibroblasts.

The functional significance of this finding is not yet certain. Measurement of the amount of an actin-like protein in cells does not indicate either the intracellular location or the state of assembly of the protein. Such knowledge is clearly important to an understanding of the role of actin in cell function. It seems unlikely that the increased actin content is primarily associated with the plasma membrane, since the protein content of this cell fraction represents only about 6% of total cell protein (Perdue & Schneider, 1970) and the actin content of senescent fibroblasts is 11.5% of total protein. Increased actin content does not necessarily imply an increase in the amount of actin in the form of microfilaments, but if an equilibrium exists between the amount of actin in the globular and microfilament states, more actin may be an indication of increased amount of microfilaments. In addition, whether the relationship between increased actin content and loss of proliferative capacity and low cell density at confluence is direct or coincidental awaits further study.

Actin is a major protein constituent of a variety of cells and its function may vary with cell type. Thus actin in macrophages is thought to be important in phagocytosis (Hartwig & Stossel, 1975). Microfilaments are thought to function in the secretion of hor-

mones stored in membrane-covered vesicles (Ostlund *et al.*, 1977) and in controlling the mobility and distribution of cell-surface components (Edelman & Yahara, 1976; Albertini & Anderson, 1977). Alterations in the amount, state of assembly or intracellular location of actin may therefore have an effect on a variety of cell processes. It will therefore be of interest to determine if age-related alterations in actin occur in cell types other than chicken-embryo fibroblasts.

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