Age-specific nuclear proteins in the nematode worm *Caenorhabditis elegans*

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The nematode worm *Caenorhabditis elegans* is known to undergo characteristic morphological as well as physiological signs of senescence. Two-dimensional gel electrophoresis shows that alterations also occur in the pattern of the nuclear proteins as a function of age. Non-histone proteins whose level exhibits a steep fall with age are egg-specific and not involved in senescence. However, a distinct set of non-histones accumulates with age and can be considered as senescence markers. Some of these are glycoproteins, as shown by their concanavalin A-binding properties. One age-specific polypeptide, called 'protein S-28', was further characterized by peptide mapping and determination of its *N*-terminal amino acid sequence.

INTRODUCTION

The existence of a multitude of theories advanced to explain aging and senescent death at the molecular level is itself an indication that the mechanism of biological aging is still a mystery. Nevertheless, two alternative views can be distinguished. In the first view aging can be considered to be the result of a genetically programmed determination (Hill & Franks, 1977; Hayflick, 1980a). In the second view aging is thought to result from progressive deterioration at the epigenetic level; faulty macromolecules would accumulate as a consequence of random damage and inevitably reach a cytotoxic level. Faulty macromolecules can be generated by different mechanisms, e.g. somatic mutation (Curtis, 1963) or post-translational modification of correct macromolecules through reaction with superoxide and free radicals (Harman, 1981) or simply as a consequence of 'wear and tear' due to their normal thermodynamic instability (Rothstein, 1975, 1982). Perhaps the most popular non-genetic theory of aging is the error-catastrophe theory (Orgel, 1963, 1973), which holds that random errors in translating the genetic code will propagate fresh errors at an exponential rate until the amount of faulty macromolecules reaches proportions that are incompatible with normal life.

A major problem in aging research is the lack of a suitable model system. Mice and rats have been favourite organisms, although their life expectancy is long (over 2 years) and the final costs per animal are high. In addition, organs and tissues are usually composed of several cell types, some of which may be post-mitotic, whereas other cells are still able to divide. Thus one must always be careful when interpreting biochemical data obtained from 'young' and 'old' tissue. The study of post-mitotic cells such as neurons is more straightforward, but generally implies careful and laborious purification procedures. Alternatively, much work has been done with cultured cells, since it was found that

normal human and animal cells (e.g. fibroblasts) undergo a definite number of population doublings *in vitro* (Hayflick & Moorhead, 1961). Several biochemical alterations occur during the many cell divisions before the cessation of mitotic activity, and many of these resemble those manifestations of senescence seen in the whole animal (Hayflick, 1980b). However, the possible exchange of 'age signals' between cell types, tissues and organs, aging as an integrative function, cannot be studied in cultured cells, and it remains to be established whether aging in cell culture bears sufficient relevance to aging *in situ*.

Free-living nematodes were first introduced in the early 1970s as suitable model systems for basic aging research (Gershon, 1970). Their advantages include a short life span of 2–3 weeks, distinct signs of senescence, ability to be grown in either monoxenic or axenic culture conditions, small size (approx. 1 mm in length) and feasibility to be handled as individuals for microscopic examination or in bulk for biochemical study. In addition they retain a constant number of somatic cells after maturity. The germ cells proliferate during the reproductive period, then progressively atrophy, so that old worms consist only of old cells. One of the favourite species, *Caenorhabditis elegans*, offers further genetic advantages: because it is hermaphrodite it is possible to perform aging studies on large populations which have a uniform genetic background. The current intensive study of genetics and developmental and molecular biology of *C. elegans* offers the potential of being co-ordinated with the aging research.

By using techniques of two-dimensional polyacrylamide-gel electrophoresis we show here that highly reproducible age-related alterations occur in the pattern of the nuclear proteins. The abundance of a distinct set of proteins increases considerably with age. One of these proteins, designated 'S-28', was characterized in more detail to permit further study of the regulation of its expression.

Abbreviations used: NEPHGE, non-equilibrium pH-gradient gel electrophoresis; PEG, poly(ethylene glycol) 6000; PAGE, polyacrylamide-gel electrophoresis.

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EXPERIMENTAL

Strain and culture conditions

Caenorhabditis elegans strain Bristol (N2) was obtained from Dr. H. F. Epstein (Department of Neurology and Biochemistry, Baylor College of Medicine, Houston, TX, U.S.A.). The worms were grown at room temperature (21–23 °C) under axenic culture conditions in a medium consisting of 3% (w/v) dry yeast extract, 3% (w/v) soy peptone and haemoglobin (500 µg/ml; Serva, Heidelberg, Germany) as described previously (Meheus & Vanfleteren, 1986).

Age synchronization

Under the experimental conditions described the average generation time and median life span were about 5–6 and 18–22 days respectively. Adults harvested 10 days after inoculation, at which time the cultures were still in exponential growth phase, were therefore assumed to be between 5 and 10 days old (neglecting the relatively very small number of adults introduced along with the inoculum). They were separated from the juvenile stages by repeated sedimentation at unit gravity in S buffer (0.1 M-NaCl/0.05 M-potassium phosphate, pH 6.0). This treatment was repeated until the final worm population comprised at least 95% adults. Senescing cultures were established by adding 50 µM-fluorodeoxyuridine to 10-day-old cultures. At this low level the drug prevented reproduction and stopped larval development without interfering with normal vitality and longevity (Mitchell *et al.*, 1979; Gandhi *et al.*, 1980), and the adult morphology at harvest was perfectly normal. The cultures were harvested after 10 days of exposure to the drug. The adults, then 15–20 days old, were collected as described above. As dead nematodes sedimented more slowly than live worms, the occurrence of dead worms in old cultures was not a problem.

Freshly collected worms were suspended in twice the volume of homogenization buffer [1.7 M-sucrose/0.5% (v/v) Nonidet P40/5 mM-CaCl₂/50 mM-Tris/HCl, pH 7.4] and immediately dripped in liquid N₂ and stored at –196 °C until use.

Preparation of chromosomal proteins

Nuclei were purified as outlined previously (Meheus & Vanfleteren, 1986). They were extensively washed in 0.14 M-NaCl/50 mM-Tris/HCl (pH 7.4)/1 mM-phenylmethanesulphonyl fluoride and frozen at –18 °C for at least 1 h. After thawing, washing was repeated three times to extract nucleoplasm from the broken nuclei as much as possible. The chromatin was subsequently solubilized in 2 M-NaCl/10 mM-Tris/HCl, pH 7.4, for 1 h and clarified in an Eppendorf centrifuge. Nucleic acid and protein were separated by their differential solubility in an aqueous two-phase system consisting of PEG (100 mg/ml), dextran T-500 (40 mg/ml), 5 M-NaCl and 10 mM-Tris/HCl, pH 7.4, as described by Bidney & Reeck (1977). The proteins were recovered from the PEG phase by precipitation with trichloroacetic acid (20%, w/v, final concn.); PEG was next extracted with acetone containing 0.05 M-HCl. Further washings were performed with acetone and acetone containing 1% (v/v) triethylamine. The samples were finally dried under reduced pressure.

Electrophoretic analysis

NEPHGE-SDS/PAGE was performed as described by O'Farrell *et al.* (1977), with slight modification. The first dimension was in slab gels (0.5 mm thick) coated on to PAG-Gelbond film (LKB, Bromma, Sweden) and run horizontally on the Multiphor II apparatus (LKB). Only ampholytes pH 3.5–10 (LKB) were used. The protein samples were dissolved in lysis buffer (O'Farrell *et al.*, 1977) and loaded on to glass-fibre (GF/C; Whatman, Maidstone, Kent, U.K.) application pieces placed along the anodic side. The electrophoretic conditions were 200 V for 1 h, 400 V for 2 h and 800 V for 4 h in this sequence. After electrophoresis in the first dimension the gels were fixed in 20% (w/v) trichloroacetic acid for 30 min, washed with distilled water (3 × 10 min) and stained in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 10% (v/v) acetic acid 50% (v/v) methanol. For electrophoresis in the second dimension, the appropriate lanes (still attached to PAG-Gelbond film) were cut out and equilibrated against 2.3% (w/v) SDS/62.5 mM-Tris/HCl, pH 6.8, for 3 × 10 min. Subsequently they were carefully shifted between the glass plates of the second-dimension gel until close contact was obtained with the stacking gel. Electrophoresis in the second dimension was essentially performed as described by Laemmli (1970), except that the running buffer contained 0.05 M-Tris base, 0.384 M-glycine and 0.1% (w/v) SDS. The slab gels (16 cm × 20 cm × 0.1 cm) were run overnight at 70–90 V in the Protean II cell (Bio-Rad, Richmond, CA, U.S.A.). The gels were stained with silver as described by Ansorge (1985).

Detection of glycoproteins

Nuclear proteins from old worms were separated by NEPHGE-SDS/PAGE and transferred to nitrocellulose (30 µm pore size; Millipore, Milford, MA, U.S.A.), essentially as described by Towbin *et al.* (1979). The gel was equilibrated in 25 mM-Tris base/20% (v/v) methanol/0.2 M-glycine, pH 8.3. The gel and the nitrocellulose sheet were then properly sandwiched between the electrodes and inserted in the buffer chamber of the LKB 2005 Transphor unit. With the current adjusted to 0.7–1.0 A, electrophoretic transfer was achieved in 4 h. The buffer chamber was continuously refrigerated to keep the buffer temperature below 5 °C. Glycoprotein detection was performed as described by Clegg (1982), except that the concanavalin A incubation buffer was phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.2) containing 10 µM-Mn²⁺ and 0.5% (v/v) Triton X-100 with no Ca²⁺ or Mg²⁺ added.

Peptide mapping

Peptide mapping of protein S-28 and its satellite spot was performed as described by Cleveland *et al.* (1977). The appropriate spots were punched out from a two-dimensional gel, equilibrated with sample buffer and loaded in the sample wells of a 15% (w/v) polyacrylamide/SDS gel. Digestion was effected with 0.05 µg of *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) in each sample well for 30 min at room temperature. The resulting pattern of peptide fragments was detected by silver staining.

Amino-acid-sequence determination of protein S-28

Protein S-28 was prepared in quantities sufficient for automated Edman degradation by using a procedure of

preparative two-dimensional electrophoresis. The protein sample was loaded on a 0.5 cm × 10 cm strip of glass fibre (GF/C; Whatman). Otherwise the first-dimension gel was handled as described previously. After electrophoresis the gel was stained with Coomassie Blue R-250, and the band which contained protein S-28 was excised and equilibrated against the sample buffer of the second dimension. The strip was next cut into gel pieces no more than 1 cm long, and these were placed on top of the stacking-gel portion of the second-dimension gel. By using this procedure it was possible to resolve ten duplicate protein S-28 spots in one second-dimension gel. These were punched out and electrophoretically eluted by the method of Hunkapiller *et al.* (1983). Automated Edman degradation was carried out by using the Applied Biosystems 470 gas-phase sequencer. The phenylthiohydantoin derivatives of the amino acids were analysed as described by Hunkapiller & Hood (1983).

RESULTS

Age-related alterations in non-histone proteins

Fig. 1 reveals the considerable increase of the level of some non-histone proteins as a function of age. In order to eliminate small fluctuations due to differences among nematode batches and the slight variability which is intrinsic to cell-fractionation and sample-preparation methods, each protein sample to be analysed was a mixture of approximately equal amounts taken from three independent sample preparations. The two-dimensional pattern displayed under these conditions proved very reproducible.

It should be noted that young adult nematodes bear many eggs, which contain all stages of embryonic

development. Thus polypeptides whose abundance decreases with age might be involved in embryonic development and quite unrelated to the aging process. We have therefore also included chromosomal proteins prepared from eggs in our study (results not shown). We have marked (e) five non-histones in Fig. 1(a) that were highly abundant in eggs and which are also observed among the nuclear proteins prepared from young adults, but are hardly detectable in aged worms. These polypeptides might either be markers of development and as such also present among the non-histones of young gravid adults, or they might be expressed in both eggs and young-adult tissue.

Proteins whose abundance undergoes sharp increases with age are marked with S (for senescence) in Fig. 1(b). The most important one on the basis of mass has an M_r of approx. 28000 and was therefore designated 'S-28'. Protein S-28 is remarkable in being present among the non-histones of young adults, although at levels beyond the detection limit of Coomassie Blue R-250. However, this protein was consistently easily distinguished on Coomassie Blue-stained gels loaded with a comparable amount of 'old' nuclear proteins. Its increase as a function of age is underestimated from silver-stained gels like those shown in Fig. 1, as it readily exceeds the limits of linearity between protein concentration and stain intensity.

Characterization of protein S-28

As can be seen from Fig. 1, protein S-28 is accompanied by a slightly more acidic satellite spot. This spot actually represents a modified form of the major polypeptide, as shown by a Cleveland-digest experiment (Fig. 2). Consequently, both spots were combined for

Fig. 1. Two-dimensional gels (NEPHGE-SDS/12% PAGE) of chromosomal proteins of *Caenorhabditis elegans* stained with silver

About 400 µg of protein was applied. (a) Young adult stage; (b) aged worms. e, Egg-specific protein, very abundant in eggs, much less abundant in young gravid adults and virtually extinct in the aged post-reproductive stage; s, senescence-specific nuclear protein, hardly detectable in young adults, but quite obvious in the aged stage; sg, senescence-specific concanavalin A-binding glycoproteins; H1, H2A, H2B, H3 and H4 are histones.

Fig. 2. Cleavage digest of protein S-28 and its satellite spot

The 15% Polyacrylamide/SDS gel was stained with silver. Lane a, *Staphylococcus aureus* V8 proteinase (0.05 μ g), not detected with silver; lane b, peptide pattern of protein S-28 generated by digestion with 0.05 μ g of *S. aureus* V8 proteinase; lane c, satellite spot of protein S-28 cleaved by *S. aureus* V8 proteinase (0.05 μ g).

further characterization of this potentially important protein. Approx. 10–15 μ g of protein were purified by semi-preparative two-dimensional electrophoresis, starting from approx. 20 mg of chromosomal proteins, and used for sequencing. The *N*-terminal amino acid sequence of protein S-28:

Lys-Tyr-Val-Ala-Lys-Asp-Gly-Lys-Asp-Gly-Lys-Asp-Gly-Lys-Ala-(Leu)-Gly-Ala-Glu-Lys-Val-(?)-His- . . .

exhibits some unusual properties (amino acid residues given in parentheses are those for which only weak experimental evidence was obtained). First, it has a four-fold mutated repeat of the tripeptide Gly-Lys-Asp. Secondly, it contains an exceptionally large amount of charged amino acids almost in balance (6 Lys, 3 Asp and 1 Glu among the first 20 residues). The stretch of charged residues is interspersed with the small amino acid residues alanine and glycine. This conformation is also observed at the *N*-terminus of calf thymus HMG (high-mobility-group protein) 17 (Walker *et al.*, 1977), although there is no clear sequence similarity. We have screened a data bank (Lipman & Pearson, 1985) to check whether any similarity exists with the *N*-terminal region of any of the 3557 stored polypeptide sequences (updated to 28 February 1986), but found none. Approx. 50% homology exists with internal polypeptide fragments of histidine permease inner-membrane receptor protein and DNA-directed DNA polymerase, but this similarity is probably meaningless.

Some age-specific non-histones are glycoproteins

A set of five senescence-specific polypeptides can be seen in Fig. 1 which are located just above and beneath protein S-28 and exhibit an elongated shape in the vertical direction. Since this kind of streaking is a known property of glycoproteins, a procedure was performed which allows the detection of concanavalin A-binding proteins on a nitrocellulose transfer (Clegg, 1982). The original gel was then stained with silver so as to enable more straightforward identification of carbohydrate-containing proteins. The results of this experiment (Fig. 3) provide firm evidence that three out of the five putative glycoproteins marked in Fig. 1 indeed contain carbohydrate. Consequently they were marked 'sg'. Two more polypeptides which have about the same M_r as protein S-28, but are slightly more basic, also carry carbohydrate, but their abundance is low and they are

Fig. 3. Evidence that a subset of senescence-specific nuclear proteins are glycoproteins

(a) Western blot of nuclear proteins from aged worms, resolved by NEPHGE-SDS/PAGE. Detection of concanavalin A-binding protein with horseradish peroxidase and aminoethylcarbazole in the presence of H_2O_2 (Clegg, 1982). ←, Glycoprotein probes (cellobiohydrolases I and II). (b) Original gel after electrophoretic transfer of the proteins. The residual proteins were stained with silver. Senescence-specific glycoproteins (▶) are located close to protein S-28.

not readily detectable among the proteins resolved in Fig. 1(b). The glycoprotein nature of two age-specific polypeptides slightly more acidic than protein S-28 and having M_r values of approx. 20000 and 30000 was not detected by this method. However, it should be noted that the binding assay with concanavalin A can provide only confirmation, but not negation, about the carbohydrate content of polypeptides, since some glycoproteins may not bind to this lectin.

DISCUSSION

By using techniques of two-dimensional electrophoresis and silver staining we clearly demonstrated that changes in the pattern of the nuclear proteins indeed do occur as the nematodes grow old. These results are in disagreement with those of Johnson & McCaffrey (1985), who found no changes in the spectrum of proteins resolved in worms of different ages. However, the experimental conditions used were largely different: (1) they compared the complete protein content of worms of various ages, i.e. cytoplasmic and nuclear as well; (2) they used conventional two-dimensional electrophoresis with isoelectric focusing in the first direction; (3) worms were fed on ^{35}S -labelled *Escherichia coli* cells and the labelled proteins were detected by fluorography, i.e. the rates of synthesis were measured rather than the actual levels; (4) interpretation of the data was difficult, because of the drastic reduction in the incorporation of label by old worms.

Since we compare the actual levels of proteins in young and old by staining with silver, from a formal point of view we cannot distinguish whether the level of any given protein increases with age as the result of a higher synthesis rate or a slowed degradation rate. However, the increases of the levels of the putative and proven glycoproteins in aged worms, as discussed above, are most likely the result of a higher synthesis rate, since they are virtually absent in the young adult stage.

The abundance of the age-specific proteins suggests that they play a structural rather than a regulatory role. One of these, protein S-28, was characterized in more detail, and it should be possible now to isolate the structural gene in the near future and to study its regulation.

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