The turnover of vimentin in Ehrlich ascites tumour cells

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The turnover of vimentin and vimentin-derived peptides has been examined in logarithmically growing Ehrlich ascites tumour cells. Cells were pulse-labelled with [^{35}S]methionine for 30 min and then chased for up to 60 h. It was found that the specific radioactivity of the main isoelectric variant of vimentin decreased to half the original value in 15.3 h which was close to the division time of the cells (16 h). The protein moiety of the phosphorylated variant of vimentin also turned over very slowly, in contrast to the turnover rate of the phosphate group itself which has a half-life of 1.4 h. The role of the intermediate filament-specific, Ca^{2+}-activated proteinase has been considered in relationship to the slow turnover of vimentin.

Vimentin *Intermediate filaments* *Protein turnover* *Ca^{2+}-activated proteinase*

1. INTRODUCTION

Intermediate filaments containing the protein vimentin are found in the cytoplasm of most in vitro cultured vertebrate cells and, in tissues, in cells derived from the mesenchyme [1–3]. Vimentin-containing intermediate filaments are thought to fulfill a structural role in the cell [4,5], but some doubt has been cast on this view by the observation that injection of a vimentin-directed antibody into fibroblasts has no effect on cell shape, movement or division [6].

Nevertheless, the very ubiquity and conservation of vimentin suggests that this protein plays an important role in cellular physiology. This view is strengthened by the discovery of an intermediate filament-specific, Ca^{2+}-activated proteinase which, like vimentin, is present in many tissues and culture cells and is similarly highly conserved [8,9]. It has not, however, been demonstrated that vimentin is degraded by this proteinase in vivo. We have shown that some cells containing the proteinase in extractable amounts nevertheless do not appear to synthesize vimentin [10] suggesting that the proteinase may have a function or functions in addition to the degradation of vimentin.

We have further investigated the interrelationship of vimentin and the intermediate filament-specific, Ca^{2+}-activated proteinase by examining the turnover of vimentin in logarithmically growing Ehrlich ascites tumour cells. These cells contain considerable amounts of both vimentin and the proteinase [11].

It is possible that the cellular function of vimentin involves its degradation at some point, a degradation which might be mediated by the proteinase. Since vimentin is found to be a major protein in EAT cells, extensive degradation would be required to be balanced by a relatively high rate of synthesis. The high levels of vimentin mRNA in these cells could potentially allow for such a high rate of synthesis [10]. We were interested to discover if there was indeed a high rate of degradation; i.e., if vimentin turns over rapidly.

Another interesting feature of vimentin is the presence of a small proportion as a phophorylated variant [11–13]. The function of this phosphorylated variant is not known but it has been shown that its cellular concentration increases

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during mitosis and under the influence of hormones [14–16]. Consequently, we have investigated the turnover of the protein moiety of the phosphorylated variant and compared this with both the turnover rate of the main isoelectric variant and with the turnover rate of the phosphate group itself. This latter value has been determined in CHO cells to be ~1 h [13,17].

2. MATERIALS AND METHODS

2.1. *Materials*

Reagent grade chemicals and biochemicals were obtained from Merck AG (Darmstadt). Ampholines were obtained from LKB (Bromma). Phenylmethylsulphonyl fluoride (PMSF) was purchased from Serva Feinbiochemica (Heidelberg). *Nα-p*-Tosyl-L-lysine chloromethyl ketone (TLCK) and acetyl-L-leucyl-L-leucyl-L-argonal (leupeptin) from Sigma (St Louis MO). [^{35}S]Methionine (>800 Ci/mmol) and En^{3}Hance were obtained from New England Nuclear (Dreieich), [^{32}P]orthophosphate (10 mCi/ml, carrier-free) was obtained from Radiochemical Centre (Amersham), Soluene 350 was obtained from Packard Instruments Company Inc. (Downers Grove IL) and X-ray film from Fuji Photo Film (Tokyo). The Bio-Rad Protein Assay Kit was supplied by Rio-Rad (Richmond CA)

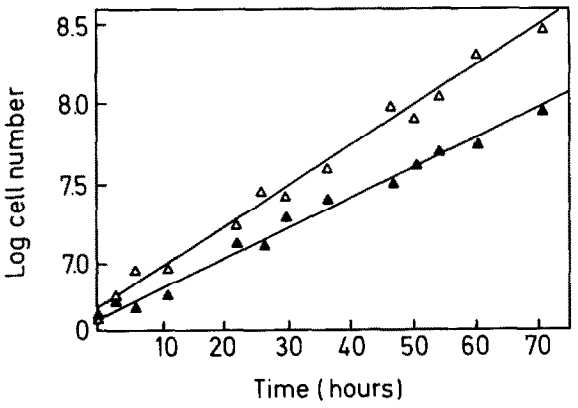

Fig. 1. Growth kinetics of EAT cells after labelling with [^{35}S]methionine. A 10 ml culture of EAT cells was labelled for 30 min in 2 ml methionine-free medium containing 50 μCi[^{35}S]methionine/ml. The cells were recovered by centrifugation and resuspended in 10 ml normal medium and growth was continued for 60 h. Aliquots were removed at intervals and the cell density determined: (▲—▲) cells labelled with [^{35}S]methionine; (△—△) control culture which was treated as above with the exception of the addition of [^{35}S]methionine.

2.2. *Cell culture*

Ehrlich ascites tumour (EAT) cells were grown in suspension culture using minimal essential medium supplemented with 5% foetal calf serum as in [18]. Cells from 10 ml culture at 5×10^5 cells/ml were labelled in 2 ml of methionine-free medium at 50 μCi/ml [^{35}S]methionine for 30 min or in 2 ml of phosphate-free medium at 1 mCi/ml, recovered by centrifugation at $225 \times g_{av}$ for 5 min and resuspended in 10 ml normal medium. Growth was continued for up to 60 h, during which time the culture was maintained in log phase by dilution with fresh medium. At intervals, 0.5 ml aliquots were withdrawn and added to 1 ml frozen medium containing 1 mM EGTA, 1 mM PMSF, 1 mM TLCK and 0.1 mM leupeptin. For ^{32}P-labelled cells the frozen medium contained, additionally, 25 mM NaF and 1 mM ATP to inhibit phosphatase activity [15]. Cells were recovered by centrifugation at $225 \times g_{av}$ for 5 min at 0°C, washed with 0.15 M NaCl, 10 mM Tris–HCl (pH 7.5) for ^{35}S-labelled cells or 0.15 M NaCl, 10 mM phosphate buffer (pH 7.4) for ^{32}P-labelled cells, in both cases containing the inhibitors as above. The cells were then repelleted and frozen in liquid nitrogen.

2.3. *Analysis of labelled proteins*

Each sample was resuspended in 100 μl 9.5 M urea, 2% NP40, 5% 2-mercaptoethanol and the inhibitors as above, shaken for 15 min at room temperature and undissolved material removed by centrifugation at $8000 \times g_{av}$ for 5 min. Portions (10 μl) of each sample were analysed by two-dimensional gel electrophoresis after addition of 1 μl ampholines and 1 μg purified vimentin as in [10]. The ampholines used were a mixture varying from pH 2.5–11 (70% 5–7; 20% 3.5–10; 5% 2.5–5; 5% 9–11). Quantitative analysis and fluorography were as in [10].

The protein concentration in an aliquot of each sample was determined using a Bio-Rad protein determination mixture and the specific activity was measured by diluting a 1 μl aliquot of each sample into 1 ml 0.2 mg bovine serum albumin/ml, adding 1 ml 40% methionine, 10% trichloroacetic acid, incubating at 100°C for 15 min and filtering

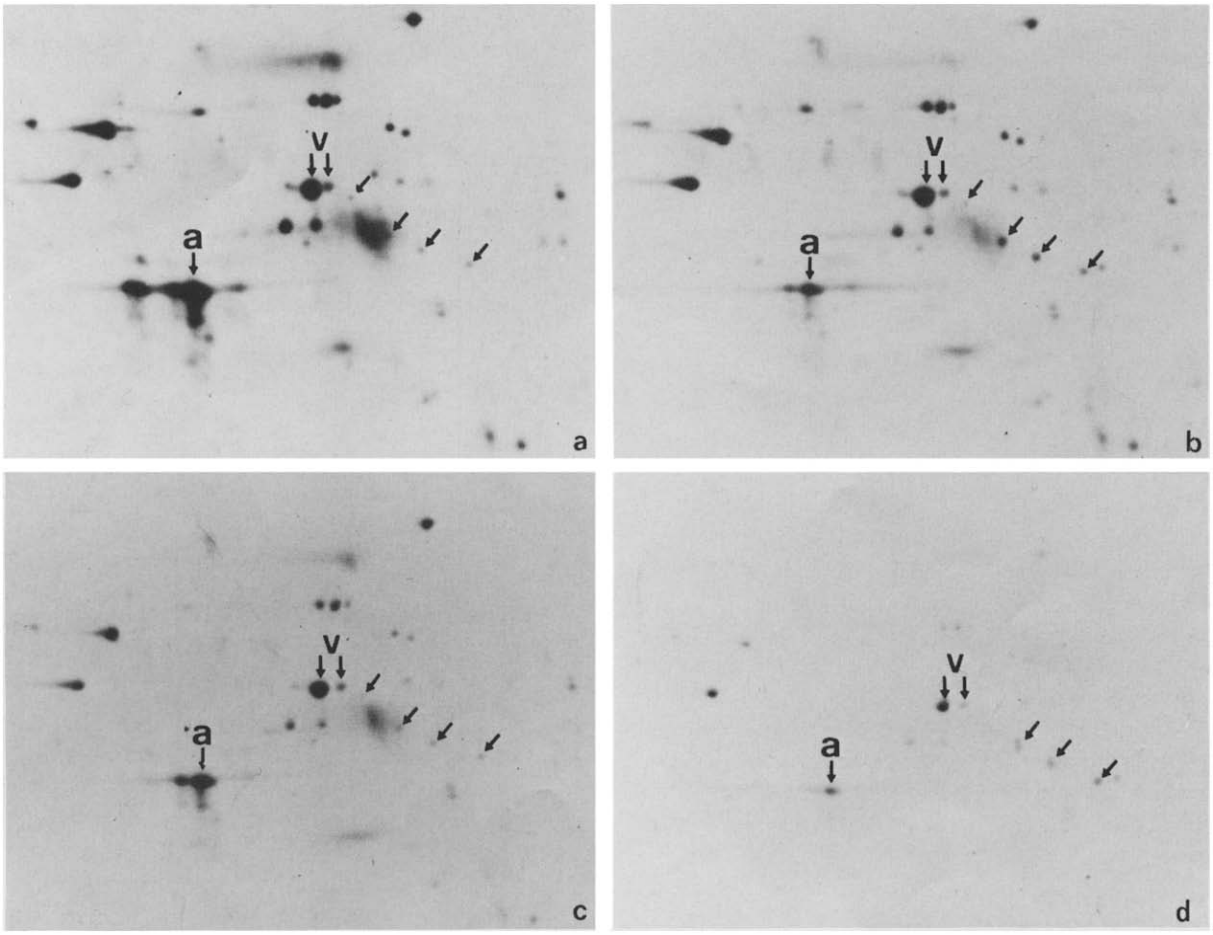

Fig. 2. Fluorographs of 2-dimensional polyacrylamide gels of proteins from cells pulse-labelled with [^{35}S]methionine followed by a cold chase in normal medium. Aliquots were taken at 4 different times as in section 2 and cells dissolved in 100 μl 9.5 M urea, 2% NP40, 5% 2-mercaptoethanol. Samples of 10 μl were electrophoresed in 2-dimensional gels after the addition of 1 μl (pH 2.5–11) ampholines. After electrophoresis, the gels were fixed in 10% acetic acid, soaked in En^{3}Hance and water, dried and exposed to X-ray film for 5 days. Only the vimentin-containing section of the fluorograph is shown. Vimentin (v) and its degradation products are indicated by arrows; actin (a) is also indicated: (a) 0 h; (b) 10 h; (c) 25 h; (d) 45 h.

through a glass fibre filter which was counted in 5 ml toluene-based scintillation cocktail.

3. RESULTS AND DISCUSSION

Cells were labelled for 30 min in methionine-free medium containing 50 μCi[^{35}S]methionine/ml. It was found that the use of higher concentrations of [^{35}S]methionine affected subsequent growth rates during the cold chase, resulting in non-logarithmic growth kinetics. After labelling in the above conditions, the cells grew throughout the cold chase with a division time of 16 h (fig. 1). Although constant, this was nevertheless slower than the control culture, which has a division time of 12 h. This effect of [^{35}S]methionine on cell growth and/or division may be due to radioactive emission.

The turnover of vimentin during a cold chase is shown in fig. 2. Aliquots of samples taken at 4 different time intervals have been analysed by two-dimensional gel electrophoresis, followed by fluorography. Vimentin is one of the most prominent, labelled proteins. The acidic variant of vimentin can be seen as well as a characteristic

Fig. 3. A fluorograph of two-dimensional gel of proteins from cells labelled with [^{35}S]methionine for 30 min and electrophoresed with vimentin digested with the Ca^{2+}-activated proteinase for 2 min. (→) Positions of the degradation products on the stained gel.

'staircase' of degradation products. Vimentin clearly has a rather long half-life, there still being significant label in the vimentin spot 45 h after the beginning of the cold chase. The intensity of labelling of vimentin relative to that of other proteins does not appear to change radically throughout the chase period suggesting that vimentin is neither particularly long-lived nor rapidly turned over in comparison to other proteins. The same is true also

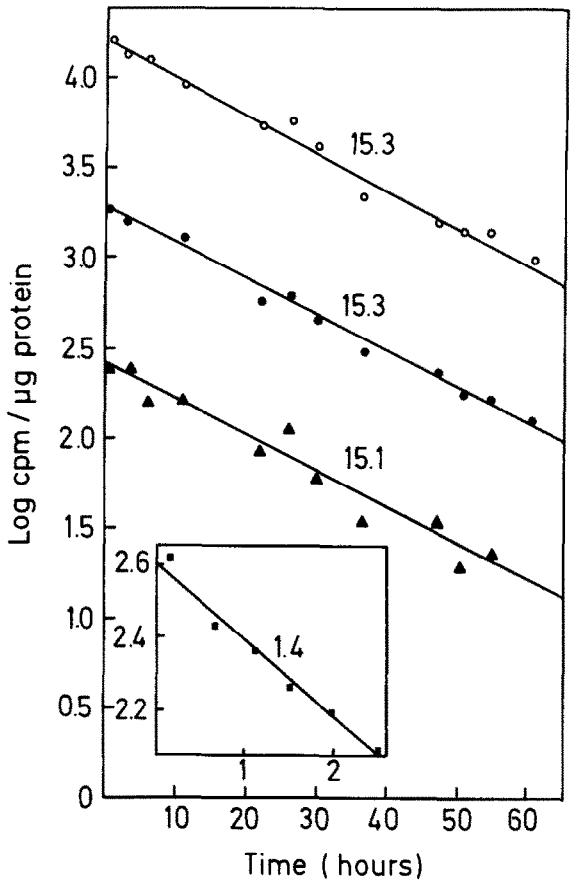

Fig. 4. A quantitative analysis of the turnover of total trichloroacetic acid-precipitable protein, the major isoelectric variant of vimentin and the phosphorylated variant of vimentin. Cells were labelled and analysed by 2-dimensional gel electrophoresis as in section 2 with the addition of 1 µg purified vimentin prior to electrophoresis. After electrophoresis of the IEF gels, the vimentin containing sections were excised and the pieces applied to the second-dimensional gel. The gel was stained in Coomassie blue, destained and appropriate spots were cut out, soaked in 1 ml Soluene 350 overnight and counted in 10 ml toluene-based scintillation cocktail. Each point is expressed as cpm/µg protein applied to the gel and is an average of 3 determinations. The slope of each line was determined by regression analysis and the half-lives of each protein species are indicated on the figure: (○—○) total trichloroacetic acid-precipitable proteins; (●—●) vimentin (main isoelectric variant): (▲—▲) vimentin (phosphorylated variant); (■—■), turnover of ^{32}P in vimentin from cells labelled with [^{32}P]orthophosphate. The values were determined as above.

for the acidic variant of vimentin, the relative intensity of which does not appear to change dramatically during the cold chase. A certain amount of degradation products of vimentin is seen. This was a rather small amount which varied in a random fashion between different samples and which may be due to the partial degradation of vimentin during the harvesting of the cells. A close examination of a fluorograph of in vivo labelled protein co-electrophoresed with purified vimentin pre-digested with the intermediate filament-specific, Ca^{2+}-activated proteinase showed no labelled, lower-M_r degradation products (fig. 3). The inclusion of the small amount of degradation products in the quantitative analysis of vimentin turnover did not affect the value obtained but added only to the scatter of the points and has not been further considered.

We have analysed the turnover of vimentin more quantitatively by excising stained spots and counting the radioactivity directly in scintillation vials. The specific activity of total trichloroacetic acid-precipitable material decreases to half the original value in 15.3 h, which is close to the division time of the cells. Under the growth conditions used, a completely stable protein (i.e., one not undergoing any degradation) would be expected to have a half-life of 16 h since in this time the amount of protein will have doubled resulting in a reduction of the specific activity to half the original value. The value of 15.3 h which is not significantly different from 16 h would suggest that most proteins are very stable, undergoing little or no degradation. The value of 15.3 h is, however, a maximal estimate since all pulse–chase experiments are subject to the problem of precursor reutilisation [19–21]. However, the error introduced by such reutilisation is greater for rapidly turned over proteins than for proteins with a longer half-life [22], and would not alter the conclusion that the majority of proteins are rather stable.

Vimentin would appear to be one of these stable proteins since, by quantitative analysis, the main isoelectric variant of vimentin also has a half-life of 15.3 h and is therefore very stable in logarithmically growing cells. A half-life of 15.3 h would give a value for the turnover rate of 1.5% generation. The acidic variant, which accounts for 10% of the labelled vimentin, has a half-life of 15.1 h which is not significantly different from the value of 15.3 h for the main form.

The turnover of the phosphate group in vimentin was determined by labelling a culture with [^{32}P]orthophosphate and similarly analysing the labelled cells. In contrast to the slow turnover of the protein moiety, the phosphorylation of vimentin has a half-life of 1.4 h (fig. 4, insert). This is in agreement with data on CHO cells which give values for the half-life of 60–70 min [13,17]. The value obtained is subject to error introduced by reutilisation of the labelled precursor which, in the case of [^{32}P]orthophosphate, may be extensive. The true value may therefore be <1 h. Nevertheless, it is clear that the turnover of the phosphate group and the turnover of the protein moiety are markedly different and would suggest that phosphorylation or dephosphorylation is not associated with simultaneous degradation of vimentin.

A difference in the half-life of a protein and the half-life of the phosphate group on the phosphorylated derivative is also found in acidic nuclear proteins in HeLa cells [23]. Such a discrepancy suggests either that a subpopulation of protein undergoes cycles of phosphorylation and dephosphorylation or that each molecule is phosphorylated for a short period during its life span. This latter possibility, in the case of vimentin, cannot be an entirely cell cycle-dependent event since, although the degree of phosphorylation of vimentin increases during mitosis [14, 15], there is always a certain proportion of vimentin in the phosphorylated form throughout the cell cycle. The phosphorylation and dephosphorylation of a number of enzymes appears to be an important regulatory mechanism [24]. This may extend also to the regulation of intermediate filaments many of which contain phosphorylated subunit variants [25–27].

The role of the intermediate filament-specific, Ca^{2+}-activated proteinase in the turnover of vimentin remains unclear. Since the bulk of the vimentin turns over very slowly, if at all, it is apparent that the proteinase, if its function is to degrade vimentin, cannot be acting on more than a small proportion of the vimentin, perhaps either on a localised subpopulation (for example, the ends of the filaments) or under certain metabolic conditions (for example, in localised areas of high calcium concentration which may occur near the plasma membrane). The presence of a certain

amount of degradation products characteristic of those produced by degradation of vimentin with the proteinase would suggest that some proteinase activity is occurring in vivo. However, it is difficult to exclude the possibility that these degradation products are formed during the handling of the culture before the proteinase inhibitors can enter the cell. Nevertheless, the presence of these degradation products may be a real phenomenon and it is possible that a subpopulation of vimentin is degraded very rapidly to form these degradation products which would appear to be subsequently stable.

Only high-M_r products are observed, although, in vitro, vimentin can be degraded into a complex pattern of degradation products with a broad M_r range. Most of these contain methionine [10] and should be detectable in vivo if present in significant amounts. Their absence in vivo would suggest that degradation of vimentin may be less extensive than in vitro. The possibilities that low-M_r products are produced in very low amounts or that they are extremely unstable or are modified in some way, cannot, of course, be excluded. The role of the intermediate filament-specific, Ca^{2+}-activated proteinase must be further investigated by examining, for example, the turnover of vimentin in cells in which the Ca^{2+}-metabolism is disturbed.

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