Degradation of peptides and proteins of different sizes by homogenates of human MRC5 lung fibroblasts

Aged cells have a decreased ability to degrade shortened proteins

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The degradation of haemoglobin and haemoglobin-derived peptide fragments by homogenates of MRC5 fibroblasts has been investigated. Results show that the smaller fragments were degraded more rapidly than larger substrates at both pH 5.5 and pH 7.5. Only the smallest of the soluble cyanogen bromide peptides (M_r 3500) was degraded at pH 7.5. Degradation at pH 5.5 proceeded more rapidly than that at pH 7.5 for all substrates tested but was more marked with the larger substrates. Homogenates prepared from aged cells degraded puromycyl peptides and, to a lesser extent, cyanogen bromide peptides at a slower rate, at pH 7.5, than those prepared from younger cells. We suggest that cytosolic degradation is less selective and at least one cytosolic proteolytic activity decreases as cells age.

MRC5 fribroblast *Proteolysis* *Homogenate* *Puromycin* *Aging* *Globin*

1. INTRODUCTION

Protein degradation is important in regulating intracellular protein concentration [1,2]. Intracellular proteolysis has been studied in erythroid cells [3,4], hepatocytes [5] and fibroblasts in culture [6–8]. In fibroblasts the majority of endogenous proteins are degraded cytosolically but a significant proportion is degraded lysosomally, particularly upon serum deprivation [6,7]. Exogenous proteins are thought to be degraded principally in the lysosomes [9]. Previous studies have shown that proportionally more short half-life proteins are degraded in the cytosol than long half-life proteins [6–8,10]. In addition, abnormal proteins containing amino acid analogues [6,7,10] and puromycyl peptides (prematurely terminated endogenous proteins) [8] are preferentially degraded in the cytosol. Thus, the nature of the endogenous substrates make a significant contribution to their site of degradation. Although it is possible to differentiate between lysosomal and cytosolic degradation by using lysosomotropic agents [11] or serum-free media [2,6,7], an alternative approach is to prepare homogenates of cells and to measure degradation at neutral pH (which primarily measures lysosomal degradation) and acidic pH (which primarily measures lysosomal degradation because of the acid pH optima of the majority of lysosomal proteinases). Hendil [12] has used a similar system to investigate the degradation of haemoglobin in BHK-21 hamster fibroblasts. Here we have studied the degradation of haemoglobin, globin and smaller globin-derived peptides to see if there is any correlation between the subcellular site of proteolysis and the substrate molecular mass in fibroblast homogenates. Our laboratory has extensively used these substrates in the reticulocyte system [3,13–15]. We also investigated any

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passage number/degradation relationships as it has been reported that neutral proteinase activity decreases in ageing cultured cells [16].

2. MATERIALS AND METHODS

All chemicals were of analytical grade and were purchased from BDH (Poole, Dorset) or Sigma (Poole, Dorset). Cell culture material was purchased from Flow Laboratories (Irvine, Scotland). Rabbit haemoglobin, containing L-[4,5-^{3}H]leucine or [U-^{14}C]leucine was prepared in rabbit reticulocytes as in [13]. Globin [14], cyanogen bromide peptides of globin [14], and puromycyl peptides prepared from washed isolated polysomes [15] were all prepared as previously described. Cell culture was as in [7], and protein was assayed by the method of Lowry et al. [17] using bovine serum albumin as a standard. Homogenates of fibroblasts were prepared as follows: approx. 4×10^6 confluent MRC5 fibroblasts were trypsinised and twice washed with Eagle's MEM containing 10% (v/v) foetal calf serum to inactivate the trypsin. The cells were then washed 5 times in serum-free MEM. All subsequent steps were carried out at 4°C. The resulting cells were resuspended in 2 ml homogenising buffer (5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol), 0.2 ml of 1% (w/v) digitonin was added to lyse the cells, and after 45 min the homogenate was centrifuged at $1000 \times g$ for 5 min to remove nuclei and unlysed cells. Twenty μl of 0.1% (v/v) Triton X-100 was then added to solubilise the remaining cell organelles and after 30 min the homogenate was centrifuged at $10000 \times g$ for 5 min. The supernatant was analysed for protein content, frozen in liquid nitrogen, and stored at −70°C. Protein degradation was detected as the appearance of radiolabel soluble in 10% (w/v) trichloroacetic acid as in [13]. Incubations consisted of 100 μg homogenate protein, either 30 nmol puromycyl peptides of globin, 35 nmol cyanogen bromide peptides of globin, 2.9 nmol globin, or 4.5 nmol haemoglobin. An ATP generating system (1 mM ATP, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase) was also included in the incubation mix because the homogenization would probably disrupt mitochondria and dilute glycolytic enzymes and co-factors, and thus perturb ATP synthesis. We felt that addition of the ATP-generating system was a useful expedient, should some of the proteases be ATP-stimulated, although we did not investigate this possibility in fibroblasts. Penicillin and streptomycin were added to prevent bacterial growth. The volume was made up to 1 ml, with either 0.1 M Tris-HCl buffer (pH 7.5) or 0.1 M sodium acetate buffer (pH 5.5). Degradation was determined after 24 h incubation at 37°C. Rod gel electrophoresis was carried out as in [14] following the method of Swank and Munkres [18].

3. RESULTS

Most studies in which the metabolism of a number of proteins have been compared have employed a variety of disparate proteins and polypeptides. In the present study we have taken a different but equally valid approach in which the proteins catabolized are all related: haemoglobin – the holoprotein, globin – the apoprotein, and puromycyl peptides and cyanogen bromide peptides of globin which represent incomplete chains, have been used as putative substrates. Catabolism of these polypeptides in reticulocyte extracts has revealed different breakdown characteristics, particularly with regard to the involvement of ATP [15]. Previous studies with cultured fibroblasts have demonstrated that differences in polypeptide chemistry (especially chain length and amino acid composition) rather than half-life, profoundly affect the subcellular site of degradation [7,8]. This study was undertaken to determine whether degradation in cell-free preparations reflected that in whole cells, in particular if the incomplete polypeptides were more readily degraded by cytosolic (neutral) proteinases than longer complete molecules.

Fig.1 shows the effect of the fibroblast homogenate proteolytic activity on globin cyanogen bromide peptides when incubated at pH 5.5 and 7.5. The cyanogen bromide peptides were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) into essentially 3 components (fig.1a), the two amino terminal fragments of the globin chains (slices 16–19 and 21–24, cyanogen bromide cleaves exclusively at methionine residues and there is one methionine in each of the globin chains) plus a smaller amount of uncleaved globin (slices 10–13). Previous studies showed that the

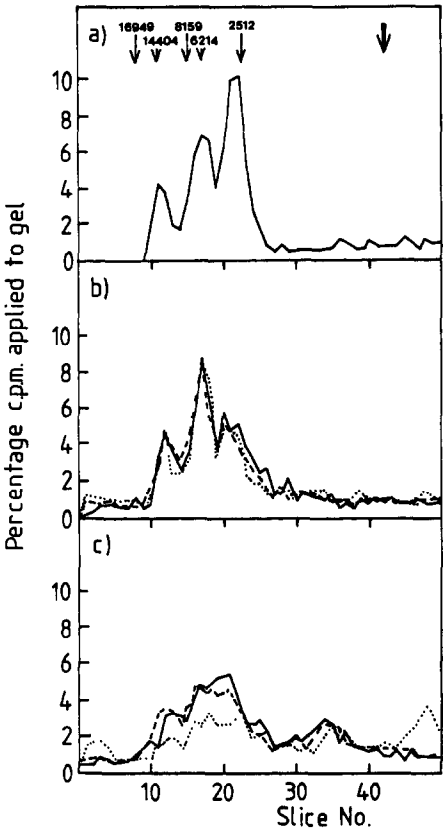

Fig.1. Effect of pH upon the degradation of cyanogen bromide peptides by fibroblast homogenates. SDS electrophoretic profiles of globin cyanogen bromide peptides after incubation for 24 h with: (a) no enzyme; (b) homogenate at pH 7.5; and (c) homogenate at pH 5.5; homogenates prepared from cells at passage numbers 33 (——), 39 (– – –), 45 (···). Incubations consisted of 59 μg substrate and 65 μg, 69 μg and 100 μg homogenate protein prepared from cells of passage numbers 33, 39 and 45, respectively. The position and molecular mass of standard peptides are shown, and the position of the marker dye, Bromophenol blue, is shown by the large arrow. The gels contained 12.5% acrylamide and 8 M urea [14]; and electrophoresis followed the procedure of Swank and Munkres [18].

carboxyl terminal fragments were essentially insoluble at neutral pH [14]. Fig.1b clearly shows that at pH 7.5 the homogenate exclusively degraded the smallest peptide (the 3.5-kDa fragment from the α-chain [14]). In contrast, incubation of the homogenate at pH 5.5 resulted in a much more complex electrophoretic pattern (fig.1c) which indicates the presence of a number of degradative intermediates resulting from the catabolism of all three polypeptide species. These observations suggest that the neutral (cytosolic) proteolysis is more selective than the acidic (lysosomal) degradation, which may result from differences in specificity between the cytosolic and lysosomal proteinases and the possibility that susceptible bonds in the larger peptides are only made available for enzymic cleavage after unfolding at the lower pH.

No age/degradation relationship was detectable from the passage 33, 39 and 45 cells (fig.1b) because the amount of lysate which was added to the substrate had to be varied as the experiment was designed to achieve approximately equal proteolysis using the different homogenates from the three cell age groups. In separate experiments we therefore measured proteolysis per mg homogenate protein, at both pH 5.5 and 7.5 using haemoglobin, globin, globin puromycin-peptides and the globin cyanogen bromide peptides. Table 1 shows the substrate degraded (in nmol) after 24 h, per mg homogenate protein, and the ratios of degradation at pH 5.5 to pH 7.5. Time course experiments showed that degradations were approximately linear with volumes of homogenate and time, and in no case had the extent of degradation reached a limiting value; i.e., the enzyme concentration was rate-limiting (not shown). At both pH 5.5 and pH 7.5, the cyanogen bromide peptides were the most rapidly degraded, puromycyl peptides were degraded more slowly followed by globin and haemoglobin. Degradation of haemoglobin at pH 5.5 proceeded at 1.4×10^{-4} nmol/h per μg protein, in good agreement to that obtained by Hendil [12] (5.5×10^{-4} nmol/h per μg protein) with BHK hamster fibroblasts at pH 5.0, although the hamster fibroblast homogenate was prepared in a way which did not involve digitonin or the removal of cell debris. The above results indicate that in general the smaller the protein, the more rapidly it is degraded. However, it must be borne in mind that we are measuring the appearance of trichloroacetic acid-soluble material, and the shorter the initial peptide the more likely it is that the resulting fragments are trichloroacetic acid-soluble, although studies with the reticulocyte have shown that free amino groups are generated more rapidly from small peptides

Table 1

The degradation of the various substrates by fibroblast homogenates at different pH values prepared from cells of different passage number

Substrate	Cell passage no.	pH 5.5	pH 7.5	Ratio pH 5.5/pH 7.5
Cyanogen bromide globin peptides	33	250	100	2.50
	39	250	79.1	3.16
	45	199	63.2	3.15
Puromycyl peptides of globin	33	38.5	59.7	0.64
	39	37.1	22.8	1.63
	45	32.8	11.6	2.83
Globin	33	17.4	2.82	6.16
	39	16.1	3.07	5.23
	45	12.0	1.46	8.22
Haemoglobin	33	3.54	0	–
	39	3.50	0	–
	45	3.60	0.08	45.00

Experimental details are given in the text. Results are expressed as nmol degraded after 24 h per mg homogenate protein

than larger ones, even taking into account the different molecular masses [13].

The relative difference between the proteolysis at pH 5.5 (primarily lysosomal) and pH 7.5 (primarily cytosolic) also shows an interesting size-degradation relationship. As we are dealing with a ratio of degradation, the possible problem involving the validity using trichloroacetic acid solubility as a criterion of proteolysis will not arise as one degradation serves as an internal control for the other. The complete globin chains (haemoglobin and globin) were almost undegraded by the cytoplasmic system but were susceptible to the acidic proteinases. In these cases therefore, we can infer that degradation occurs primarily by the lysosomal route. In contrast, the shortened peptides were degraded significantly at both pH values. This is due to the selective degradation of the smallest polypeptide species as shown in fig.1b. We have previously shown that puromycyl peptides of endogenous proteins are preferentially degraded in the cytosol of intact MRC-5 fibroblasts [8].

Some evidence for age-related changes in degradation was detected, particularly with the puromycyl peptides at pH 7.5, and a smaller increase in the pH 5.5/pH 7.5 proteolysis ratio with age was detected with the cyanogen bromide peptides. We have already shown in fig.1b that only the smaller of the cyanogen bromide peptides is degradable at pH 7.5, thus the decline in activity at neutral pH must be due to a decreased catabolism of this particular polypeptide.

4. DISCUSSION

The advantage of using a homogenate system to investigate proteolysis lies in the certainty that the degradation of like substrates can be compared. The results presented clearly indicate that proteolysis of the shortened globin chains occurs readily with both the neutral and the acidic proteases, whereas breakdown of the longer polypeptides (globin and haemoglobin) occurs primarily with the acidic enzymes, observations which may suggest different catabolic routes for the normal length and shortened molecules in fibroblasts. A disadvantage of using a homogenate system at different pH values is that the control of access to the lysosomes is lost, and indeed we find that the incomplete globin chains are readily degraded at both pH 5.5 and 7.5. However, one can envisage that in the whole cell, incomplete protein chains (which could arise by premature release of the

polypeptide from the ribosome or the action of a cytoplasmic endoproteinase attack upon a normal or abnormal protein) would be exposed to cytoplasmic proteinases prior to their entry into the lysosome (should the cytoplasmic degradation be insufficiently rapid). Therefore one cannot ascribe with any certainty a lysosomal role in the degradation of shortened polypeptides. Our findings that the degradation of endogenous puromycin peptides in fibroblasts is relatively unaffected by lysosomal-tropic reagents and serum deprivation [8] supports the suggested cytosolic proteolysis of certain shortened proteins.

Our results suggest that when assayed with incomplete globin chains (cyanogen bromide-peptides and puromycin peptides) there is an age-related decline in the ability to degrade some of them by certain neutral proteinases. These results are in partial agreement with Bosmann et al. [16] who have shown that general neutral proteinase activity decreases in ageing WI-38 fibroblasts. Lavie et al. [19] have found that the livers of aged mice possess a decreased ability to degrade puromycin-peptides. In the maturing reticulocyte it has been shown that a number of neutral proteolytic activities decrease, e.g., the degradation of puromycyl peptides and proteins with amino acid analogues incorporated into them [20], peptidase activity [3] and ATP-dependent proteolysis [4]. Our results show, however, that the decrease in proteolytic activity is not a general phenomenon but that only certain proteinases which degrade certain substrates are involved. Thus many possible age-related effects may remain undetected because the incorrect substrates are being used to monitor activity. Indeed, others have detected quite different effects in whole cells, which, as discussed by Okada and Dice [21], may be consequent upon the employment of a large variety of different protocols to label endogenous protein. Attempts to compare proteolytic activity in 'young' and 'old' fibroblasts are also limited by the uncertainty as to whether the cells synthesise the same proteins in exactly the same quantities during senescent and pre-senescent stages [21]. Furthermore, in whole cell studies it is uncertain whether enzyme, or substrate is the rate-limiting factor in any measurement of proteolysis. Such problems do not apply to the studies with homogenates described in this report.

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