Abnormal proteins of shortened length are preferentially degraded in the cytosol of cultured MRC5 fibroblasts

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Puromycyl peptides were degraded in MRC5 fibroblasts more rapidly than normal proteins labelled for the corresponding length of time for both long and short labelling periods. The degradation of the puromycyl peptides occurred almost exclusively in the cytosol of the cells. Even when the half-lives of normal and puromycyl peptides were manipulated to be similar, proportionally more of the normal proteins were degraded in the lysosomes. The rapid degradation of the puromycyl peptides was not due to the inhibition of protein synthesis brought about by puromycin but was due to the structure of the substrates themselves. The degree and intracellular site of degradation of puromycyl peptides closely mimic those of abnormal (missense) proteins containing amino acid analogues.

Puromycin *MRC5 fibroblast* *Proteolysis* *Lysosome*

1. INTRODUCTION

It is important for the cell to be able to remove potentially deleterious aberrant molecules. Two such abnormal molecules which have been studied are proteins with either missense or nonsense errors, models of which are, respectively, proteins containing amino acid analogues and proteins prematurely terminated by the action of puromycin. It is known that puromycyl proteins are rapidly degraded in *Escherichia coli* [1,2], reticulocytes [3–5], and other mammalian cells [6,7]. However little work has been carried out on the site and mechanism of degradation. Our laboratory has established that the ability to degrade puromycyl peptides decreases with reticulocyte maturation [8]. It was shown in [6] that degradation varies with the growth state in 3T3 fibroblasts, not because of differences in the degradative machinery but because different peptides are synthesised and degraded according to the growth phase. Short half-life proteins are preferentially degraded in the cytosol of mammalian cells [9,10]. Abnormal proteins containing amino acid analogues are also rapidly degraded and are preferentially broken down in the cytosol of cultured cells [9,10]. These findings have been extended by authors in [11] who have established that even when abnormal proteins of longer half-lives than some normal proteins are generated, they are still preferentially degraded in the cytosol. This means that the site of degradation is not wholly dependent upon the half-life of the protein but upon the configurational nature of the proteins. Here we report that similar conditions apply for the degradation of puromycyl peptides of various half-lives.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Methylamine, puromycin dihydrochloride, cycloheximide and sodium fluoride were purchased from Sigma (Poole), phosphate-buffered saline tablets (Dulbecco A) were obtained from Oxoid (Basingstoke) and L-[4,5-^{3}H]leucine (spec. act. 64 Ci/mmol) from Amersham International, Amersham. All tissue culture materials were pur-

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chased from Flow Laboratories, Irvine, as were Linbro 'Space-Saver' multiwell plates in which the degradation experiments were carried out.

2.2. *Methods*

Cell culturing was performed as in [11] using confluent cells of passage numbers of between 30 and 40. Measurement of protein degradation was also as in [11] with the following differences: [^{3}H]leucine was used as a radiolabel instead of valine with appropriate changes in the labelling (leucine-free) and degradation (containing 10 mM non-radioactive leucine) media. Separate experiments have shown that both radiolabels gave very similar results (unpublished). For the preparation of endogenous puromycyl peptides, cells were labelled with [^{3}H]leucine for either 30 min or 18 h in the presence of either 10 or 2.5 μg/ml puromycin dihydrochloride, respectively. Amino acid incorporation was measured, correcting for cell number, as in [12].

3. RESULTS

3.1. *Degradation of long- and short-labelled proteins and puromycyl peptides*

Fig.1 shows the percentage degradation of 30 min and 18 h labelled normal proteins and puromycyl peptides in normal medium containing a 10% (v/v) supplement of foetal calf serum, and in serum-free medium. By prolonging the labelling period proteins with longer half-lives tend to be preferentially labelled [13] and this is shown in fig.1 with the longer-labelled normal proteins having longer half-lives in serum-containing medium (a) than short-labelled normal proteins (c). Prolonging the labelling period also increases the half-life of puromycyl peptides so that the half-life of the long-labelled puromycyl peptides (b) is similar to that of the short-labelled normal proteins (c). The increase in the degree of proteolysis brought about by the incubation with puromycin was similar for long- and short-labelled peptides (2.63- and 2.62-fold, after 5 h of degradation). The concentrations of puromycin used, 2.5 μg/ml for long-labelled proteins, and 10 μg/ml for short-labelled proteins, were chosen because they inhibited the incorporation of [^{3}H]leucine into protein by the same amount, approx. 70%. Neither

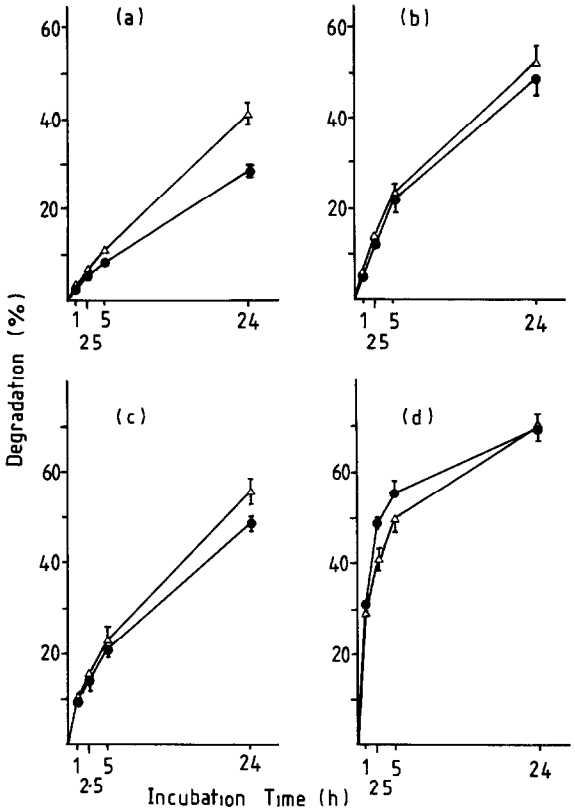

Fig.1. The degradation of long-labelled and short-labelled normal proteins and puromycyl peptides in serum-containing and serum-free media. Approx. 2 × 10^5 cells were labelled for 30 min (short label) or 18 h (long label) with [^{3}H]leucine in 0.5 ml leucine-free Eagles minimal essential medium supplemented with 10% (v/v) foetal calf serum and 20 mM Hepes buffer with or without puromycin (2.5 μg/ml long label; 10 μg/ml short label). Degradation was measured over 24 h in high (10 mM) leucine MEM with and without serum and expressed as a percentage total radioactivity which is trichloroacetic acid soluble. (●) Serum-containing medium, (△) serum-free medium. Results are means of 3 determinations ± SD. (a) Long-labelled normal proteins, (b) long-labelled puromycyl peptides, (c) short-labelled normal proteins, (d) short-labelled puromycyl peptides.

concentration had any effect upon cell viability as determined by trypan blue exclusion (not shown).

3.2. *The degree of lysosomal proteolysis as determined by an increase in degradation in serum-free medium*

When fibroblasts are placed in serum-free

medium the lysosomal contribution to degradation is increased [14], thus the extent of the increase in proteolysis will give an indication of the degree of lysosomal involvement in the degradation. Normal long-labelled proteins (fig.1a) have the largest lysosomal involvement in their degradation. Normal short-labelled proteins and long-labelled puromycyl peptides show (fig.1c,b) very similar (small) amounts of lysosomal involvement as determined by this method. Short-labelled puromycyl peptides show negligible lysosomal involvement in their degradation (fig.1d). The possibility that proteolysis of the puromycyl peptides is partly lysosomal in serum-containing medium is not excluded by this experiment (but see section 3.3).

3.3. *The degree of lysosomal proteolysis as determined by the inhibition by methylamine*

Another method of assessing lysosomal degradation is by use of lysosomotropic agents which accumulate in lysosomes, raising their pH and thus inactivating those lysosomal proteinases which have acid pH optima [15]. Therefore the greater the inhibition brought about by these agents, the greater is the lysosomal contribution to degradation. The lysosomotropic agent used in this study was 10 mM methylamine. Table 1 shows the percentage inhibition brought about by methylamine compared to controls in serum-containing medium and serum-free medium for the various classes of labelled proteins. For normal long-labelled proteins the inhibition was greater in serum-free medium than in serum-containing medium which was to be expected as the increase in protein degradation brought about by serum-free medium is lysosomal in origin. After 24 h incubation methylamine suppressed degradation to similar levels in serum-free medium and serum-containing medium for normal long-labelled proteins (i.e., to 22.8 and 24.7%, respectively) and normal short-labelled proteins (to 40.0 and 38.6%, respectively). The percentage inhibition is less for short-labelled normal proteins than long-labelled normal proteins, showing an increased lysosomal contribution in the degradation of the latter. The results for both species of puromycyl peptides, on the other hand, show very little inhibition by methylamine and in fact a slight stimulation is observed in some cases. Therefore, as judged by this criterion, puromycyl peptides show much less lysosomal involvement in their degradation than normal proteins in both the serum-stimulated and serum-deprived states.

3.4. *The effect of protein synthesis inhibitors during the labelling period*

Puromycin is a protein synthesis inhibitor, therefore the amount of labelled polypeptide generated during the labelling period would be decreased. To check that the increase in the degradation of puromycyl peptides was not due to a decreased substrate: enzyme ratio, but due to actual alterations in the substrate molecules

Table 1

Inhibition of degradation of normal proteins and puromycyl peptides by 10 mM methylamine

	% Inhibition of degradation			
	Normal protein		Puromycyl peptides	
	5 h	24 h	5 h	24 h
Long-labelled proteins				
Serum-containing medium	18.5	21.3	3.1	5.8
Serum-free medium	32.4	36.9	−9.6	7.1
Short-labelled proteins				
Serum-containing medium	17.0	17.7	−11.5	−1.1
Serum-free medium	21.3	28.8	−4.1	9.4

Experimental details are as described in the text. Results are means of two determinations. A negative value denotes a stimulation of protein degradation

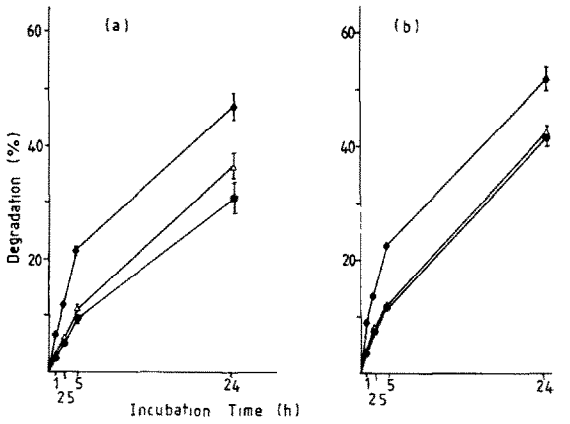

Fig.2. The degradation of long-labelled proteins which were labelled in the presence of puromycin and cycloheximide. Protocol was the same as in fig.1 for long-labelled proteins. (●) Control, (◆) 2.5 μg/ml puromycin, (△) 1.2 μM cycloheximide. Results are means of 3 determinations ± SD. (a) Serum-containing medium, (b) serum-free medium.

themselves, the effects of cycloheximide, an elongation inhibitor, upon protein degradation were tested. Puromycin inhibited the incorporation of [^{3}H]leucine by 73% in the long-labelled experiments. In separate experiments it was found that 1.2 μM cycloheximide inhibited incorporation by 72% and thus this concentration was used in subsequent experiments. Proteins were labelled for 18 h in the presence of either puromycin or cycloheximide, and the extent of protein degradation was measured as in previous experiments. Fig.2 shows that puromycin increased protein degradation to a much greater extent than cycloheximide, particularly at shorter incubation (chase) times. This effect was greater in serum-free medium than in serum-containing medium. Puromycin and cycloheximide had no effect upon cell viability as determined by trypan blue exclusion.

4. DISCUSSION

These results show that, in agreement with [1–7], puromycyl peptides are degraded faster than normal proteins labelled for the corresponding length of time. To our knowledge, this is the first time such an observation has been made with MRC5 cells. By altering the labelling period it was possible to label preferentially puromycyl peptides and normal proteins of different half-lives. The percentage increase in degradation brought about by the action of puromycin was similar in long- and short-labelled proteins. The same has been found to be the case with abnormal proteins brought about by the inclusion of canavanine instead of arginine [11]. The results with serum-free medium and with methylamine show that puromycyl peptides are degraded to a greater extent in the cytosol than normal proteins labelled for the same length of time. The results with methylamine and, to a lesser extent serum-free medium, indicate that, even when the half-lives of the proteins are similar as is the case with normal short-labelled and long-labelled puromycyl peptides, a greater proportion of the puromycyl peptides are degraded in the cytosol. The increase in the percentage degradation brought about by puromycin was not due to the protein synthesis inhibitory effects of puromycin which could have resulted in a decrease in the synthesis of potential substrate and an increase in the degradation rate of any substrate produced simply as a consequence of a decrease in the substrate:enzyme ratio. It seems therefore that the rapid degradation of the puromycyl peptides is due to their abnormal structure. This is borne out by the finding that in reticulocyte lysates, where substrate and enzyme concentrations can be controlled, puromycyl peptides of globin are degraded rapidly [4,5]. The different cellular locations of the degradation of puromycyl peptides and normal proteins, as well as canavanine-containing proteins and normal proteins [11], indicate there are differences in the proteolytic pathways of abnormal and normal proteins. However, in our studies we are only measuring the rate-limiting step of proteolysis and many steps could be common to both pathways. Differences in the steps of the degradation of amino acid analogue-containing proteins, and the activity of certain proteinases towards short peptides have been detected in the reticulocyte [4,5,8] and *E. coli* [2].

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