Both endocytic and endogenous protein degradation in fibroblasts is stimulated by serum/amino acid deprivation and inhibited by 3-methyladenine

Klavs B. HENDIL,*† Anne-Marie B. LAURIDSEN* and Per O. SEGLEN†

*August Krogh Institute, 13 Universitetsparken, DK 2100 Copenhagen O, Denmark, and †Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway

Incubation of BHK-21 hamster fibroblasts in a serum- and amino acid-deficient medium caused a 3-fold increase in the degradation of endogenous protein, a doubling of the degradation of endocytosed epidermal growth factor, and an eightfold increase in the degradation of endocytosed α_2 -macroglobulin. 3-Methyladenine (3MA) inhibited the deprivation-induced lysosomal degradation of both endogenous and endocytosed protein, but had no effect on basal (non-induced) degradation. 3MA also inhibited deprivation-induced protein degradation in human IMR-90 fibroblasts. Some inhibition of protein synthesis and of endocytic uptake of α_2 -macroglobulin was observed in 3MA-treated BHK-21 cells, whereas cellular ATP levels were unaffected. These results are different from those obtained with isolated hepatocytes, and suggest that in some cells both endogenous and endocytic protein degradation may be accelerated as part of a general deprivation response.

INTRODUCTION

Most of the protein degradation in growing cells takes place in the cytoplasm, whereas a lysosomal pathway is responsible for the enhanced intracellular proteolysis in growth-inhibited cells (Mortimore & Pösö, 1987; Rechsteiner, 1987; Seglen, 1987). An inhibitor of the lysosomal pathway would therefore be of considerable pharmacological interest for the potential control of tissue-wasting disorders.

In rat hepatocytes 3-methyladenine (3MA) inhibits the autophagic sequestration of cell proteins (Seglen & Gordon, 1982; Seglen *et al.*, 1986b, 1989), i.e. the first step in the lysosomal pathway of protein degradation, while having no effect on the non-lysosomal breakdown of endogenous proteins. The species and tissue specificity of 3MA is little known. In the present work we have therefore investigated the effects of the inhibitor on two fibroblastic cell lines, hamster BHK-21 and human IMR-90 cells.

MATERIALS AND METHODS

Materials

3MA was purchased from Fluka A.G., Buchs, Switzerland, and Sigma, St. Louis, MO, U.S.A.; BSA (fraction V) and soya-bean trypsin inhibitor were from Boehringer, Mannheim, Germany; tryptose phosphate broth (TPB) was from Difco, Detroit, MI, U.S.A.; growth medium and serum for cell culture were from Gibco, Paisley, Scotland, U.K. Unless otherwise stated, other biochemicals were purchased from Sigma. Isotopically labelled compounds were produced by Amersham International (Amersham, Bucks, U.K.). Epidermal growth factor (EGF, murine; receptor grade, from Sigma) was labelled with ^{125}I (Vlodavski *et al.*, 1978) to 26 TBq/mmol. α_2 -Macroglobulin ($\alpha_2\text{M}$) was purified from fresh human plasma (Sottrup-Jensen *et al.*, 1983). ^{125}I - $\alpha_2\text{M}$ (10 mg) was prepared by the chloramine-T method (Hunter & Greenwood, 1962), desalted by gel filtration and then allowed to react for 5 min at room temperature with 0.5 mg of trypsin before the surplus of trypsin

was inactivated with 1 mg of soya-bean trypsin inhibitor. The specific radioactivity was 0.58 TBq/mmol of $\alpha_2\text{M}$. Human plasma was treated for 5 min with bovine trypsin and then with soya-bean trypsin inhibitor (2.5 mg of each/ml). Phosphate-buffered saline (PBS) contained 137 mM-NaCl, 2.7 mM-KCl, 4.1 mM- Na_2HPO_4 , 0.73 mM- KH_2PO_4 , pH 7.4.

Methods

Cell culture. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 . BHK-21 hamster fibroblasts were grown in Eagle's minimal essential medium (MEM; Eagle, 1959) with 5% (v/v) newborn-calf serum and 5% (v/v) TPB, and IMR-90 human fibroblasts were grown in MEM with 5% newborn-calf serum and 5% foetal-calf serum. Cultures for experiments were inoculated with 4×10^4 cells/cm² the previous day. All experiments were performed at least three times with similar results.

Protein synthesis. Cultures in 9 cm² Petri dishes were incubated for 2 h in 1.5 ml of MEM with 4 mM-leucine but without other amino acids or in MEM with 4 mM-leucine, 5% calf serum and 5% TPB. The medium contained 3MA as indicated. For determination of protein synthesis, 10 μl of [^3H]leucine (37 MBq/ml) was added to each culture; 2 h later the cells were rinsed in 2×2.5 ml of PBS and dissolved in 1 ml of 0.1 M-NaOH/0.4% sodium deoxycholate. Total and trichloroacetic acid-soluble radioactivity in the cell lysate and growth medium was determined as described by Slot *et al.* (1986).

Degradation of cell protein. This was determined from the release into the medium of trichloroacetic acid-soluble radioactivity from cells that had been labelled by incubation for 18 h in medium with 0.2 mM- $^{3\text{H}}$ leucine (74 kBq/ml) (Slot *et al.*, 1986). It is calculated as a percentage of the initially incorporated radioactivity.

Abbreviations used: 3MA, 3-methyladenine (6-amino-3-methylpurine); $\alpha_2\text{M}$, α_2 -macroglobulin; EGF, epidermal growth factor; MEM, Eagle's minimal essential medium; TPB, tryptose phosphate broth; PBS, phosphate-buffered saline.

† To whom correspondence should be addressed.

Degradation of α_2 M and EGF. Cultures in 25 cm² dishes were briefly rinsed in PBS and incubated for 2 h in 3 ml of MEM with 0.5 % BSA and 20 μ g of ¹²⁵I- α_2 M-trypsin/ml or 5 ng of ¹²⁵I-EGF/ml. For experiments with BHK-21 cells the medium also contained 5 % TPB. The cultures were then washed in 4 × 5 ml of PBS with 0.1 mM-CaCl₂ and 1 % calf serum, and incubated in 4 ml of fresh medium. The degradation of ¹²⁵I-labelled protein was then measured as described for endogenous cell protein.

Uptake of α_2 M. Cell cultures (25 cm²) were rinsed once in PBS and incubated in 4 ml of Eagle's medium with 4 mM-leucine, 5 % TPB, 0.5 % BSA and 3 % trypsin-treated human plasma. At intervals, duplicate cultures were rinsed in 3 × 5 ml of PBS with 1 mM-CaCl₂ and 1 % bovine serum, then once in PBS. The cells were then scraped into 2 ml of buffer (PBS with 5 mM-EDTA and 0.05 % Tween 20) with a rubber spatula and frozen. The cell suspension was rapidly thawed and 200 μ l was taken for protein analysis. The remaining cell suspension was mixed with 100 μ l of calf serum, sonicated for 10 s, diluted in PBS, and analysed for α_2 M by an enzyme-linked immunosorbent assay employing rabbit antibody (Dakopatts, Copenhagen, Denmark) and peroxidase-conjugated monoclonal antibody (Slot & Hendil, 1987).

Other methods. Protein was determined by a modified Lowry procedure upon co-precipitation with sodium deoxycholate/trichloroacetic acid (Peterson, 1983), with BSA as a standard. ATP was measured in neutralized HClO₄ extracts by a luciferin/luciferase method (Rasmussen & Nielsen, 1968). Radioactivity was determined by liquid-scintillation counting. Differences in counting efficiencies between samples were determined by internal standardization (³H) or with an external standard (¹²⁵I).

RESULTS

Deprivation of amino acids and serum more than doubled the degradation rate of intracellular proteins in cultured fibroblasts, as shown in Fig. 1. 3MA, an inhibitor of autophagy (Seglen & Gordon, 1982), had no effect on the basal protein degradation in complete medium, but the deprivation-induced degradation was decreased by 75–85 % in BHK-21 cells (Fig. 1a) and IMR-90 human fibroblasts (Fig. 1b). Chloroquine, a lysosome inhibitor (Wibo & Poole, 1974), which did not inhibit the basal protein degradation, had only a small effect in addition to that of 3MA under deprivation conditions (Fig. 1).

In order to investigate whether the uptake and degradation of endocytosed protein could be affected by 3MA, we followed the cellular uptake of α_2 M into BHK-21 fibroblasts. The cells were incubated in the presence of human plasma that had been treated with trypsin to convert the α_2 M into proteinase complexes, since the latter are rapidly taken up by receptor-mediated endocytosis (van Leuven *et al.*, 1978; Ney *et al.*, 1985). As shown in Fig. 2, a time-dependent accumulation of α_2 M could readily be demonstrated, the accumulation being inhibited markedly by 3MA. The uptake of α_2 M was strongly temperature-sensitive, being decreased by more than 95 % at 4 °C.

The concentration of α_2 M in the medium was practically constant (0.1 mg/ml) during the incubations, so the cellular uptake is also expected to be constant with time. The degradation of α_2 M in lysosomes follows first-order kinetics (van Leuven *et al.*, 1978), so the change in the amount L of intracellular α_2 M with time t is expected to be

$$dL/dt = k_e - k_d L \quad (1)$$

Fig. 1. Inhibition of endogenous protein degradation by 3MA

(a) BHK-21 cells; (b) IMR-90 cells. The degradation of labelled protein was measured in growth medium with 4 mM unlabelled leucine but without other amino acids and without TPB and serum (Δ, ▲, ■) or with (○, ●) amino acids, 10 % serum and (in a) 10 % TPB. In some of the cultures the medium contained 10 mM-3MA (▲, ●) or both 10 mM-3MA and 0.1 mM-chloroquine (■). The values are means from two cultures that did not deviate by more than 3 %.

Fig. 2. Effect of 3MA on endocytosis of trypsin- α_2 M complexes

The BHK 21 cells were incubated in medium without (○) or with 10 mM-3MA (●). The values are means from two cultures. The curves were fitted to eqn. (2) (see the text), with $k_e = 1.466 \pm 0.053$ μ g/h per mg of cell protein (mean $\pm$ S.D.), $k_d = 0.270 \pm 0.016$ h⁻¹, $r^2 = 0.999$ in the absence of 3MA, and $k_e = 1.011 \pm 0.081$ μ g/h per mg of cell protein, $k_d = 0.253 \pm 0.035$ h⁻¹, $r^2 = 0.994$ in 10 mM-3MA.

where k_e is the rate of endocytosis and k_d is the first-order rate constant for degradation of α_2 M.

Since $L = 0$ at $t = 0$,

$$L = \frac{k_e}{k_d} (1 - e^{-k_d t}) \quad (2)$$

Fig. 3. Effects of 3MA on the degradation of ^{125}I - $\alpha_2\text{M}$ (a), EGF (b) and endogenous cell protein (c), and on protein synthesis (d)

BHK-21 cells were incubated in growth medium with serum amino acids and TPB (○), or in medium with leucine but without other amino acids, serum and TPB (●). The values are means from two cultures that did not deviate by more than 7%. (a) Degradation of $\alpha_2\text{M}$ was measured as the increase in trichloroacetic acid-soluble radioactivity in cells and medium between 15 min and 2 h after an incubation with ^{125}I - $\alpha_2\text{M}$ and expressed as first-order rate constants (%/h). The release of trichloroacetic acid-insoluble ^{125}I into the growth medium during the 2 h period was 4% in complete medium and 14% in medium without serum, amino acids and TPB. (b) Degradation of EGF was measured as described for $\alpha_2\text{M}$. The release of acid-insoluble ^{125}I during the 2 h incubation was 6% in complete medium and 12% in incomplete medium. (c) Protein degradation was measured as the increase in acid-soluble radioactivity in medium and cells from 15 min to 6 h after an 18 h labelling period with ^3H leucine, and expressed as %/h. (d) The cells were preincubated for 2 h with 3MA before protein synthesis was determined as the incorporation of ^3H leucine.

A fit of the data in Fig. 2 to eqn. (2) suggested that 3MA inhibited receptor-mediated endocytosis by $32 \pm 4\%$ (mean $\pm$ S.E.M. of three experiments).

Since $\alpha_2\text{M}$ degradation in these experiments could be estimated only indirectly, and by a relatively insensitive method (change in immunoreactivity), we made more direct degradation measurements by recording the release of acid-soluble radioactivity from endocytosed $\alpha_2\text{M}$. After a 2 h uptake period, $\alpha_2\text{M}$ was degraded at a rate of 5.4 %/h (first-order rate constant; $t_{1/2} = 13$ h) by BHK-21 cells under basal conditions, i.e. in a complete medium. In a medium deficient of amino acids, serum and TPB, $\alpha_2\text{M}$ degradation was accelerated to 42 %/h ($t_{1/2} = 1.7$ h). The degradation of EGF, which is also taken up by receptor-mediated endocytosis (Carpenter & Cohen, 1976), was increased from 22 %/h ($t_{1/2} = 3.2$ h) to 49 %/h ($t_{1/2} = 1.4$ h) when BHK-21 cells were incubated in a medium without amino acids, serum and TPB rather than in complete medium. 3MA had no effect on the basal degradation of endogenous or endocytosed proteins, but it inhibited the deprivation-induced degradation of endogenous protein, $\alpha_2\text{M}$ and EGF (Fig. 3).

In similar experiments with IMR-90 human fibroblasts the rate of degradation of ^{125}I - $\alpha_2\text{M}$ was 27 %/h ($t_{1/2} = 2.6$ h) in cells in complete medium and 110 %/h ($t_{1/2} = 0.6$ h) in a medium without amino acids and serum. 3MA had no effect on protein degradation in IMR-90 cells incubated in complete medium, but the deprivation-induced degradation of ^{125}I - $\alpha_2\text{M}$ was inhibited by 50% by 15 mM-3MA.

Protein synthesis in BHK-21 cells was also inhibited by 3MA, but considerably less than were the degradation processes

Fig. 4. Effect of 3MA on cellular ATP

BHK-21 cells were incubated for 3 h in complete medium (a) or in medium without serum, amino acids and TPB (b): □, no 3MA; ▨, +10 mM-3MA. Values represent means $\pm$ S.E.M. from four cultures.

(Fig. 3c). In these experiments the size of the intracellular pool of ^3H leucine did not change with culture conditions or concentration of 3MA. The measurement of protein synthesis was therefore not influenced by any differences in rates of amino acid transport.

The inhibitory effects of 3MA seem not to reflect general cytotoxicity. ATP levels were significantly decreased by incubation of cells under deprivation conditions, but hardly by 3MA (Fig. 4).

DISCUSSION

In hepatocytes, degradation of endocytosed asialofetuin is

unaffected by amino acid deprivation under conditions where degradation of endogenous protein is enhanced 3-fold (Seglen *et al.*, 1980, 1981; Grinde & Seglen, 1981). The increase in degradation of endocytosed α_2 M and EGF is therefore somewhat surprising. The results suggest that the deprivation response may encompass different assemblies of catabolic pathways in different cell types. In rabbit macrophages, amino acid deprivation stimulated the degradation of albumin taken up by endocytosis, while having no effect on endogenous protein degradation (Besterman *et al.*, 1983). In a mouse fibroblast line, the degradation of endocytosed α_2 M was slightly inhibited by insulin (Ney *et al.*, 1984). Insulin can at least partially substitute for serum in suppressing the deprivation response in IMR-90 fibroblasts (Auteri *et al.*, 1983).

The cellular contents of lysosomal proteinases increase during deprivation for serum and amino acids, but too slowly (Kaplan, 1978) to account for the enhancement of degradation seen here. Instead, the serum and amino acid deprivation may promote utilization of the proteolytic potential of lysosomes, for instance by stimulating fusions of lysosomes with pre-lysosomal vesicles that contain endocytosed protein. In macrophages, the point of transfer of pinocytosed material from a recycling to a non-recycling compartment appears to be subject to regulation by protein kinase C, since the transfer is stimulated by phorbol ester (Swanson *et al.*, 1985). It should furthermore be noted that endosomes can fuse with autophagosomes before their fusion with lysosomes (Gordon & Seglen, 1988); if this autophagy-dependent endocytic pathway predominates in fibroblasts, it may well account for the correlated control of autophagic and endocytic protein degradation observed in the present study. Functionally, an accelerated utilization of exogenous protein to compensate for the lack of amino acids would make sense. It would be interesting to learn if deprivation induction of endocytic protein degradation in BHK-21 and IMR-90 cells is a general phenomenon, or is restricted to a few protein categories such as EGF and α_2 M. The species or tissue specificity of induced degradation of endocytosed protein should also be investigated. 3MA inhibited the degradation of endocytosed α_2 M and EGF in nutrient-deprived BHK-21 fibroblasts. In rat hepatocytes Seglen's group found that lysosomal degradation of endocytosed asialofetuin, whether preloaded or continuously taken up, is totally unaffected by 3MA (Gordon & Seglen, 1982; Seglen & Gordon, 1982; Schwarze *et al.*, 1985). In contrast, Winkler & Segal (1984) reported a significant inhibition by 3MA in similar experiments. However, the rate of asialofetuin degradation found in their study was less than 10% of the rate observed in Seglen's experiments, complicating any comparison. In both BHK-21 and IMR-90 cells the deprivation-induced degradation of endogenous protein was inhibited by about 80% by 3MA. In rat hepatocytes, 3MA behaves as a relatively specific inhibitor of autophagy. It inhibits the initial sequestration step virtually completely (Seglen *et al.*, 1986a, 1989), thus effectively shutting down the whole autophagic-lysosomal degradation pathway (Seglen *et al.*, 1986b).

Practically nothing is known about the mechanisms of 3MA action. Although inhibition of glycolysis and a slight increase in hepatocytic cyclic AMP levels have been reported, these changes appear to be unrelated to inhibition of autophagy (Caro *et al.*, 1988). Some general toxicity has been observed at high 3MA concentrations in human lymphocytes (Seglen *et al.*, 1984) and HeLa cells (Knowles *et al.*, 1989), but the intracellular ATP level is maintained in both rat hepatocytes (Seglen & Gordon, 1982) and BHK-21 cells. The inhibition of protein synthesis seen in BHK-21 cells was not observed in hepatocytes (Seglen & Gordon, 1982), although very many purine derivatives tend to be protein-synthesis inhibitors, independently of their effect on protein

degradation (Gordon & Seglen, 1982). It is noteworthy that the high specificity of 3MA for inhibition of protein degradation versus protein synthesis observed in hepatocytes cannot automatically be extended to other cell types. 3MA appears to be a very effective autophagy inhibitor in all vertebrate cell types examined (Seglen & Gordon, 1982; Seglen *et al.*, 1984; Zeman *et al.*, 1985; Wildenthal & Wakeland, 1985; Ballard, 1985; Chandler & Ballard, 1988; Punnonen & Reunanen, 1988; Knowles *et al.*, 1989), but it may have various additional effects, depending on cell type. Thus, although 3MA resistance can, with some confidence, be equated with autophagy independence, 3MA sensitivity does not unequivocally imply that autophagy is involved. This is an argument which applies to all inhibitor studies, since absolute specificity cannot be proved, and indeed is unlikely to occur.

We thank Dr. Poul Bennekou and Dr. Palle Christophersen for help with the curve fitting and with ATP analysis, respectively. This work has been supported by the Norwegian Council for Science and Humanities.

REFERENCES

- Auteri, J. S., Okada, A., Bochaki, V. & Dice, J. F. (1983) *J. Cell. Physiol.* **115**, 167–174
- Ballard, F. J. (1985) *Exp. Cell Res.* **157**, 172–180
- Besterman, J. M., Airhart, J. A., Low, R. B. & Rannels, D. E. (1983) *J. Cell Biol.* **96**, 1586–1591
- Caro, L. H. P., Plomp, P. J. A. M., Wolvetang, E. J., Kerkhof, C. & Meijer, A. J. (1988) *Eur. J. Biochem.* **175**, 325–329
- Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171
- Chandler, C. S. & Ballard, F. J. (1988) *Biochem. J.* **251**, 749–755
- Eagle, H. (1959) *Science* **130**, 432–437
- Gordon, P. B. & Seglen, P. O. (1982) *Arch. Biochem. Biophys.* **217**, 282–294
- Gordon, P. B. & Seglen, P. O. (1988) *Biochem. Biophys. Res. Commun.* **151**, 40–47
- Grinde, B. & Seglen, P. O. (1981) *Biochim. Biophys. Acta* **676**, 43–50
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
- Kaplan, J. (1978) *Arch. Biochem. Biophys.* **187**, 376–386
- Knowles, S. E., Hopgood, M. F. & Ballard, F. J. (1989) *Biochem. J.* **259**, 27–33
- Mortimore, G. E. & Pösö, A. R. (1987) *Annu. Rev. Nutr.* **7**, 539–564
- Ney, K. A., Gidwitz, S. & Pizzo, S. V. (1984) *Biochemistry* **23**, 3395–3403
- Ney, K. A., Gidwitz, S. & Pizzo, S. V. (1985) *Biochemistry* **24**, 4586–4592
- Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95–119
- Punnonen, E. L. & Reunanen, H. (1988) *Conf. Ser. Inst. Phys. No. 93* **3**, 223–224
- Rasmussen, H. & Nielsen, R. (1968) *Acta Chem. Scand.* **22**, 1745–1756
- Rechsteiner, M. (1987) *Annu. Rev. Cell Biol.* **3**, 1–30
- Schwarze, P. E., Tolleshaug, H. & Seglen, P. O. (1985) *Carcinogenesis* **6**, 777–782
- Seglen, P. O. (1987) in *Proteolysis and Lysosomal Function* (Glaumann, H. & Ballard, F. J., eds.), pp. 369–414, Academic Press, London
- Seglen, P. O. & Gordon, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1889–1892
- Seglen, P. O., Gordon, P. B. & Poli, A. (1980) *Biochim. Biophys. Acta* **630**, 103–118
- Seglen, P. O., Gordon, P. B., Grinde, B., Solheim, A. E., Kovács, A. L. & Poli, A. (1981) *Acta Biol. Med. Germ.* **40**, 1587–1598
- Seglen, P. O., Munthe-Kaas, A. C. & Dybedal, M.-A. S. (1984) *Exp. Cell Res.* **155**, 121–128
- Seglen, P. O., Gordon, P. B., Tolleshaug, H. & Høyvik, H. (1986a) *Exp. Cell Res.* **162**, 273–277
- Seglen, P. O., Gordon, P. B. & Høyvik, H. (1986b) *Biomed. Biochim. Acta* **45**, 1647–1656
- Seglen, P. O., Gordon, P. B. & Høyvik, H. (1989) in *Intracellular Proteolysis: Mechanisms and Regulations* (Katunuma, N. & Komimami, E., eds.), pp. 419–426, Japan Scientific Societies Press, Tokyo
- Slot, L. A. & Hendil, K. B. (1987) *Scand. J. Clin. Lab. Invest.* **47**, 393–397

- Slot, L. A., Lauridsen, A.-M. B. & Hendil, K. B. (1986) *Biochem. J.* **237**, 491–498
- Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Lønblad, P. B., Kristensen, T., Mortensen, S. B., Petersen, T. E. & Magnusson, S. (1983) *Ann. N.Y. Acad. Sci.* **421**, 41–60
- Swanson, J. A., Yirinec, B. D. & Silverstein, S. C. (1985) *J. Cell Biol.* **100**, 851–859
- van Leuven, F., Cassiman, J. J. & van den Berghe, H. (1978) *Exp. Cell Res.* **117**, 273–282
- Vlodavski, I., Brown, K. D. & Gospodarowicz, D. (1978) *J. Biol. Chem.* **253**, 3744–3750
- Wibo, M. & Poole, B. (1974) *J. Cell Biol.* **63**, 430–440
- Wildenthal, K. & Wakeland, J. R. (1985) in *Intracellular Protein Catabolism* (Khairallah, E. A., Bond, J. S. & Bird, J. W. C., eds.), pp. 511–520, Alan R. Liss, New York
- Winkler, J. R. & Segal, H. J. (1984) *J. Biol. Chem.* **259**, 1958–1962
- Zeman, R. J., Kameyama, Y., Matsumoto, K., Bernstein, P. & Etlinger, J. D. (1985) *J. Biol. Chem.* **260**, 13619–13624

Received 15 March 1990/27 July 1990; accepted 13 August 1990