Changes in the proteolytic activities of proteasomes and lysosomes in human fibroblasts produced by serum withdrawal, amino-acid deprivation and confluent conditions

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INTRODUCTION

In both prokaryotic and eukaryotic cells, proteins are in a dynamic equilibrium in which degradation is potentially as important as protein synthesis in the control of protein mass. There are various proteases involved in the intracellular degradation of proteins, but proteasomes [1] and lysosomes [2] represent the main proteolytic pathways in mammalian and in other eukaryotic cells. Previous studies investigated the relative importance of lysosomal and non-lysosomal pathways of intracellular protein degradation in different cell types simply by inhibiting lysosomal degradation with weak bases (NH₄Cl, chloroquine, etc.) and/or inhibitors of lysosomal cathepsins [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane cell-permeant derivatives, leupeptin, etc.; see e.g. [3–5] for reviews], and it was assumed that the portion of intracellular proteins whose degradation remained resistant to these inhibitors was degraded by non-lysosomal pathways. On the basis of these studies, it was generally believed that in most mammalian cells growing under optimal nutrient conditions degradation of intracellular proteins occurred mainly by non-lysosomal proteolysis, whereas lysosomal degradation became important only in the enhanced degradation of intracellular proteins produced by starvation or deprivation of certain hormones and nutrients.

More recently, a substantial body of experiments firmly established the ubiquitin–proteasome system as the major non-lysosomal pathway for the degradation of intracellular proteins in eukaryotic cells (see [6] for a recent review). In this pathway, proteins are targeted for destruction by the assembly, on surface-exposed lysine residues, of a polyubiquitin degradation signal that is subsequently recognized by one or more subunits of the 19 S regulatory complex within the 26 S proteasome [7]. In addition, proteasomes, which exist in several different molecular forms, can degrade proteins by ubiquitin-independent mechanisms [8,9]. Although proteasomes have also been reported to function in the degradation of most of the long-lived proteins [10,11], many of the studies on these proteolytic complexes have focused on their regulated degradation of misfolded, abnormal and specific short-lived proteins [6,12,13].

Another well-established pathway of intracellular protein degradation involves segregating structures which sequester large areas of cytoplasm, typically including whole organelles, to form autophagic vacuoles which fuse with lysosomes or late endosomes to degrade its content [14,15]. This lysosomal process, called (classical) autophagy or macroautophagy, has been studied extensively in the last few years, mainly due to the availability of yeast mutants [16,17]. But, in addition to macroautophagy, it has become evident that lysosomes can also participate in intracellular protein degradation by other mechanisms, including endocytosis, cristophagy, microautophagy and chaperone-mediated autophagy (see e.g. [18,19] for reviews). Finally, other non-lysosomal and non-proteasomal proteolytic pathways operate in the cells.
including soluble proteases such as calpains [20] and proteases associated with particular organelles (e.g. mitochondria [21]). However, compared with macroautophagy, much less is known concerning the relative importance of these other lysosomal and non-lysosomal pathways in the overall degradation of intracellular proteins.

Examining the contribution of the different cellular proteolytic pathways to the degradation of the various intracellular proteins in intact cells should help to increase the knowledge about their various functions. The availability of pharmacological inhibitors of proteasomes [22], together with the use of the well-established macroautophagic inhibitor 3-MA (3-methyladenine) [23] in combination with other amply used lysosomal inhibitors [5], allows an analysis of the role of the different proteolytic pathways under various situations. In particular, it is now possible to investigate separately the contributions of macroautophagy, other lysosomal pathways, proteasomes and other non-lysosomal and non-proteasomal pathways to the degradation of intracellular proteins. In a previous study [24], we investigated the effect of some of these inhibitors on the degradation of short-lived proteins. However, the effects of the various inhibitors now available have not been systematically analysed either in that study or in any of the studies conducted so far. Such an analysis will be quite useful, since it may provide a guide for the rational use of inhibitors of the various proteolytic pathways when investigating their role in the breakdown of specific proteins or in other cellular processes in response to various physiological and pathological situations.

The objective of the present study has been to assess the relative importance of macroautophagy, other lysosomal pathways, proteasomes and other non-lysosomal and non-proteasomal pathways in the overall degradation of intracellular proteins in human fibroblasts under various growth conditions. Since long-lived proteins represent the vast majority of proteins in the cell, we analysed here the degradation of these proteins. Among other observations, we found that some still widely used proteasome inhibitors also inhibit lysosomal pathways with the same efficiency. Surprisingly, we also found that the proteasomal activity increases in exponentially growing cell cultures deprived of serum and/or amino acids, that confluent conditions decrease the activity of proteasomes by affecting the levels of various proteasomal subunits, and that lysosomal pathways different from macroautophagy are more important in protein degradation than is usually recognized, especially under confluent conditions.

**EXPERIMENTAL**

**Reagents**

Sources of chemicals were as described previously [24] with the following additions: the aldehyde Z-Leu-Leu-Leu-H (referred to as MG132 hereafter; Z represents benzoyloxycarbonyl) was obtained from Sigma (St. Louis, MO, U.S.A.). MG132 was also purchased from Affiniti (Technion-Israel Institute of Technology, Haifa, Israel) or purchased from Affiniti (clone FK2) respectively. The secondary antibodies were anti-mouse and anti-rabbit IgG–alkaline phosphatase from goat (Sigma). Other reagents were of the best analytical quality available.

**Cell culture and intracellular protein degradation of long-lived proteins**

Normal human skin fibroblasts were obtained and cultured as described previously [24]. Cell viability, checked by Trypan Blue exclusion, was always above 95% at the end of incubations. To label long-lived proteins, fibroblasts were incubated for 48 h in complete MEM (minimal essential medium; Gibco Invitrogen Corporation, Prat de Llobregat, Barcelona, Spain) containing 20% (v/v) FCS (foetal calf serum) and with 5 μCi/ml L-[3H]leucine or 5 μCi/ml L-[3H]valine. Before starting the proteolysis measurements, the cells were washed once with complete MEM, containing 2 mM L-leucine or 10 mM L-valine, and chased for 24 h at 37°C in this medium to degrade short-lived proteins. For prolonged starvation, the cells were washed and chased as above but in a medium without FCS. To measure protein degradation, all cultures were incubated in a fresh complete medium containing 2 mM L-leucine or 10 mM L-valine with or without [under serum withdrawal (SW) conditions] 20% FCS and the indicated additions. For experiments of amino-acid deprivation, labelling and washing of the cells were performed as above, but using only L-[3H]valine and 10 mM L-valine. To measure protein degradation under amino-acid deprivation, the cells were incubated in Krebs–Henseleit medium (118.4 mM NaCl/4.75 mM KCl/1.19 mM KH2PO4/2.54 mM MgSO4/2.44 mM CaCl2 · 2H2O/28.5 mM NaHCO3/10 mM glucose, pH 7.4) containing 10 mM L-valine with or without (under SW) 20% FCS, and the indicated additions. Preliminary experiments suggested the convenience of analysing protein degradation 1 h after the addition of the different inhibitors used in the present study and for a period of only 3 additional hours. This ensures optimal inhibition and should avoid possible secondary effects of the drugs, which may compromise cell viability.

Intracellular protein degradation was measured as the net release of trichloroacetic acid-soluble radioactivity from the labelled cells into culture medium, as described in [24]. Radioactivity released in the non-soluble trichloroacetic acid fraction of the medium was negligible (at all the experimental times it was always 0.3% h−1 of the initial non-soluble trichloroacetic acid radioactivity). Proteolytic rates were calculated by least-squares regression of semi-logarithmic plots.

**Electron microscopy and morphometric analysis**

Cells were washed with ice-cold PBS and fixed for 15 min in the dishes by direct addition of a mixture of 2% (v/v) glutaraldehyde and 1% formaldehyde (EM grade; Polysciences, Warrington, PA, U.S.A.) buffered with 0.05 M sodium cacodylate (pH 7.4). The cells were detached from the dishes with a rubber policeman and further fixed as above for 45 min. Then, the cells were washed three times for 30 min in 0.05 M sodium cacodylate buffer, post-fixed in a mixture of 1% osmium tetroxide (Polysciences) and 1% potassium ferrocyanide for 60 min, incubated for 1 min with...
0.15 % tannic acid in 0.1 M sodium cacodylate buffer and washed overnight in 0.1 M sodium cacodylate buffer. All these treatments were performed at 0–4 °C. The following day, the fixed cells were stained with 2 % (w/v) uranyl acetate for 2 h at 20 °C and dehydrated, embedded in Poly/Bed 812 resin (Polysciences) and polymerized according to the manufacturer’s instructions. Ultrathin sections of approx. 60–70 nm were cut with an LKB 4801 A ultramicrotome, mounted on copper grids, stained with Reynold’s lead citrate and examined in a Philips CM-10 electron microscope at 60 kV.

Morphometric analysis of lysosomes was performed in randomly selected electron micrographs (18 cm × 24 cm) at a final magnification of ×10 500 by standard methods [26]. For each measurement, the fractional volume of lysosomes was estimated by the point counting method in 30 electron micrographs from three different experiments. Lysosomes were identified using morphological criteria described previously [26].

Western blot

For immunoblot analysis with an alkaline phosphatase detection system, lysates of cells were prepared in RIPA buffer (150 mM NaCl/1 % Nonidet P40/0.5 % sodium deoxycholate/0.1 % SDS/50 mM Tris, pH 8.0) containing 0.1 mM leupeptin and 1 mM PMSF. Proteins (50 µg) from the various lysates were separated on 6 or 8 % (for experiments with anti-ubiquitin antibodies) and 15 % (for experiments with anti-proteasome antibodies) acrylamide slab gels and transferred to nitrocellulose membranes (electrophoresis grade, 0.45 µm pore size; Amersham Biosciences, Little Chalfont, Bucks., U.K.) for 16 h at 30 V, using a blotting-tank transfer cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The membranes were stained for 2 min with 0.2 % (w/v) Ponceau S (Sigma) solution in 3 % (w/v) trichloroacetic acid, destained with PBS for 5 min, blocked with 5 % (w/v) skimmed milk (in PBS) and reacted at 20 °C for 16 h with the various antibodies. Primary and secondary antibodies were applied in 3 % (w/v) BSA in PBS plus 0.02 % sodium azide. Secondary antibodies were incubated for 1 h at 20 °C. Between incubations, membranes were rinsed three times for 5 min with PBS. After the last wash, the blots were incubated for 10 min in alkaline phosphatase buffer and stained with Nitro Blue Tetrazolium and 5-bromo-5-chloro-3-indolyl phosphate p-toluidine salt (Sigma) by standard methods. The bands were quantified by densitometric analysis of the Western blots using either a 2202 Ultroscan laser densitometer from Pharmacia LKB (Uppsala, Sweden) with a Hewlett–Packard (Palo Alto, CA, U.S.A.) 3396 Series II integrator or SigmaGel software from Jandel Scientific (Ekreath, Germany). Both procedures gave similar results. The linearity of the method [27] was established for each antibody using 20 and 26 S proteasome preparations purified from rat liver [28] and recombinant PA28 [29].

Other general procedures were performed as described previously [30]. All experiments, except the morphometric analysis of lysosomes (see above), were performed at least four times with duplicate or triplicate samples. Statistical analysis was performed by the Student’s t test.

RESULTS

Analysis of various inhibitors of the proteasomal and lysosomal proteolytic pathways

The degradation rates of long-lived intracellular proteins, labelled as described in the Experimental section, were followed for 24 h in normal human fibroblasts under four different situations (exponential growth and confluent conditions, with or without serum). L-[3H]Leucine- or L-[3H]valine-labelled human fibroblasts lost protein radioactivity at a similar rate, indicating that leucine and valine residues are equally valid as labelling reagents to investigate the degradation of proteins in these cells and under the conditions analysed here (see the Experimental section). As shown in Figure 1, in confluent cultures in the presence of serum there is a slight, but significant, increase in the degradation rate of long-lived proteins as compared with exponentially growing cells. In exponentially growing cells and in confluent cultures, SW increased the proteolytic degradation rates by almost two times. Again, as in the presence of serum, degradation was also consistently higher in confluent cultures than in exponentially growing cells. In all cases, degradation of labelled proteins followed first-order exponential kinetics (not shown) with an apparent half-life (t1/2) of 130.8 ± 7.5 h (exponential), 117.5 ± 4.3 h (confluent), 63.0 ± 4.1 h (exponential/SW) and 56.8 ± 2.8 h (confluent/SW); all these values are means ± S.D. from five different experiments; differences from exponential half-life were significant at P < 0.05 or less, and differences between exponential/SW and confluent/SW half-lives were significant at P < 0.05.

Next, we tried to select specific inhibitors for the various degradation pathways of intracellular proteins. In eukaryotic cells, proteasomes and lysosomes represent the major proteolytic systems. Therefore we analysed the effects of widely employed inhibitors of both pathways on the degradation of long-lived proteins in exponentially growing cells under SW. We choose these conditions because degradation of proteins is enhanced (see Figure 1) and also because in many cells it is usually believed that lysosomes are not important for intracellular protein degradation under basal metabolic conditions [6]. Similar inhibitions were
G. Fuertes and others

Figure 2 Effects of various lysosomal and proteasomal inhibitors on the degradation of long-lived proteins in exponentially growing human fibroblasts subjected to SW for 4 h

(A) Inhibition of protein degradation by various concentrations of NH₄Cl (▼), NH₄Cl + 0.1 mM leupeptin (●) and 3-MA (▲). The inset shows the inhibition produced by various concentrations of leupeptin (▲) and leupeptin + 20 mM NH₄Cl (●). (B) Inhibition of protein degradation by various concentrations of MG132 (■), ALLN (▲), lactacystin (●) and ALLM (▼). The inset shows the inhibition produced by various concentrations of MG262 (●). (C) Inhibition of protein degradation by various combinations of inhibitors at the indicated concentrations (the ones on the right in each pair of histograms). The separate effects by each inhibitor of the combinations tested (as indicated below) and, thus, the expected additive values are also shown (the ones on the left in each pair of histograms). Inhibition is expressed as percentage, and each value is the mean ± S.D. from at least four separate experiments with duplicate samples.

obtained when using L-[³H]leucine or L-[³H]valine to label long-lived proteins. Figure 2 summarizes the more relevant observations obtained in this search.

Among various well-established inhibitors of lysosomes (bafilomycin A, concanamycin A, pepstatin A, leupeptin, chloroquine and NH₄Cl, and combinations thereof, assayed at various concentrations), we found that using NH₄Cl (20 mM) + leupeptin (0.1 mM) produced the highest inhibition of intracellular protein degradation (results not shown). Thus Figure 2(A) shows the inhibition produced by various concentrations of NH₄Cl and leupeptin (inset), each alone or in combination with a fixed concentration (0.1 mM leupeptin and 20 mM NH₄Cl respectively) of the other lysosomal inhibitor. It is clear that most of the observed combined inhibition is due to NH₄Cl, since the inhibition produced by this weak base alone (especially at 20 mM or higher concentrations) was only slightly below (< 10%) the inhibition produced by leupeptin + NH₄Cl (see also Figure 2C, the two histograms on the left). Thus most of the inhibition of protein degradation produced by leupeptin in human fibroblasts is also affected by NH₄Cl.

The main lysosomal pathway for intracellular protein degradation is macroautophagy and formation of autophagic vacuoles has been found to be completely inhibited by the drug 3-MA [23]. In human fibroblasts, 3-MA produced its maximal inhibitory effect on proteolysis at 10 mM (Figure 2A). Wortmannin, a well-known inhibitor of PI3K (phosphoinositide 3-kinase), had a similar effect at 100 nM or higher concentrations (results not shown), in good agreement with the conclusion that 3-MA affects macroautophagy in hepatocytes by inhibition of these enzymes [31]. In Figure 2(A), notice that, in serum-deprived exponentially growing cells, the inhibitory effect on proteolysis produced by 3-MA was high, but significantly (P < 0.05 or less) below the values obtained with leupeptin + NH₄Cl (or even NH₄Cl alone) when the three inhibitors were used at their optimal concentrations (see also Figure 2C, the second pair of histograms).

We also tested various agents which have been used by others to inhibit proteasomes in mammalian cells (Figure 2B). The most effective inhibitor was MG132, which, at 10 µM or higher concentrations, blocked 90% or more of the degradation of intracellular proteins in the absence of serum. This was observed with
MG132 obtained from two different sources (see the Experimental section). ALLN was also quite effective in inhibiting proteolysis when used at high concentrations. In contrast, the maximal inhibition with lactacystin was only approximately half of the inhibition obtained with MG132 and ALLN (Figure 2B). Results similar to those observed for lactacystin were obtained with clasto-lactacystin \( \beta \)-lactone and epoxomicin, another proteasome inhibitor (results not shown). We also performed experiments with MG262, a boronate derivative of MG132, which is considered more potent than MG132 against purified proteasomes [32]. As shown in Figure 2(B, inset), although MG262 produced its effect at very low concentrations, its maximal inhibition was slightly less than the maximal inhibition obtained with lactacystin.

Next, we analysed the effects produced by combinations of leupeptin + NH\(_4\)Cl with 3-MA, MG132, ALLN and lactacystin (Figure 2C). All these agents were used at their optimal concentrations to produce maximal inhibition (see Figures 2A and 2B). To facilitate comparisons, the expected additive values of the inhibitions, produced when used separately, are also indicated by a combined histogram on the left of the experimental values obtained for the various combinations of inhibitors. Thus (Figure 2C, the second pair of histograms), addition of 3-MA does not further increase the inhibition produced by NH\(_4\)Cl + leupeptin (51.8 versus 52.2\%). Therefore we assume that NH\(_4\)Cl + leupeptin fully inhibit macroautophagy and that the percentage of protein whose degradation is inhibited by them but not by 3-MA should represent the protein which is degraded by lysosomal pathways different from macroautophagy (i.e. microautophagy, chaperone-mediated autophagy, etc., see below). With proteasomal inhibitors, it is clear that, among MG132, ALLN and lactacystin, only the last (and also clasto-lactacystin \( \beta \)-lactone and epoxomicin; results not shown) produces a suitable additive effect with NH\(_4\)Cl + leupeptin (Figure 2C). The inhibitions produced by MG262 and NH\(_4\)Cl + leupeptin were also additive (Figure 2C, second to last pair of histograms). These results indicate that MG132 and ALLN, at least under the conditions investigated here, also inhibit lysosomal pathways quite efficiently. This strong inhibition of lysosomal pathways was also observed at lower concentrations of MG132 (10, 15 and 25 \( \mu \)M) and ALLN (25 \( \mu \)M), whereas, as expected, the inhibitory effects of lactacystin at lower concentrations were also additive with the inhibition produced by NH\(_4\)Cl and leupeptin (results not shown). When MG132 and ALLN were combined with lactacystin, NH\(_4\)Cl + leupeptin and/or 3-MA, it could be calculated (by comparing their effects with those produced by leupeptin plus NH\(_4\)Cl, 3-MA and/or lactacystin) that MG132 or ALLN, inhibited 95–100\% of both lysosomal (by macroautophagy or by other lysosomal pathways) and proteasomal degradation of long-lived proteins. We also tested, in similar experiments, the effect of ALLM at 50 \( \mu \)M, because it is also known to inhibit proteasomes [33]. However, it should be noted that, at 50 \( \mu \)M, ALLM produces only approx. 60\% of the maximal inhibitory effect, which is obtained at 200–300 \( \mu \)M (Figure 2B). Although there is some additive effect of ALLM with leupeptin + NH\(_4\)Cl (Figure 2C, last pair of histograms), ALLM at this concentration also inhibits approx. 77.2\% of the lysosomal degradation, but inhibits only 21.0\% of the proteasomal degradation. However, the inhibition of the proteasomal degradation increases at higher ALLM concentrations (e.g. it is approx. 68.9\% at 100 \( \mu \)M). Thus, perhaps it may be possible to discriminate among the contributions of the various proteolytic pathways to the degradation of intracellular proteins when using various concentrations of this inhibitor.

At any rate and in conclusion, in the following experiments, we used leupeptin (0.1 mM) + NH\(_4\)Cl (20 mM) to inhibit all lysosomal pathways, 3-MA (10 mM) to inhibit macroautophagic degradation and lactacystin (20 \( \mu \)M) to inhibit proteasomal pathways of protein degradation. The contribution of the non-macroautophagic lysosomal pathways was calculated by subtracting the inhibition obtained with 3-MA from the inhibition obtained with NH\(_4\)Cl + leupeptin. Finally, the contribution of non-lysosomal and non-proteasomal pathways to intracellular degradation was calculated from the degradation remaining after the combined inhibition with lactacystin and NH\(_4\)Cl + leupeptin or after treatment with MG132 (50 \( \mu \)M), which at this concentration inhibits almost totally the lysosomal and proteasomal pathways of intracellular protein degradation.

**Contribution of the main proteolytic pathways to the degradation of long-lived proteins in human fibroblasts under various conditions**

In previous experiments, we established the precise conditions for exponential growth and confluence in human fibroblasts and for treatments with inhibitors without loss of cell viability (see the Experimental section). Next, the effects of the various proteolytic inhibitors on the degradation of long-lived proteins were tested under these two conditions, with or without (0–4 h) serum. Since prolonged serum starvation in confluent cells is believed to activate the degradation of proteins by chaperone-mediated autophagy [19], we also analysed confluent cultures subjected to deprivation of serum for 24–28 h (prolonged starvation). As shown in Figure 3, clear differences were found in the effects of the different inhibitors under the various conditions. Thus in the presence of serum and under exponential conditions, 50–55\% of the long-lived proteins were degraded by proteasomes (Figure 3A). The remaining proteins were degraded by non-macroautophagic lysosomal (25–30\%) and other non-lysosomal (15–20\%) pathways. Under confluent conditions with serum, the main difference from exponential conditions was a moderate increase in the activity of the non-macroautophagic lysosomal pathways (from 28.1 ± 2.0 to 34.6 ± 3.3\%; differences significant at \( P < 0.05 \)), which fully explains the slightly higher degradation observed under these conditions (see Figure 1). The contribution of non-proteasomal and non-lysosomal pathways to the degradation of intracellular proteins remained nearly the same under exponential and confluent conditions with serum (18.4 and 17.8\% respectively). Under these two conditions, we could not detect any inhibition of the degradation of long-lived proteins by the macroautophagic inhibitor 3-MA. In fact, in the presence of serum, addition of 3-MA apparently increased the degradation rate (by approx. 1–5\%) of long-lived proteins, especially in exponentially growing cells. Although this increase in protein degradation may appear to be low, it should be pointed out that it was consistently observed.

Under SW (0–4 h), there is a large increase in macroautophagy in both exponentially growing and confluent cells, and approx. 40\% of the total degradation of long-lived proteins under these two conditions occurs by this pathway. Since, under SW, degradation of proteins increases by approx. 100\% (see Figure 1), the variations in activity of the various pathways under these conditions, when compared with cells incubated in the presence of serum, are more evident in Figure 3(B), which shows the percentage of protein degraded per hour by the various pathways. From these results, it can be calculated that macroautophagy is responsible for approx. 80\% of the SW-induced degradation. Owing to the large increase in macroautophagy, the contribution (in percentage) of proteasomes to the degradation of long-lived proteins under SW decreases moderately (Figure 3A). However, proteasomes degrade much more protein (approx. 1.7 times more in exponentially growing cells) under these conditions.
Figure 3 Contribution of various proteolytic pathways to the degradation of long-lived proteins in human fibroblasts under various conditions

The contribution of proteasomes, macroautophagy, other lysosomal pathways ('other lysosomal') and other non-lysosomal pathways different from proteasomes ('other pathways') under the indicated conditions (exponential, exponential + 4 h SW, confluent, confluent + 4 h SW and prolonged starvation) was calculated as described in the text, using lactacystin to inhibit proteasomes, 3-MA to inhibit macroautophagy and NH₄Cl + leupeptin to inhibit macroautophagy and other lysosomal pathways. The results are presented either as percentage of inhibition (A) or as percentage of the labelled protein whose degradation is inhibited per hour by the various inhibitors (B). Since MG132 inhibits both proteasomal and lysosomal pathways (see Figure 2), the contribution of other (non-lysosomal, non-proteasomal) pathways shown in (B) is also calculated from the degradation remaining after treatment with this inhibitor (MG132-insensitive). Each value is the mean from at least four separate experiments with duplicate samples. S.D. values are also shown in (B). Differences from exponential conditions were significant at *P < 0.05, **P < 0.005 and ***P < 0.0005.

(Figure 3B). In fact, in exponentially growing cells, it can be calculated that the increased degradation of long-lived proteins under SW, which is not due to macroautophagy (approx. 20%), is produced by the activity of proteasomes (Figure 3B). In contrast, in confluent cultures deprived of serum, the contribution (in percentage) of proteasomes to the degradation of long-lived proteins decreases to approx. 33% (Figure 3A) and the increase in the degradation by proteasomes is also less marked (Figure 3B). Finally, the degrading activity of other non-lysosomal and non-proteasomal pathways under SW (see Figure 3B) is either the same (as assessed by the degradation remaining after MG132 treatment, which inhibits both lysosomal and proteasomal pathways quite efficiently, as mentioned above), or it is inhibited under SW in exponentially growing cells but not in confluent cells (as assessed by the remaining degradation after treatment with lactacystin and NH₄Cl + leupeptin).

In confluent cells, prolonged starvation decreased the degradation of proteins by approx. 30% when compared with the first 4 h of SW (from 1.22 ± 0.06% h⁻¹ under confluent/SW to 0.80 ± 0.04% h⁻¹ under prolonged starvation; differences significant at P < 0.0005). This decrease is mainly due to decreased (from 39.4 ± 4.4 to 19.8 ± 2.1%; differences significant at P < 0.0005) macroautophagic degradation of proteins. The contribution of proteasomes to the degradation of proteins under prolonged starvation is little affected (it varies from 32.6 to 36.2%). Also, the small contribution of non-lysosomal and non-proteasomal pathways of protein degradation is not affected by prolonged starvation. In contrast, the relative importance of non-macroautophagic lysosomal pathways increases from 19.2 ± 3.0% during the first 4 h of SW to 35.6 ± 2.8% (differences significant at P < 0.0005) under prolonged starvation.

Changes in the levels of lysosomes and proteasomes in human fibroblasts under various growth conditions

To determine whether the observed variations in the contribution of lysosomes to the degradation of proteins could be correlated with lysosomal morphological changes, we examined the electron microscopic appearance of human fibroblasts under the various conditions investigated (Figure 4). A morphometric analysis of lysosomes was also made in the cells under each of the growth conditions investigated (Table 1). Lysosomes, which were not frequent in exponentially growing cells and confluent cells, increased in serum-deprived cells, and the increases in the lysosomal fractional
Degradation of proteins by proteasomes and lysosomes

Figure 4 Appearance of lysosomes in human fibroblasts subjected or not to serum starvation as seen by electron microscopy

Representative micrographs showing typical lysosomes in cells under the following conditions: exponential growth (A), confluent phase (B), exponential growth + 4 h SW (C) and confluent phase + 4 h SW (D). Scale bar, 0.2 µm.

Table 1 Effect of various growth conditions on the fractional volume of lysosomes in human fibroblasts

Results, which are pooled from three different experiments, are shown as means ± S.D., which are then converted into a percentage of the exponential growth values (within parentheses). These values are compared with the corresponding variations in the turnover rate $k_d$ of long-lived proteins (see Figure 1) and in the percentage of protein degradation which occurs by lysosomal pathways (see Figure 3). Differences from exponential conditions were significant at *$P < 0.05$ and **$P < 0.005$.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Lysosomal fractional volume (mm²/cm³ of cytoplasm)</th>
<th>$k_d$ (h⁻¹)</th>
<th>Lysosomal protein degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential</td>
<td>35.6 ± 2.7 (100)</td>
<td>0.0053 (100)</td>
<td>28.1 (100)</td>
</tr>
<tr>
<td>Exponential/SW</td>
<td>82.8 ± 8.4 (233)**</td>
<td>0.0110 (208)</td>
<td>52.2 (196)</td>
</tr>
<tr>
<td>Confluent</td>
<td>41.8 ± 3.4 (117)</td>
<td>0.0059 (111)</td>
<td>34.6 (123)</td>
</tr>
<tr>
<td>Confluent/SW</td>
<td>78.4 ± 6.6 (220)**</td>
<td>0.0122 (230)</td>
<td>58.6 (209)</td>
</tr>
<tr>
<td>Prolonged starvation</td>
<td>51.2 ± 4.5 (144)*</td>
<td>0.0080 (151)</td>
<td>55.4 (197)</td>
</tr>
</tbody>
</table>

The morphology of lysosomes was also different in the presence of serum (Figures 4A and 4B) and under SW (Figures 4C and 4D). In this latter case, lysosomes were larger and their morphologies correspond to typical autophagic vacuoles containing cellular material in various stages of degradation. The morphology of lysosomes confirms that, as already suggested by the effects of inhibitors, different lysosomal mechanisms of protein degradation occur in response to various conditions; i.e. macroautophagy operates only under SW, whereas lysosomal mechanisms different from macroautophagy are also operative in the presence of serum. Under prolonged starvation, the electron microscopic appearance of lysosomes (Figure 5) was similar to that of confluent cells deprived of serum for 4 h (see Figure 4D); however, autophagic vacuoles (Figure 5A) were less abundant and smaller (notice that the scale bars in Figures 4 and 5 correspond to 0.2 and 0.1 µm respectively) and lysosomes resembling residual bodies and containing abundant remnants of membranes were also frequent (Figure 5B).

To explain the observed variations in the activity of proteasomes (Figure 3), we also examined the levels of various subunits from different proteasome complexes using specific antibodies (see the Experimental section). It appears (Figure 6 and Table 2) that, under the various growth conditions investigated, there is no change in the intracellular levels of two $\alpha$-subunits (C8 and C9) of the 20 S proteasome. In contrast, we found that, under confluent conditions, with or without serum, the levels of two component subunits (p45 and S3) of the 19 S particle that forms the 26 S proteasome decreased slightly, whereas the levels of two subunits, one of the 11 S regulatory complex (PA28β) and the other of the immunoproteasome (LMP2), increased to a large extent (Table 2). Interestingly, the level of Y, the $\beta$-subunit of 20 S
proteasomes, which is interchangeable with LMP2, also decreased to the same extent as the two components of the 19 S particle (see Table 2). Under prolonged starvation, the levels of proteasome subunits do not apparently change when compared with the first 4 h of SW. Thus confluent conditions affect proteasomes in a way that resembles the effect of interferon-γ, which is known to increase the level of immunoproteasomes and PA28 proteasome complexes and to decrease the level of 26 S proteasomes (see [34] for a review). It appears therefore that, in confluent cells, some parts of the 26 S complexes present under exponential growth are substituted by PA28-immunoproteasomes (Table 2). This may explain the decreased activity (in percentage) of proteasomes in human fibroblasts under confluent conditions (see Figure 3A).

However, the increase in the degradation of proteins by proteasomes observed under SW, especially in exponentially growing cells (see Figure 3B), cannot be explained by changes in the levels of proteasomes or their complexes, which even decreased slightly (see Table 2). Therefore it is quite probably due to changes in the substrate proteins which make them more susceptible to degradation by the ubiquitin–proteasome system. In fact, immunoblot analysis with antibodies which recognize ubiquitinated proteins (Figure 7) show that, in the presence of MG132, which is amply used to reveal ubiquitinated proteins (see e.g. [33,35]), these proteins accumulate more in exponentially growing cells deprived of serum (Figure 7B, ‘Exp./SW’) than in exponentially growing cells incubated in the presence of serum (‘Exp.’).

**Contribution of the main proteolytic pathways to the degradation of long-lived proteins in human fibroblasts under amino acid starvation**

Amino acids are key regulators of proteolysis (see e.g. [36] for a review). To obtain information about the maximal capacity of
Degradation of proteins by proteasomes and lysosomes

Figure 7 Increased ubiquitination of proteins in exponentially growing human fibroblasts after serum withdrawal

Proteins (45 µg) from exponentially growing fibroblasts, incubated with serum (‘Exp.’; A, lanes 1 and 2, B, lane 1) or without serum (‘SW’; B, lane 2) for 4 h and treated (A, lane 2; B, lanes 1 and 2) or not (A, lane 1) with 50 µM MG-132 for the same incubation period, were extracted with RIPA buffer and then analysed in a Western blot developed with an antibody which recognizes ubiquitinated proteins. (A, B) show 6 and 8 % acrylamide gels respectively.

Figure 8 Contribution of various proteolytic pathways to the degradation of long-lived proteins in human fibroblasts under amino acid starvation

Experiments and calculations were performed as described in the legend to Figure 3, but in the absence of amino acids. Each value is the mean from four separate experiments with duplicate samples. S.D. values are also shown in (B). When compared with Figure 3(B), scale differences can be noticed in the ordinate values. Differences from exponential conditions were significant at *P < 0.05, **P < 0.005 and ***P < 0.0005.

Each of the proteolytic pathways under extreme conditions, MEM was replaced by Krebs–Henseleit salt medium without amino acids. Cell viability, checked by Trypan Blue exclusion, was above 95 % at the end of the 4 h incubations. During this period, degradation followed first-order exponential kinetics with turnover rates (k_d) of 0.0113 ± 0.0011 h⁻¹ (exponential), 0.0180 ± 0.0019 h⁻¹ (exponential/SW), 0.0091 ± 0.0003 h⁻¹ (confluent), 0.0139 ± 0.0005 h⁻¹ (confluent/SW) and 0.0102 ± 0.0008 h⁻¹ (prolonged starvation). All these values are means ± S.D. from four different experiments and, except for prolonged starvation, they are significantly different from exponential conditions at P < 0.05 or less. Notice that, compared with the k_d values obtained in the presence of amino acids (see Table 1), there is increased degradation, especially in exponentially growing cells.

Effects of the various proteolytic inhibitors on the degradation of long-lived proteins were then investigated (Figure 8). Under all conditions, and in contrast with experiments performed in the presence of amino acids (Figure 3), lysosomes (macroautophagy plus other lysosomal pathways) become more important than proteasomes in the degradation of proteins. Amino-acid deprivation mainly increases macroautophagy under all conditions. It also increases, to a minor extent, the activity of proteasomes (only in exponentially growing cells) and of other non-lysosomal and non-proteasomal pathways (only under confluent conditions), whereas, similarly to SW, it does not affect lysosomal pathways different from macroautophagy (cf. the amount of protein...
degraded per hour by the various pathways in Figures 3B and 8B). Moreover, most variations in the activity of the various proteolytic pathways produced by confluent conditions and SW in the absence of amino acids were qualitatively similar to those observed in the presence of amino acids. Thus proteasomes degrade more protein in exponentially growing cells under SW, whereas in confluent cells there is a decrease in their activity (from approx. 34–36% in exponentially growing cells to approx. 9–16% under confluent conditions), which is much more marked than in the presence of amino acids (cf. Figures 3A and 8A). Also, as in the presence of amino acids (Figure 3), the following significant changes in the activity of the various pathways, when compared with exponentially growing cells, were observed: (i) a large increase in macroautophagy under SW, (ii) a moderate increase in the activity of the non-macroautophagic lysosomal pathways under confluent conditions, (iii) a decrease in macroautophagy and an increase in non-macroautophagic lysosomal pathways under prolonged starvation, and (iv) an inhibition of other non-lysosomal and non-proteasomal pathways in exponentially growing cells under SW. Finally, as in the presence of amino acids, there were no significant differences in the MG132-insensitive proteolysis.

**DISCUSSION**

By employing specific inhibitors, we monitored the role of the main proteolytic pathways in the degradation of intracellular proteins in human fibroblasts under various growth conditions. With lysosomal inhibitors, we found that NH₄Cl + leupeptin is the most effective of all combinations tested in the present study. Much of this inhibition is due to ammonia, which has been shown to enter lysosomes, consuming protons and increasing the intralysosomal pH [37]. However, the peptide inhibitor leupeptin, which inhibits lysosomal cysteine proteases such as cathepsins B, H and L [37], further increases the inhibition produced by ammonia. Although some non-lysosomal proteases (e.g. calpains) are also inhibited by leupeptin under *in vitro* conditions, it has been shown that leupeptin, similarly to *trans*-epoxyoxycarbonyl-1-leucylamido-(4-guanidino)butane, does not readily permeate membranes, and thus its uptake into cells occurs by endocytosis [38]. Therefore, most probably, the additional effect of leupeptin, in the inhibition produced by NH₄Cl alone, is due to inhibition of the residual activity of lysosomal cysteine proteases at the near-neutral lysosomal pH produced by ammonia. In this regard, it should be noticed that the optimal pH of cysteine cathepsins (pH 3.5–6) is more close to neutral pH when compared with that of aspartic cathepsins, which is more acidic (pH 3–5).

To inhibit macroautophagy, we used 3-MA [23,39], which targets enzymes of the PI3K family [31,40]. Class III PI3K is probably engaged in the control of the formation of autophagic vacuoles [40], whereas the plasma-membrane-associated class I PI3K would be required to transduce a negative signal for the biogenesis of the autophagic vacuole [41]. Thus a possible explanation for the slight activation of proteolysis produced by 3-MA in the presence of FCS and amino acids is that it is responsible for half or less of this degradation. The remaining degradation occurs by lysosomal and non-lysosomal pathways. Particularly, and in agreement with results obtained by others in various cell types (see e.g. [3–5] for reviews), there is a notable increase in macroautophagic degradation of long-lived proteins under SW or amino acid deprivation. Less expected was the contribution of proteasomes to approx. 20% of the enhanced degradation of long-lived proteins in exponentially growing cells, which, at least in the absence of serum, is most probably due to activation of ubiquitin conjugation to proteins. It is usually believed that the enhanced degradation of proteins under SW or amino acid deprivation is non-selective, and since proteasomes are involved in selective protein turnover [6], it is possible that only a specific group of proteins is multi-ubiquitinated, but this remains to be investigated. In contrast, lysosomal pathways different from macroautophagy appear not to be activated by SW or amino acid starvation, whereas their contribution, but not that of macroautophagy, increases under confluent conditions and prolonged serum starvation. This suggests a different kind of regulation of macroautophagy and the pathway different from macroautophagy account for 30–40% of starvation-induced lysosomal protein degradation [42].

When we tested various proteasome inhibitors, we observed that MG132 and ALLN, two widely employed peptide aldehydes since they were introduced in 1994 [10], and which were quite efficient in inhibiting intracellular protein degradation, are not suited as specific inhibitors of proteasomes because they also inhibited lysosomal protein degradation to the same extent. In contrast, lactacystin [43], which was less effective than the other inhibitors in inhibiting protein degradation, was more adequate, since its effect was additive with that of NH₄Cl + leupeptin. The effects produced by epoxomicin [44] and clasto-lactacystin β-lactone [11] were indistinguishable from those observed with lactacystin. Therefore, when using the peptide aldehyde inhibitors (MG132, ALLN, etc.) to inhibit proteasomes because of their advantages (reversibility, inexpensiveness, etc.), it is advisable to demonstrate also similar effects with the more specific proteasomal inhibitors, as already recommended [22]. We also tested MG262, a drug belonging to the class of proteasome inhibitors which contain boronate and which includes PS-341 [32], a proteasome inhibitor used in multiple myeloma clinical trials [45]. MG262 was quite effective at very low concentrations and its inhibition was also additive with that of NH₄Cl + leupeptin. We could, however, not test the effects of PS-341 because it is not yet available commercially.

Lactacystin also interferes with lysosomal cathepsin A [46], but the protein-degradative role of this carboxypeptidase has been limited to hydrolysis of some regulatory peptides [47]. More recently, cathepsin A has been proposed to regulate chaperone-mediated autophagy by degrading lampla2a, the lysosomal receptor of this pathway [48]. However, due to the additive effects of lactacystin and lysosomal inhibitors, lactacystin does not appear to modify the lysosomal degradation of long-lived proteins under the conditions (i.e. 4 h SW) investigated here. Lactacystin at high concentrations is also known to inhibit the giant protease or tripeptidyl peptidase II [49]. Since we found that concentrations of lactacystin higher than 10 μM do not increase its inhibitory effect, and since epoxomicin [44], which is supposed to be more proteasome-specific than lactacystin, produces the same inhibition, it appears that this giant protease has here also a limited role in the overall degradation of long-lived proteins.

Changes in the growth conditions of cells involved alterations in the activities of the various proteolytic pathways. Proteasomes [6] certainly constitute an important proteolytic pathway for the degradation of long-lived proteins under all conditions; however, even in the presence of amino acids, they are responsible for half or less of this degradation. The remaining degradation occurs by lysosomal and non-lysosomal pathways. Particularly, and in agreement with results obtained by others in various cell types (see e.g. [3–5] for reviews), there is a notable increase in macroautophagic degradation of long-lived proteins under SW or amino acid deprivation. Less expected was the contribution of proteasomes to approx. 20% of the enhanced degradation of long-lived proteins in exponentially growing cells, which, at least in the absence of serum, is most probably due to activation of ubiquitin conjugation to proteins. It is usually believed that the enhanced degradation of proteins under SW or amino acid deprivation is non-selective, and since proteasomes are involved in selective protein turnover [6], it is possible that only a specific group of proteins is multi-ubiquitinated, but this remains to be investigated. In contrast, lysosomal pathways different from macroautophagy appear not to be activated by SW or amino acid starvation, whereas their contribution, but not that of macroautophagy, increases under confluent conditions and prolonged serum starvation. This suggests a different kind of regulation of macroautophagy and the
other lysosomal pathways of intracellular protein degradation. In this regard, the morphological features of lysosomes in cells grown in the presence or absence of serum are different. Pathways different from macroautophagy have been proposed (see e.g. [18,19] for reviews). The processes responsible for lysosomal degradation in the presence of serum are unknown, but they could include microautophagy, which at least in hepatocytes appears to be regulated by factors different from those controlling macroautophagy [50]. In confluent cells under prolonged starvation, it has been postulated that the chaperone-mediated autophagic pathway is activated [19]. The percentage of protein degradation, in the presence or absence of amino acids, decreases under prolonged starvation, and this is mainly due to a strong reduction in macroautophagy. In contrast, the contribution to the degradation of long-lived proteins by lysosomal pathways different from macroautophagy increases, probably because the lysosomal degradation machinery needs to be more specific in selecting substrates that do not compromise cell viability, and chaperone-mediated autophagy fulfils these requirements better than macroautophagy.

In confluent cells, the relative contribution of proteasomes to the degradation of proteins decreases, especially in amino acid-deprived cells. This decrease was also observed when analysing the degradation of short-lived proteins [24]. Although the levels of ubiquitinated proteins did not apparently change under conditions of amino acid deprivation cells. This decrease was also observed when analysing the degradation of proteins decreases, especially in amino acid-deprived cells. This decrease was also observed when analysing the degradation of proteins decreases, especially in amino acid-deprived cells. However, it is possible that some of the inhibitors used in the present study are unable to inhibit fully all the activities of their respectively affected pathways or, conversely, that some of these inhibitors also affect certain non-lysosomal and non-proteasomal pathways of protein degradation. Therefore calculation of the precise contribution of these pathways to the degradation of long-lived proteins would require the use of specific inhibitors of cytosolic (e.g. calpains) and organellar proteases [20,21].

In conclusion, our results demonstrate that: (i) certain inhibitors are more appropriate than others to investigate the participation of different proteolytic pathways in intracellular protein degradation; (ii) in human fibroblasts, lysosomes and proteasomes are equally responsible for a majority (80% or more) of the overall intracellular protein degradation; and (iii) the various proteolytic pathways are differently activated or inhibited under various growth conditions. Therefore these results should be useful to investigate the specific signals that control the activity of the various proteolytic pathways that degrade intracellular proteins.

This work was supported by the Ministerio de Ciencia y Tecnología (grant nos. BMC2001-0816 and SAF2002-00226), the Generalitat Valenciana (grant no. CTIDIB/2002/86) and the FIS (Fondo de Investigación Sanitaria) of the Instituto de Salud Carlos III, Red de Centros RDGM (G03/212), Madrid, Spain. We thank Rachael Z. Murray (University of Bristol, Bristol, U.K.) for helpful discussions and A. Montaner and D. Cerveró for technical assistance.

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