Blocking intracellular degradation of the erythropoietin and asialoglycoprotein receptors by calpain inhibitors does not result in the same increase in the levels of their membrane and secreted forms

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The erythropoietin receptor (EPO-R), a type 1 membrane glycoprotein, is degraded mainly in the lysosomes or endosomes, whereas the asialoglycoprotein receptor (ASGP-R) H2a subunit, a type 2 membrane glycoprotein, is degraded exclusively in the endoplasmic reticulum. The present study describes compounds that inhibit the intracellular degradation of these receptors in an efficient manner. However, the levels of cell-surface expression and secretion of their soluble exoplasmic domains were not enhanced to the same extent. The calpain inhibitors *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) and *N*-acetyl-leucyl-leucyl-methional (ALLM) inhibited EPO-R degradation profoundly. After 3 h of chase using Ba/F3 cells and NIH 3T3 fibroblasts expressing the EPO-R, virtually all of the receptor molecules were degraded, whereas 80% of the pulse-labelled receptor remained intact in the presence of the inhibitor. EPO-R cell-surface expression was elevated 1.5-fold after 1 h of incubation with ALLN. In the absence of protein synthesis, ALLN caused the accumulation of non-degraded EPO-R molecules in endosomes and lysosomes, as determined by double immunofluorescence labelling of NIH 3T3 cells expressing EPO-Rs. In

Ba/F3 cells expressing a soluble EPO-R, ALLN treatment increased secretion of the soluble exoplasmic domain of the EPO-R 2–5-fold. Similarly, in NIH 3T3 cells singly transfected with the ASGP-R H2a subunit cDNA, ALLN inhibited degradation of the ASGP-R H2a subunit precursor, as well as the degradation of the 35 kDa proteolytic fragment corresponding to the receptor ectodomain, by 3–6-fold. However, accumulation of secreted proteolytic fragment in the medium was augmented in the presence of ALLN by only 1.75-fold. In cells expressing the G78R mutant of the ASGP-R H2a subunit, which is not cleaved to the 35 kDa fragment [Yuk and Lodish (1993) *J. Cell Biol.* **123**, 1735–1749], degradation of the precursor was inhibited. Overall, our data suggest the involvement of cysteine proteinases located in the endoplasmic reticulum, as well as in post-Golgi compartments, in degradation of the EPO-R and the ASGP-R H2a subunit. The much lower effect of the inhibitory compounds on cell-surface and secreted forms of the EPO-R and ASGP-R H2a subunit illustrates the complexity and the tight regulation of the cellular localization and stability of membrane proteins.

INTRODUCTION

Intracellular protein degradation is an important, highly controlled, mechanism for regulating the levels of individual proteins in the cell. It takes place in various organelles, including lysosomes, autophagosomes and the endoplasmic reticulum (ER), and in the cytosol via the ubiquitin-mediated pathway and other cytosolic proteinases. Degradation of incorrectly folded or unassembled proteins in the ER has been demonstrated for several proteins, including the T cell receptor subunits [1,2] and the asialoglycoprotein receptor (ASGP-R) [3,4]. 3-Hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) undergoes regulated degradation in a pre-Golgi compartment [5,6]. Cysteine proteinases are found in the ER [7,8] and in lysosomes [9], and also in the cytosol (calpains) [10], where they are activated by increased intracellular calcium. Unlike lysosomal proteinases, which have a broad specificity, these cytosolic calcium-activated neutral proteinases catalyse the limited proteolysis of a restricted set of substrate proteins, including the epidermal growth factor receptor, protein kinase C, the platelet-derived growth factor receptor and cytoskeletal proteins, suggesting they may play a regulatory role in cellular physiology (reviewed in [11]).

The calpain inhibitors *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) and *N*-acetyl-leucyl-leucyl-methional (ALLM) are potent cell-permeable cysteine proteinase inhibitors that block degradation in the ER of HMG-CoA reductase [7] and of the unassembled T cell receptor subunits [2]. ALLN also inhibits the post-Golgi degradation of secretory IgM [12] and apolipoprotein E [13], and the proteasome-mediated degradation of cellular proteins [14]. The present study was designed to address the participation of ALLN-sensitive proteinases in the degradation pathway of two membrane-bound receptors: the erythropoietin receptor (EPO-R) and the ASGP-R H2a subunit.

The EPO-R, a type 1 membrane-spanning protein, is the cell-surface receptor for EPO, the hormone that regulates the viability, proliferation and differentiation of mammalian erythroid progenitor cells [15,16]. When the interleukin-3-dependent pro-B cell line Ba/F3 is transfected with EPO-R cDNA, the cells grow in the presence of EPO as well as interleukin-3 [17], rendering this system suitable for studying the EPO-R. In transfected Ba/F3 cells the EPO-R is synthesized as a 64 kDa endoglycosidase H (Endo H)-sensitive molecule with a single N-linked oligosaccharide. Within 1 h of synthesis approximately half of the molecules mature to a 66 kDa Golgi-processed form. This form

Abbreviations used: ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; ALLM, *N*-acetyl-leucyl-leucyl-methional; ASGP-R, asialoglycoprotein receptor; CHX, cycloheximide; Endo H, endoglycosidase H; EPO, erythropoietin; EPO-R, EPO receptor; ER, endoplasmic reticulum; FCS, fetal calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; s-EPO-R, soluble extracellular domain of the EPO-R; Tos-Lys-CH₂Cl, tosyl-Lys-chloromethane ('TLCK'); Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

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of the receptor is degraded rapidly with a half-life of about 1 h in lysosomes or other acidic intracellular vesicles [18,19]. In these transfected cells the majority of EPO-Rs are intracellular, with less than 2000 molecules on the cell surface. The low surface expression of the receptor is also characteristic of cells endogenously expressing the EPO-R (reviewed in [20]).

The human ASGP-R, a type 2 membrane-spanning protein, is a Ca^{2+} -dependent lectin which binds and removes by receptor-mediated endocytosis glycoproteins with carbohydrate side chains bearing terminal galactose residues [21,22]. The functional receptor is a hetero-oligomer consisting of two types of subunits, H1 and H2. There are two subtypes of H2: H2a and H2b [23]. In singly transfected cells, newly synthesized H2a is degraded in the ER [4,23–25], while about 30% of newly synthesized H2b matures through the Golgi. During degradation of H2a and H2b a 35 kDa fragment is formed in the ER by proteolytic cleavage in the exoplasmic domain near the transmembrane region [3,4,26]. A portion of the H2a fragment is then processed through the Golgi and secreted (G. Z. Lederkremer and H. F. Lodish, unpublished work). We showed that tosyl-lysylchloromethane (Tos-Lys- CH_2Cl ; 'TLCK') and tosylphenylalanylchloromethane (Tos-Phe- CH_2Cl ; 'TPCK') as well as iodoacetamide inhibit degradation of the ASGP-R H2a subunit, probably in a pathway that does not involve formation of the 35 kDa fragment [25]. Here we have employed H2a as a model system for a receptor subunit degraded in the ER, to determine the effect of ALLN, a cysteine proteinase inhibitor, on ASGP-R H2a subunit degradation.

We show that calpain inhibitor I (ALLN) inhibits mainly lysosomal degradation of wild-type EPO-R, increases by 50% the amount of surface EPO-R and enhances 2–5-fold the secretion of the soluble exoplasmic domain of the EPO-R. In the case of the ASGP-R H2a subunit, ALLN inhibited its ER degradation, thereby stabilizing both the intact precursor and the 35 kDa proteolytic fragment by 3–6-fold. Accumulation of the 35 kDa fragment resulted in a 75% increase in its secretion.

EXPERIMENTAL

Materials

Materials were obtained from sources previously listed [19]. In addition, we obtained ALLN and ALLM from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Purified recombinant human EPO was kindly donated by Kirin Inc., Tokyo, Japan.

Antibodies

Rabbit antibodies directed against the N- and C-terminal peptides of the EPO-R [18] were used for immunoprecipitating the EPO-R, as indicated. For immunofluorescence, these antibodies were affinity purified on a column containing the immunizing peptide. A rat monoclonal antibody (IG11) against the lysosomal membrane glycoprotein Lamp 1 [27] (kindly provided by Dr. H. Rosen, Merck Inc., Rahway, NJ, U.S.A.) was used as a specific stain for the endocytic pathway (endosomes and lysosomes) in NIH 3T3 cells. Secondary antibodies (goat antibodies conjugated to fluorescein isothiocyanate or Cy3-conjugated donkey anti-rabbit) were from Jackson ImmunoResearch Laboratories Inc. (Avondale, PA, U.S.A.). Anti-(ASGP-R H2 C-terminal peptide) antibodies were as described in Amara et al. [3].

Cells

Murine interleukin-3-dependent Ba/F3 cells, transfected with

the EPO-R cDNA in pXM [15] using the adenovirus major late promoter, were maintained in RPMI supplemented with 10% (v/v) fetal calf serum (FCS) and 0.25 unit/ml EPO. A cell line expressing the exoplasmic domain of the EPO-R was obtained by inserting a stop codon in the EPO-R cDNA at amino acid residue 221 and transfection of Ba/F3 cells with this cDNA in the vector pXM (20 μg) along with pSV2neo (1 μg). Positive clones were selected in RPMI containing 10% FCS, 10% WEHI (cell line) conditioned medium [17] as a source of interleukin-3 and 750 $\mu\text{g}/\text{ml}$ G418. This cell line was generously provided by Dr. S. S. Watowich and Dr. D. Hilton (Whitehead Institute).

NIH mouse 3T3 fibroblasts expressing the EPO-R [19], and NIH 3T3 fibroblasts expressing the ASGP-R H2a subunit [3] and the non-cleaved mutant of ASGP-R H2a, G78R [25], were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum.

Immunofluorescence microscopy

For immunofluorescence, the cell monolayers were trypsinized and split 1:10 to 1:60 into 60 mm tissue culture dishes at least 2 days before use. NIH 3T3 cells expressing the EPO-R were incubated for 5 h in the presence or absence of 100 μM ALLN and 30 μM cycloheximide (CHX). Double-labelling immunofluorescence of the cells with the rat monoclonal antibody IG11, specific for Lamp 1 [27], and anti-EPO-R antibodies was performed as described [19]. Samples were viewed under a Bio-Rad MRC600 confocal fluorescence microscope and photographed after identical exposure times to be able to compare signal intensities between samples.

Metabolic labelling, immunoprecipitation and Endo H digestion

Ba/F3 cells expressing EPO-R and soluble EPO-R, NIH 3T3 cell lines expressing EPO-R and NIH 3T3 cell lines expressing the ASGP-R H2a subunit and the ASGP-R H2a mutant (G78R, which is not cleaved to the 35 kDa fragment) were utilized.

Cells (1×10^6 cells for each time point) were labelled with [^{35}S]cysteine as previously described [3,19]. Subsequently cells were chased in the presence of non-labelled amino acids for the indicated periods of time. ALLN (100 μM) was added to the chase medium as indicated. Solubilization of the cells and immunoprecipitation were performed as previously described [19]. For Endo H digestion, the Protein A-agarose pellets were boiled in 60 μl of 50 mM sodium citrate buffer, pH 6.0, and 1% SDS for 3 min. The supernatant was divided into two equal aliquots which were incubated overnight at 37 °C in the presence or absence of 2 μl of Endo H (stock solution 1 unit/ml), before separation on SDS/10%-polyacrylamide gels [28].

To immunoprecipitate soluble ASGP-R H2a, 2 mM PMSF and 5 $\mu\text{g}/\text{ml}$ of both aprotinin and leupeptin were added to the culture medium prior to addition of anti-(ASGP-R H2 C-terminal peptide) antibody cross-linked to Protein A-agarose [15 μl of a 1:1 (v/v) slurry in PBS].

For N-Glycanase treatment of immunoprecipitated ASGP-R samples, the Protein A-agarose pellets were boiled in 20 μl of 50 mM sodium citrate buffer, pH 6.0, and 0.5% SDS for 5 min. The supernatants were transferred to a new tube and supplemented with 100 mM sodium phosphate, pH 8.0, 20 mM EDTA, pH 8.0, 1.5% N-octyl glucoside and 40 m-units of N-Glycanase (Boehringer) and incubated overnight at 37 °C. Sample buffer (2-fold concentrated) was added to the samples; they then were boiled for 5 min and separated by SDS/12%-PAGE.

Radioiodination of EPO

Recombinant EPO was labelled using the iodine monochloride method [29], and had a specific radioactivity of 4×10^6 c.p.m./pmol. Briefly, 2.5 μ l of a 1.25 mg/ml solution of EPO in PBS was labelled by the addition of 100 μ l of 200 mM sodium phosphate, 0.02% (v/v) Tween 20, pH 7.4, and 1 mCi of Na¹²⁵I and, while vortex-mixing, two 5 μ l aliquots of 200 mM ICl in 2 M NaCl were added at 1 min intervals. Radioiodinated EPO (¹²⁵I-EPO) was separated from free ¹²⁵I by gel filtration.

¹²⁵I-EPO binding

Cells were incubated at 37 °C in the presence or absence of ALLN for the indicated periods of time. Cells were cooled to 4 °C and washed in binding medium (RPMI/10% FCS), prior to binding of ¹²⁵I-EPO for 6 h at 4 °C. Each binding reaction contained $(0.5\text{--}1.0) \times 10^6$ cells in a final volume of 100 μ l. After sedimentation of cells through ice-cold FCS, cell-associated radioactivity and free ¹²⁵I-EPO were measured. Specific binding was determined by subtracting the cell-associated radioactivity in the presence of unlabelled EPO (100 nM) from that associated with the cells in the absence of excess unlabelled ligand.

RESULTS

ALLN blocks degradation of the EPO-R in Ba/F3 cells and NIH 3T3 cells

To determine the effect of ALLN on degradation of the EPO-R, Ba/F3 cells expressing the EPO-R were labelled for 30 min with [³⁵S]cysteine and chased in medium containing unlabelled cysteine for up to 5 h, in the presence or absence of 100 μ M ALLN. Cell viability was not affected by ALLN (results not shown), as previously demonstrated [2]. As shown previously [18,19], after the pulse most of the receptor was sensitive to Endo H (Figure 1A, lanes 1 and 2 and Figure 1C). By 1 h of chase, ~50% of the remaining EPO-R was Endo H-resistant, by 2 h of chase ~90% of the receptor was degraded, and by 3 h of chase essentially all of it had been degraded (Figure 1C). The 39 and 46 kDa C-terminal fragments of the EPO-R (Figure 1A, lanes 4 and 5, and 7 and 8) are formed in course of receptor degradation [19]. The presence of ALLN in the medium throughout the chase significantly prolonged the half-life of the EPO-R; after 5 h of chase in the presence of ALLN, ~70% of the Endo H-resistant EPO-R molecules remained intact (Figure 1A, compare lanes 21 and 22 to lanes 1 and 2; Figure 1C). At this time EPO-R molecules which had accumulated in the presence of ALLN contained mainly complex sugars, as they were resistant to digestion with Endo H. ALLN also seemed to stabilize the Endo H-sensitive form of EPO-R, but to a much lesser extent. Similar results were obtained with calpain inhibitor II (ALLM; results not shown). Noteworthy is the fact that the pattern of proteolytic fragments formed in the course of EPO-R degradation was altered in the presence of ALLN. Whereas in control cells the 39 kDa and 46 kDa fragments were detected (Figure 1A, lanes 7 and 8), in the ALLN-treated cells two different C-terminal fragments of the EPO-R, of 49 kDa and 42 kDa, were detected after 2 h of chase (Figure 1A, lanes 9 and 10) and remained throughout the 5 h chase period.

Similarly, ALLN inhibited EPO-R degradation in NIH 3T3 fibroblasts stably expressing the EPO-R. Briefly, NIH 3T3 fibroblasts expressing EPO-R cDNA were labelled for 30 min with [³⁵S]cysteine, and chased for up to 5 h in the presence or absence of the inhibitor. Figure 1B and our previous data [19] show that EPO-R metabolism is generally similar in NIH 3T3 and Ba/F3 cells. After 1 h of chase about half of the Endo H-

Figure 1 Maturation and degradation of the EPO-R in the presence of ALLN

Ba/F3 cells expressing the full-length EPO-R (A), or NIH 3T3 fibroblasts expressing the EPO-R (B), were pulse-labelled with [³⁵S]cysteine for 30 min and then chased for the indicated periods of time in medium either with no addition or with 100 μ M ALLN, as indicated. Detergent extracts of the cells were immunoprecipitated with antiserum directed against the EPO-R C-terminus. The immunoadsorbed material was divided into two aliquots which were incubated overnight either with no addition (—) or with Endo H (+). Samples were then subjected to SDS/PAGE, fluorographed and subjected to autoradiography. The closed and open arrows indicate the Endo H-resistant and Endo H-sensitive forms of the EPO-R respectively. Molecular masses (kDa) of protein standards are on the right. Panel (C) represents a densitometric scan of the bands corresponding to EPO-R in (A), normalized to the amount of EPO-R present at 0 h of chase. Symbols are as follows: Endo H-sensitive (○) and Endo H-resistant (●) EPO-R in the presence of ALLN; Endo H-sensitive (■) and Endo H-resistant (□) EPO-R in the absence of ALLN.

sensitive and Endo H-resistant receptor molecules were degraded (Figure 1B, lanes 3 and 4), and by 2 h of chase the vast majority of receptor molecules had been degraded. EPO-R molecules were barely detectable in the control samples as of 3 h of chase (Figure 1B, lanes 11 and 12). In contrast, in ALLN-treated cells the level of EPO-R remained high throughout the chase period (Figure 1B, lanes 21 and 22). Again, in the presence of ALLN, mainly Endo H-resistant EPO-Rs accumulated. The Endo H-sensitive form of the EPO-R was also stabilized, but to a much lesser

Figure 2 ALLN elevates cell-surface EPO-R

(A) Ba/F3 cells expressing the EPO-R were incubated at 37 °C for the indicated periods of time in the presence (□) or absence (●) of 100 μ M ALLN. Subsequently cells were cooled to 4 °C and incubated in a volume of 100 μ l with 100 000 c.p.m. of ¹²⁵I-EPO in the presence or absence of 100 nM unlabelled EPO for 5 h at 4 °C. ¹²⁵I-labelled EPO bound to the cells was measured; results represent means $\pm$ S.D. ($n = 3$). These values were obtained after subtracting the radioactivity bound in the presence of unlabelled competitor ligand from that obtained in its absence. The Figure depicts one out of three experiments which yielded essentially similar results. (B) Ba/F3 cells expressing the wild-type EPO-R were incubated for 5 h at 37 °C in the presence (□) or absence (●) of ALLN. Subsequently cells were incubated with various concentrations of ¹²⁵I-EPO in the presence or absence of 100 nM unlabelled EPO for 16 h at 4 °C. Scatchard plots of the data are shown.

extent. Similar proteolytic fragments were detected in the NIH 3T3 cells as in the Ba/F3 cells, but were not as obvious due to the lower expression of EPO-R.

The amount of cell-surface EPO-R is not increased to the same extent as intracellular EPO-R in the presence of ALLN

Since ALLN markedly inhibits the degradation of Endo H-resistant EPO-R, we asked whether ALLN treatment could similarly affect the amount of plasma membrane EPO-R. To investigate this possibility, cells were incubated for increasing periods of time at 37 °C with ALLN. Subsequently, cells were

Figure 3 The inhibition of degradation of EPO-R by ALLN is reversible

Ba/F3 cells expressing the EPO-R were pulsed for 30 min with [³⁵S]cysteine (lanes 1 and 2) and chased for 3 h in the presence of ALLN (lanes 3–10). After this 3 h chase the cells were washed to remove the inhibitor (lanes 3 and 4) and further incubated for 1 h (lanes 5 and 6) 2 h (lanes 7 and 8) or 3 h (lanes 9 and 10) in the absence of ALLN. Lanes 11 and 12 represent control cells chased for 6 h without inhibitor, and lanes 13 and 14 are cells chased in the presence of ALLN throughout the entire 6 h period. At all time points detergent extracts of the cells were immunoprecipitated with anti-(EPO-R C-terminal peptide) antibodies, and incubated overnight at 37 °C in the presence (+) or absence (–) of Endo H. Samples were then subjected to SDS/PAGE and fluorography. Molecular masses (kDa) of protein standards are shown on the right.

cooled to 4 °C, washed in medium, resuspended in binding medium, and ¹²⁵I-EPO binding to the cells was examined. As demonstrated in Figure 2A, the number of EPO binding sites increased with time of incubation with ALLN, reaching after 1 h a plateau 1.5-fold above the level of control samples. Scatchard analysis demonstrated that ALLN did not alter the affinity of EPO for the EPO-R (Figure 2B), as demonstrated by the similar dissociation constant of 150 pM in control and ALLN-treated cells. As previously demonstrated, the EPO-R displays a single-affinity for EPO, with a dissociation constant in the range 200–1000 pM [18]. Compared with the profound effect ALLN had on the intracellular pools of EPO-R, its effect on cell-surface EPO-R was moderate.

The ALLN block of EPO-R degradation is reversible

To determine whether the effect of ALLN on the degradation of the EPO-R in Ba/F3 cells is reversible, the following experiment was performed. Cells were pulsed with [³⁵S]cysteine for 30 min and chased in the presence of ALLN for 3 h. After this 3 h chase, the inhibitor was removed. Cells were then further chased for 4, 5 and 6 h with no inhibitor. As shown in Figure 3, the stabilization effect of ALLN on the EPO-R was abolished upon removal of the inhibitor. Confirming Figure 1A, Figure 3 (lanes 3 and 4) shows that after 3 h of chase in the presence of ALLN most of the EPO-R was Endo H-resistant. After removal of the inhibitor, the Endo H-resistant EPO-R was rapidly degraded. In control cells the 39 kDa fragment appeared after 1 h of chase (Figure 1, lanes 3 and 4). Note that the 39 kDa fragment of the EPO-R, which was not observed in the chase in the presence of ALLN (Figure 3, lanes 3 and 4), appeared after 1 h of chase following removal of ALLN (Figure 3, lanes 5 and 6), and accumulated in the subsequent hours after removal of the inhibitor (Figure 3, lanes 6–10). For comparison, cells were chased in the absence or presence of ALLN for 6 h (Figure 3, lanes 11 and 12 and 13 and 14 respectively); the EPO-R was stabilized upon exposure to ALLN in the chase medium for 6 h, whereas it was entirely degraded in untreated samples.

Figure 4 Confocal double label immunofluorescence of EPO-R and lysosomes plus endosomes

Fixed permeabilized NIH 3T3 cells expressing the EPO-R were processed as previously described [19]. Panels (a) and (b) represent double labelling of control cells with rabbit polyclonal affinity-purified anti-(EPO-R C-terminal peptide) antibodies and rat monoclonal anti-(Lamp 1) antibodies (labelling endosomes and lysosomes) respectively. Panels (c) and (d) represent double labelling with anti-(EPO-R C-terminal peptide) and anti-(Lamp 1) respectively of cells incubated for 5 h with 30 μ M CHX and 100 μ M ALLN. Cy3-labelled donkey anti-rabbit antibodies recognizing EPO-R and fluorescein isothiocyanate-labelled goat anti-rat antibodies recognizing Lamp 1 were employed. Solid and empty arrows indicate similarities and differences respectively in the staining pattern. Bar = 36 μ m.

EPO-R accumulates in endosomes and lysosomes in NIH 3T3 fibroblasts treated with ALLN and CHX

The immunofluorescence experiment in Figure 4 addressed the localization of EPO-R in ALLN-treated cells. Immunofluorescence staining of control untreated NIH 3T3 cells expressing the EPO-R with anti-(EPO-R C-terminal peptide) antibodies generated punctate perinuclear staining (Figure 4a, and [19]). On double immunofluorescence labelling of the cells, part of the punctate staining co-localized with the lysosomes and endosomes, detected with the rat monoclonal antibody IG11 directed against Lamp 1 (Figures 4a and 4b, solid arrows), whereas part was unique to the receptor (open arrows).

After incubation for 5 h in the presence of ALLN plus CHX

(to block synthesis of new proteins), immunofluorescence staining with the anti-(EPO-R C-terminal peptide) antibody was entirely punctate (Figure 4C) and overlapped to a large extent with the staining pattern visualized with the anti-(Lamp 1) antibody (Figure 4D, solid arrows). Hence it appears that ALLN blocks degradation of the EPO-R, leading to its accumulation mainly in endosomes and lysosomes. Similar results were obtained with EPO-R-expressing Ba/F3 cells (not shown).

ALLN increases secretion of the soluble extracellular domain of the EPO-R (s-EPO-R)

What effect does ALLN have on the metabolism of a secreted extracellular form of the EPO-R? Is its degradation also inhibited

Figure 5 ALLN augments the secretion of s-EPO-R

Ba/F3 cells expressing s-EPO-R were pulse-labelled for 30 min with [35 S]cysteine and chased in the presence or absence of ALLN for the indicated periods of time. s-EPO-R molecules were immunoprecipitated from detergent extracts of the cells (A) and medium (B) using antibodies specific for the N-terminus of the EPO-R [18]. Immunoprecipitated s-EPO-R was incubated overnight at 37 °C in the presence (+) or absence (−) of Endo H. Samples were then subjected to SDS/PAGE, fluorographed and subjected to autoradiography. The Endo H-resistant, Endo H-sensitive and non-glycosylated s-EPO-R are indicated with closed and open arrows and an arrowhead respectively. Note that the non-glycosylated form of the s-EPO-R is obvious in ALLN-treated cells only in the lanes that were not treated with Endo H, as it is masked by the deglycosylated s-EPO-R seen in the lanes treated with Endo H. Molecular masses (kDa) of protein standards are shown on the right. (C) Densitometric scans of the autoradiograms in (A) and (B): top panel, cellular s-EPO-R; bottom panel, secreted s-EPO-R. Symbols: Endo H-sensitive s-EPO-R in the presence (○) or absence (□) of ALLN; Endo H-resistant s-EPO-R in the presence (●) or absence (■) of ALLN.

by ALLN? If so, does it accumulate intracellularly, as for wild-type EPO-R, is its level of secretion augmented? In an attempt to answer these questions we used Ba/F3 cells expressing the s-EPO-R, obtained by insertion of a stop codon at amino acid 221. The cells were labelled for 30 min with [35 S]cysteine and chased in medium containing non-radioactive amino acids in the presence or absence of ALLN. The s-EPO-R content in the cells (Figure 5A) as well as in the culture medium (Figure 5B) was determined by immunoprecipitation with antibodies specific for the N-terminus of the EPO-R and separation on SDS/PAGE, followed by autoradiography. The bands on the autoradiograms were quantified by densitometric scanning (Figure 5C). After the pulse virtually all of the s-EPO-R was Endo H-sensitive (Figure 5A, lanes 1 and 2; Figure 5C, top panel), and no s-EPO-R was detected in the medium (Figure 5B, lanes 1 and 2; Figure 5C, bottom panel). At 1 h of chase the amount of pulse-labelled Endo H-sensitive s-EPO-R had declined by 50%, and after 3 h only trace amounts of Endo H-sensitive s-EPO-R remained. This decrease in Endo H-sensitive s-EPO-R may reflect either its maturation to the Endo H-resistant form or its degradation. Only a minor fraction (less than 10%) of the pulse-labelled s-EPO-R acquired resistance to Endo H cleavage (Figure 5A, lanes 4, 6, 8 and 10; Figure 5C, top panel). ALLN somewhat stabilized the Endo H-sensitive s-EPO-R (Figure 5A, compare

lanes 14, 16 and 18 to lanes 6, 8 and 10), and slightly prolonged its half-life from 1 to 1.5 h (Figure 5C, top panel), suggesting that it may inhibit its degradation in the ER. In addition, ALLN treatment resulted in intracellular accumulation of a non-glycosylated s-EPO-R (Figure 5A, solid arrowhead, lanes 11, 13, 15 and 17), which was not apparent in the corresponding control samples (Figure 5A, lanes 3, 5, 7 and 9). The amount of intracellular Golgi-processed s-EPO-R was essentially similar in control and ALLN-treated cells (Figure 5A, compare lanes 12, 14, 16 and 18 with lanes 4, 6, 8 and 10; Figure 5C, top panel). In contrast, the level of secreted s-EPO-R increased approx. 3-fold (Figure 5B, compare lanes 17 and 18 to lanes 9 and 10; Figure 5C, bottom panel) in the presence of ALLN. The secreted form of EPO-R contains complex oligosaccharides, as it was resistant to cleavage by Endo H.

ALLN blocks degradation of the ASGP-R H2a subunit

ALLN inhibits mainly the degradation of mature, Endo H-resistant EPO-R. However, it is possible that a fraction of EPO-R molecules remain trapped in the ER, or in a pre-Golgi compartment, and are degraded there. To examine the involvement of ALLN-sensitive proteinases in ER degradation *per se*, we explored the effect of ALLN on the degradation of the

Figure 6 ALLN blocks degradation of the ASGP-R H2a

NIH 3T3 fibroblasts expressing ASGP-R H2a (**A**) or its G78R mutant (**B**) were pulsed for 15 min with [³⁵S]cysteine (lanes 1 and 2), and chased for the indicated periods of time (lanes 3–10) in the presence or absence of the calpain inhibitor ALLN. Labelled receptor molecules were immunoprecipitated from detergent extracts of the cells and incubated overnight at 37 °C in the presence (+) or absence (–) of Endo H. Samples were then subjected to SDS/PAGE, fluorographed and subjected to autoradiography. Molecular masses (kDa) of protein standards are shown on the right. In (**A**) the solid arrows indicate the full-length uncleaved precursor H2a and the open arrow indicates the fragment produced by proteolytic cleavage near the transmembrane region. In (**B**) only the full-length precursor is observed.

ASGP-R H2a subunit in the ER [4,23,26]. The ASGP-R H2a subunit is synthesized and inserted into the ER membrane; a portion is cleaved to a 35 kDa fragment that corresponds to the ectodomain [3,4]. NIH 3T3 cells expressing either the ASGP-R H2a subunit [23] or a mutant of ASGP-R H2a (G78R) which does not generate a 35 kDa cleaved fragment [25] were pulsed for 15 min with [³⁵S]cysteine, and chased in the presence or absence of ALLN for the indicated periods of time. Cells were solubilized and ASGP-R H2a immunoprecipitated with specific antibodies [3]. As seen in Figure 6A, ALLN significantly inhibited degradation of the ASGP-R H2a subunit in the ER, but not the cleavage to the 35 kDa fragment. In control cells after 5 h of chase, less than 10 % of the pulse-labelled precursor and trace amounts of the 35 kDa fragment remained (Figure 6A, lanes 7 and 8), whereas in the presence of ALLN the amounts of pulse-labelled receptor and 35 kDa fragment were increased 3–6-fold (Figure 6A, lanes 9 and 10).

Degradation of the G78R H2a mutant follows similar kinetics to that of the wild-type H2a [25]. Here we employed the G78R mutant to determine whether ALLN inhibits precursor degradation to the same extent in the absence of formation of a 35 kDa fragment. Figure 6B demonstrates that ALLN significantly

Figure 7 Secretion of ASGP-R H2a is enhanced by incubation with ALLN

(**A**) NIH 3T3 cells expressing H2a (2-18 cell line) were pulsed for 30 min with [³⁵S]cysteine, followed by chase with complete medium for the indicated periods of time in the presence or absence of ALLN. Cell lysates (cells) or supernatants (medium) were immunoprecipitated with anti-(H2 C-terminal peptide), treated with N-Glycanase and run on SDS/12%-PAGE. The gel was fluorographed and subjected to autoradiography. As a control, non-transfected 3T3 cells (lanes 8 and 15) were studied following a pulse and a 7.5 h chase. (**B**) The autoradiogram in (**A**) was quantified by densitometry. The ratio was calculated for the amount of H2a precursor in the presence of ALLN to the amount in the absence of ALLN. (**C**) As in (**B**) but for the H2a intracellular fragment; (**D**) as in (**B**) but for the H2a secreted fragment.

blocked degradation of the G78R mutant (Figure 6B, compare lanes 7 and 9), in a similar manner to that observed for wild-type ASGP-R H2a. As the 35 kDa fragment is not formed from the G78R mutant, the effects of ALLN are probably due to inhibition of ER degradation which is not dependent on the proteolytic cleavage that generates the 35 kDa fragment [25]. Accumulation of the 35 kDa fragment in the presence of ALLN may result either from a direct block of its degradation by ALLN or from accumulation of the precursor which is then cleaved to generate the 35 kDa fragment.

Secretion of the 35 kDa ASGP-R H2a fragment in the presence of ALLN is not elevated to the same extent as the accumulation of intracellular forms

In NIH 3T3 cells expressing H2a, some of the 35 kDa fragment formed in the ER is secreted (G. Z. Lederkremer and H. F. Lodish, unpublished work). To determine whether ALLN affected the secretion of the 35 kDa H2a fragment, 3T3 cells expressing H2a (cell line 2-18) were pulse-labelled for 30 min with [³⁵S]cysteine and chased for different times (Figure 7A). In order to facilitate quantification, all samples were treated with N-Glycanase, as the secreted fragment contains complex-type N-linked oligosaccharides (G. Z. Lederkremer and H. F. Lodish, unpublished work). After a 7.5 h chase there was a reproducible 1.75-fold increase in secretion of the H2a 35 kDa fragment in the

presence of ALLN (Figure 7A, lanes 13 and 14; Figure 7D). This increased secretion accounted only partially for the almost 3-fold inhibition of the intracellular degradation of the fragment already visible at 2.5 h of chase (Figure 7A, lanes 6 and 7; Figure 7C). Therefore there may be an additional ALLN-insensitive proteinase that degrades the 35 kDa fragment before it can be secreted. Alternatively, there may be additional mechanisms which govern the targeting of the 35 kDa fragment for secretion. In the case of soluble H2a, a Golgi-processed fragment is never seen intracellularly in the presence or absence of ALLN, indicating rapid secretion after exit from the ER.

DISCUSSION

This study shows a potent effect of the calpain inhibitors ALLN and ALLM on intracellular degradation of the EPO-R and the ASGP-R H2a subunit. Mature, Endo H-resistant EPO-R is degraded mainly in the lysosomes [19], but it is possible that Endo H-sensitive EPO-R is degraded in the ER [18,19]. Both Endo H-sensitive and Endo H-resistant EPO-Rs are extremely labile (half-life approx. 40–60 min) [18,19]. We have previously demonstrated that proteolytic fragments of 39 kDa and 46 kDa are formed from a small fraction of EPO-R molecules in the course of their degradation [19]. The ASGP-R H2a subunit is degraded exclusively in the ER. ER degradation has been demonstrated for a number of proteins, generally unfolded, or unassembled polypeptides [30,31]. For both the T cell receptor and HMG-CoA reductase the existence of charged amino acid residues in the transmembrane regions serves as a signal for degradation in the ER [1,32]. In the case of the ASGP-R H2a subunit, a stretch of five amino acids containing charges at the C-terminus of the transmembrane domain enhances ER degradation by not allowing exit of the polypeptide from the ER [23].

Lysosomal degradation has been shown for a variety of growth factor receptors [33], and is a central process in the regulation of their cell-surface levels. The intracellular sorting motifs which regulate receptor distribution between the plasma membrane, early endosomes, late endosomes and lysosomes are beginning to emerge [34]. At steady state only a minor fraction of Golgi-processed EPO-R molecules is present on the cell surface, a feature characteristic of cells endogenously expressing the EPO-R and of cells transfected with EPO-R cDNA [20]. To gain insight into the mechanisms driving the cellular distribution of the EPO-R, we increased the intracellular levels of the receptor by inhibiting its degradation. If surface expression is determined by the stability of the receptor molecules, then one would expect it to increase to the same degree in response to an elevation of intracellular EPO-R. This, however, was not the case, indicating the complexity of the regulation of intracellular trafficking through the constitutive secretory pathway.

ALLN stabilized both the EPO-R and the ASGP-R H2a subunit expressed in singly transfected cells. The observation that ALLN inhibited degradation of Endo H-resistant EPO-R and ER degradation of the ASGP-R H2a subunit highlights the multiplicity of cellular compartments in which the inhibitor exerts its effect, as also demonstrated by its ability to inhibit ER [2,7,35], post-ER [12,13] and lysosomal degradation [36] and degradation catalysed by the proteasome [14]. It is of interest that the intracellular pathways of the EPO-R and the ASGP-R H2a subunit were not altered in the presence of ALLN, since ALLN inhibited degradation of both proteins but not their final cellular destinations.

Interestingly, the pattern of proteolytic fragments of the EPO-R was different between control and ALLN-treated cells (Figure 1). The presence of the C-terminal EPO-R 42 kDa and 49 kDa

fragments in chases with ALLN may indicate either that they are precursors of the 46 kDa and 39 kDa fragments [19] respectively, or that in the presence of ALLN alternative pathway(s) of degradation may occur. In any case, both in control non-treated cells and in ALLN-treated cells the proteolytic fragments are derived from a small fraction of the pulse-labelled precursor molecules, indicating that their formation is probably not the major pathway of EPO-R degradation. ALLN mainly blocks degradation of Endo H-resistant EPO-Rs (Figure 1). The action of ALLN may be attributed to direct inhibition of lysosomal cathepsins [36] or to inhibition of other cytosolic proteinases, including calpains [10]. These may degrade the cytoplasmic tail of the EPO-R, possibly a prerequisite for subsequent degradation of the receptor in lysosomes. Based on our immunofluorescence experiments one would assume that, after 3 h of chase with ALLN, most of the receptors had exited the Golgi and were present either on the plasma membrane in endocytic vesicles or in lysosomes. Formation of the characteristic EPO-R proteolytic fragments upon removal of ALLN may suggest that they are formed in endosomes *en route* to the lysosomes, or in lysosomes themselves.

The effect of ALLN on intracellular Endo H-resistant EPO-R is marked, but its effect on the level of plasma membrane receptors and the secretion of s-EPO-R is smaller. Cell-surface EPO-R and secreted s-EPO-R increased only 1.5- and 3-fold respectively in the presence of ALLN (Figure 2), suggesting the existence of additional regulatory mechanisms involved in cell-surface expression of the EPO-R. These may include a requirement for accessory proteins, or additional as yet unidentified receptor subunits [37].

The stabilization of Endo H-resistant s-EPO-R by ALLN may reflect direct inhibition of its degradation. Alternatively, or in addition, ALLN may inhibit ER degradation of the Endo H-sensitive s-EPO-R, and consequently more molecules may be able to attain proper folding, exit the ER and be secreted. Once leaving the ER, s-EPO-R molecules are mainly secreted, as the major form of intracellular s-EPO-R is Endo H-sensitive. Generally, the stabilization effect of ALLN on intracellular Golgi-processed wild-type EPO-R was far more pronounced than that observed on Golgi-processed (both intracellular and secreted) s-EPO-R. This may suggest that the transmembrane and/or cytoplasmic sequences enhance the susceptibility of EPO-R to ALLN-sensitive proteinases.

The stabilizing effect of ALLN on the ASGP-R H2a subunit (Figure 6), which is degraded in the ER, indicates the possibility that ALLN can also inhibit ER degradation *per se*, as has also been demonstrated for HMG-CoA reductase [7], the unassembled T cell receptor subunit [2] and apolipoprotein B [35]. One cannot exclude the possibility that the inhibition of ASGP-R H2a subunit degradation may also originate from inhibition of proteolysis by cytosolic enzymes (e.g. calpains) that degrade its cytoplasmic tail. This may be a necessary step for its further degradation in the ER. Proteolysis of the cytoplasmic tail might destabilize the transmembrane anchoring of the protein, leading to its translocation into the ER lumen and further degradation, as shown for the T cell antigen receptor α chain [38].

In the case of the ASGP-R H2a subunit, the fragment corresponding to the entire exoplasmic domain (35 kDa intracellular fragment; Figure 7) can be secreted. Addition of ALLN leads to increased secretion of the 35 kDa proteolytic fragment. This effect is mild (75% increase after 7.5 h chase; Figures 7A and 7D) compared with the inhibitory effect of ALLN on the intracellular degradation of the fragment (over 3-fold reduction of degradation after 5 h chase), suggesting the

existence of another degradative step before secretion or additional mechanisms participating in the regulation of fragment secretion. Thus elevated amounts of intracellular fragment are not sufficient to determine their secretion. Similarly, ALLN profoundly inhibited the intracellular degradation of apolipoprotein B, but did not lead to a similar increase in its secretion [39], supporting the notion that inhibition of degradation by ALLN is not sufficient to increase secretion by the same degree. This situation contrasts with that observed with the s-EPO-R, where ALLN treatment led to a 3-fold accumulation of secreted s-EPO-R, much greater than the increase in the intracellular amounts of s-EPO-R. The fact that the intracellular accumulation of the EPO-R and the ASGP-R H2a subunit in the presence of ALLN is not affected to the same extent as either their membrane expression or the secretion of their soluble forms may indicate that additional enzymes and/or pathways unaffected by ALLN participate in their cellular trafficking.

Although Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl block degradation of the ASGP-R precursor [4,26], they do not stabilize the 35 kDa fragment [25]. Hence their mode of action probably differs from that of ALLN, which results in the accumulation of both the precursor and the 35 kDa fragment of the ASGP-R H2a subunit. Noteworthy is the finding that the ER 72 kDa cysteine proteinase is also inhibited by ALLN, but not Tos-Phe-CH₂Cl or Tos-Lys-CH₂Cl [8]. Finally, Tos-Lys-CH₂Cl was found to cause accumulation mainly of unfolded forms of the ASGP-R H2b [24]. Our preliminary data suggest that, in contrast, in the presence of ALLN mainly folded forms of the ASGP-R H2a accumulate, suggesting that these inhibitors may have different modes of action.

ALLN has proved to be a useful tool for inhibiting the degradation of the EPO-R and the ASGP-R H2a subunit, in which lysosomal and ER intracellular sites of degradation are involved. It may prove to be a general tool in the inhibition of intracellular degradation and the promotion of the secretion of soluble protein derivatives. Nevertheless, the present study illustrates the complexity of the regulation of the intracellular degradation of membrane proteins and their soluble forms. This regulation affects the intracellular traffic of the proteins and their arrival at their final destinations, which is not merely a reflection of their intracellular levels.

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