Involvement of the Ubiquitin/Proteasome System in Sorting of the Interleukin 2 Receptor β Chain to Late Endocytic Compartments

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Down-regulation of cell surface growth factor receptors plays a key role in the tight control of cellular responses. Recent reports suggest that the ubiquitin system, in addition to participating in degradation by the proteasome of cytosolic and nuclear proteins, might also be involved in the down-regulation of various membrane receptors. We have previously characterized a signal in the cytosolic part of the interleukin 2 receptor β chain (IL2R β) responsible for its targeting to late endosomes/lysosomes. In this report, the role of the ubiquitin/proteasome system on the intracellular fate of IL2R β was investigated. Inactivation of the cellular ubiquitination machinery in ts20 cells, which express a thermolabile ubiquitin-activating enzyme E1, leads to a significant decrease in the degradation rate of IL2R β , with little effect on its internalization. In addition, we show that a fraction of IL2R β can be monoubiquitinated. Furthermore, mutation of the lysine residues of the cytosolic region of a chimeric receptor carrying the IL2R β targeting signal resulted in a decreased degradation rate. When cells expressing IL2R β were treated either by proteasome or lysosome inhibitors, a significant decrease in receptor degradation was observed. Our data show that ubiquitination is required for the sorting of IL2R β toward degradation. They also indicate that impairment of proteasome function might more generally affect intracellular routing.

INTRODUCTION

After endocytosis, many growth factor and hormone receptors are transported to late endocytic compartments and degraded. This leads to the down-regulation of receptors, which is important to control their cell surface expression, and thereby the transduction of signals delivered in response to growth factors and hormones. This process requires sorting of receptors in early endocytic compartments and further transport to late compartments, where they are degraded (Mukherjee *et al.*, 1997).

We recently investigated the intracellular fate of a growth factor receptor, the interleukin 2 receptor (IL2R). The cytokine interleukin 2 (IL2) is produced by activated helper T lymphocytes and acts as a potent proliferation and differentiation factor on a variety of cells of the immune system.

High-affinity IL2 receptors ($K_d \approx 10$ –100 pM) are composed of three distinct components, the α , β , and γ chains, that are associated in a noncovalent manner (Minami *et al.*, 1993). Both the β and γ chains, but not the α chain, belong to the cytokine receptor superfamily (Bazan, 1990). This hematopoietic cytokine receptor family includes receptors for several cytokines such as erythropoietin, the granulocyte colony-stimulating factor, the granulocyte-macrophage colony-stimulating factor, the leukemia inhibitory factor, growth hormone, prolactin, and ciliary neurotrophic factor. Many receptor subfamily members share at least one component; thus, the receptors for IL2, 4, 7, 9, and 15 have a common γ chain, and the receptors for IL2 and IL15 share the β chain (reviewed by Sugamura *et al.*, 1996).

One of the early events following IL2 binding to high-affinity receptors on the cell surface is the efficient internalization of IL2 receptor complexes (Duprez *et al.*, 1988; Subtil *et al.*, 1994). After endocytosis, the three subunits are sorted differently: the α chain recycles to the plasma membrane, whereas the β and γ chains are targeted to late endocytic compartments (Hémar *et al.*, 1995).

Little is known about the molecular mechanisms controlling intracellular sorting of the subunits. We have previously shown that a motif of 10 amino acids in the cytosolic tail of IL2R β is sufficient to retarget a normally recycling receptor

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Abbreviations used: ALLN, *N*-acetyl-L-leucyl-norleucinal; GHR, growth hormone receptor; IL2, interleukin 2; IL2R α , interleukin 2 receptor α chain; IL2R β , interleukin 2 receptor β chain; Lamp-1, lysosome-associated membrane protein-1; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TfR, transferrin receptor.

toward degradation compartments (Subtil *et al.*, 1997). Accordingly, deletion of this signal strongly impaired IL2R β degradation. Further molecular characterization of this motif showed that it was different from the well-documented tyrosine and di-leucine families of trafficking signals (Subtil *et al.*, 1998).

What are the cellular mechanisms responsible for the sorting of the β subunit to late compartments and its final degradation? It is known that cells degrade proteins generally by two major systems: the lysosomes and the ubiquitin/proteasome system. Lysosomes are in general responsible for the degradation of membrane and extracellular proteins that enter cells by endocytosis. The ubiquitin/proteasome pathway is involved in the degradation of cytosolic and nuclear proteins (Weissman, 1997). Ubiquitin is a 76 amino-acid polypeptide expressed in all eucaryotic cells and highly conserved from yeast to human. Classically, it is reported that degradation of proteins by the proteasome involves two distinct and successive steps (reviewed by Herskho and Ciechanover, 1998). First, multiple ubiquitin molecules are covalently linked to the target protein via a lysine residue, and then the ubiquitinated protein is degraded by the 26S proteasome. Ubiquitin molecules are added to target proteins through the sequential action of three enzymes; ubiquitin is first activated in an ATP-dependent manner by a specific activating enzyme (E1) and then transferred to an ubiquitin carrier protein (E2). Next, activated ubiquitin is linked to a lysine residue of the substrate protein, either directly or in cooperation with an ubiquitin ligase (E3). In most species, only one E1 enzyme but multiple E2 and E3 enzymes have been described.

Recently, it has been shown that besides its role in cytosolic and nuclear protein degradation, the ubiquitin-conjugating machinery and the proteasome may play a role in the sorting of various membrane anchored-proteins (reviewed by Bonifacino and Weissman, 1998; Hicke, 1999; Strous and Govers, 1999). A clear link between the ubiquitin/proteasome system and several membrane proteins' down-regulation has been established. Involvement of ubiquitin/proteasome pathway in the down-regulation of some mammalian cell surface receptors was demonstrated (reviewed by Bonifacino and Weissman, 1998; Hicke, 1999; Strous and Govers, 1999). Ubiquitination of some of these mammalian plasma membrane proteins might serve as a signal for their entry into the endocytic pathway. In yeast, mono- or diubiquitination in this process has been shown to be sufficient for several membrane proteins (Galan and Haguenaer-Tsapis, 1997; Terrell *et al.*, 1998; Lucero *et al.*, 2000). In some cases a direct role of the ubiquitin/proteasome system in the intracellular degradation of membrane proteins was suggested (Laing and Beyer, 1995; Mori *et al.*, 1995; Jeffers *et al.*, 1997; Staub *et al.*, 1997).

In the present article, we have investigated the role of ubiquitination as well as of proteasomes in the down-modulation of IL2R β and of a chimera carrying its degradation targeting signal.

MATERIALS AND METHODS

Cells, Monoclonal Antibodies, and Reagents

IARC 301.5 is a subclone from a cell line derived from a human T lymphoma, which expresses high- and low-affinity IL2 receptors

(Duprez *et al.*, 1985, 1988). YT12881, a subclone from the natural killer cell line YT, which expresses the IL2R β and γ chains, was provided by Dr. Kendall Smith (Dartmouth Medical School, NH) (Teshigawara *et al.*, 1987). IARC301.5, YT, and erythroleukemia K562 cells were grown in suspension in RPMI 1640. The Chinese lung cell line ts20 used in this study was kindly provided by Dr. G.J. Strous (Utrecht University, The Netherlands) and is characterized by an inactive ubiquitin conjugating system at the nonpermissive temperature of 42°C (Kulka *et al.*, 1988). ts20 cells were maintained in culture at 33°C in DMEM. All culture media were supplemented with 10% decomplemented fetal calf serum and 2 mM L-glutamine. Stably transfected K562 or ts20 cells were grown in the same medium supplemented with 1.5 mg/ml G418 (Geneticin; Life Technologies, Gaithersburg, MD).

Mouse monoclonal antibodies (mAbs) 7G7B6 (IgG2a) and 2A3A1H (IgG1) directed against the α chain of the IL2 receptor (IL2R α) were obtained from the American Type Culture Collection (Rockville, MD). Mouse mAb341 (IgG1) and 561 (IgG2a), directed against the β chain, were kind gifts from Dr. R. Robb (Dupont Merck Pharmaceutical, Wilmington, DE) (Voss *et al.*, 1993). The rabbit polyclonal anti-ubiquitin antiserum was purchased from Sigma (St. Louis, MO), the mouse monoclonal anti-ubiquitin antibody (IgG1) was from Zymed Laboratories (San Francisco, CA), and the anti-human lysosome-associated membrane protein-1 (Lamp-1) H4A3 was from the Developmental Studies Hybridoma bank (University of Iowa, Iowa City, IA). Phycoerythrin-conjugated goat F(ab')₂ anti-murine IgG was obtained from Immunotech (Marseille, France) and peroxidase-conjugated (sheep) anti-mouse immunoglobulins from Amersham Pharmacia Biotech (Uppsala, Sweden). Fluorescein-labeled anti-mouse IgG1 and Texas Red-labeled anti-mouse IgG2a were from Southern Biotechnology (Birmingham, AL.). Human transferrin was labeled with Cy5 dye (Amersham Pharmacia Biotech), by using the CyDye Fluorolink reactive dye kit according to the manufacturer's instructions. Enhanced chemiluminescence and enhanced chemifluorescence-Western Blotting detection reagents were from Amersham Pharmacia Biotech.

Chloroquine, leupeptin, and N-acetyl-L-L-leucyl-norleucinal (ALLN) were purchased from Sigma, MG132 from Calbiochem (La Jolla, CA), and lactacystin from Alexis (COGER, France). Lactacystin and ALLN were dissolved in ethanol and dimethyl sulfoxide, respectively. Throughout the experiments, control cells were preincubated with the same solvent concentration in the culture medium at a final concentration not exceeding 0.5%.

Plasmids

The cDNA coding for the IL2R β chain was isolated from plasmid pdKCR β , kindly provided by Dr. T. Kono (Osaka University, Osaka, Japan) and subcloned in NT vector, a gift from Dr. C. Bonnerot (Institut Curie, Paris, France) as previously described (Subtil *et al.*, 1997). The $\alpha\gamma\beta_{18-27}$ chimera was generated by polymerase chain reaction (PCR). Briefly, the transferrin receptor YTRF internalization signal and the β chain sorting signal were inserted in the cytosolic part of the IL2R α chain, slightly modified by the introduction of a cloning cassette (Subtil *et al.*, 1997). The primary sequence of the cytosolic part of the $\alpha\gamma\beta_{18-27}$ chimera is TWQRRQRKSEPLSYTRFQASSPDPSKFFSQL. Mutations of the two lysine residues at position 8 and 26 in the cytosolic portion of the chimera, introducing, respectively, a glutamic acid and an alanine at these positions, were also performed by PCR. The resulting mutated chimera $\alpha\gamma\beta_{18-27}$ (K8E/K26A) will be referred to as Δ Lys.

Cell Transfection

To generate stably transfected K562 or ts20 cells, 7×10^6 cells were washed once in DMEM, 4.5 g/l glucose, and resuspended in 800 μ l of the same medium, with 20 μ g of the plasmid of interest. Electroporation was performed using the Easyject electroporator (Eurogentec, Seraing, Belgium) with a single pulse, 240 V, 1500 μ F. Selection

with 1.5 mg/ml G418 was initiated 2 d after transfection, and the cells were cloned in 96-well dishes. G418-resistant clones were assayed for expression by flow cytometry by using anti- α (2A3A1H) or anti- β (341) antibodies. The expression levels of recombinant proteins in all clones tested were the same as or less than their normal level in activated lymphocytes.

Internalization Assays

Internalization of 2A3A1H or 341 antibodies directed, respectively, against the chimeras and the β chain were quantitated by flow cytometry as previously described (Subtil *et al.*, 1998). Briefly, cells were incubated at 4°C for 60 min with 2A3A1H or 341 antibody (1/2000 ascites fluid). The cells were washed in chilled phosphate-buffered saline (PBS) at 4°C, and then they were incubated at 37°C for the indicated times to allow internalization, rapidly cooled to 4°C, and washed twice in cold PBS, 2% fetal calf serum. The cells were then incubated at 4°C for 1 h with phycoerythrin-conjugated goat F(ab)'2 anti-murine IgG and washed once at 4°C. Expression of receptors remaining at the cell surface was assayed using a FACS-can flowcytometer (Becton Dickinson, San Jose, CA). Internalization was monitored by the decrease in mean fluorescence as a function of time at 37°C. For the mutant ts20 cells, after 1-h preincubation at 30°C or at 42°C, the internalization assays were performed at the same temperatures. The background fluorescence intensity, determined with cells incubated only with the secondary antibody, was <10% and was subtracted.

Cell Surface Half-Life Measurements

To measure the half-life on the cell surface of IL2R β or of the chimeras, cells were incubated with 50 μ M cycloheximide (Sigma) to prevent the synthesis of new receptors. After different times of incubation at 37°C in culture medium with cycloheximide, the cells were cooled to 4°C and cell surface expression of the remaining receptors was assayed by flow cytometry as described (Hémar and Dautry-Varsat, 1990). Time zero on the graph corresponds to a 30-min incubation in the presence of cycloheximide, a time length necessary for the newly synthesized IL2 receptor to reach the cell surface (Duprez and Dautry-Varsat, 1986). For the ts20 cell line, cells were preincubated 1 h at 30°C or at 42°C, and half-life measurement performed at these same temperatures. All experiments were done at least four times. The means $\pm$ SE are shown.

Immunofluorescence and Confocal Microscopy

K562 cells transfected with $\alpha\gamma\beta_{18-27}$ or with the Δ Lys mutant were collected and incubated for 4 h at 37°C with or without various reagents, and then cycloheximide was added in the presence of Cy5-conjugated human transferrin for the last 30 min. The cells were washed twice in cold PBS and fixed in 3.7% paraformaldehyde and 0.03 M sucrose as previously described. Cells were then incubated with anti- α (7G7B6) and anti-Lamp-1 (H4A3) mAb diluted in the permeabilizing buffer. After two washes in permeabilizing buffer, the cells were further incubated for 1 h in the permeabilizing buffer containing labeled secondary antibodies. After washes and sample mounting in 100 mg/ml Mowiol (Calbiochem), 100 mg/ml 1,4-diazabicyclo(2.2.2)octane (Sigma), 25% glycerol (vol/vol), 100 mM Tris-HCl, pH 8.5, the cells were examined under a confocal microscope (LSM 510; Zeiss, Jena, Germany). Fluorescein, Texas-Red and Cy5 dyes were excited by laser light at 488, 543, or 633 nm, respectively. To avoid bleed-through effect in multiple staining experiments, each dye was scanned independently using the multitracking function of the LSM 510 unit. Images were merged and colocalization evaluated by the LSM 510 software. Quantification of colocalization is expressed as the percentage of green pixels corresponding to the $\alpha\gamma\beta_{18-27}$ or the Δ lys signal, which also contain the staining corresponding to the transferrin receptor or Lamp-1 signal relative to the total number of green pixels.

Immunoprecipitation and Western Blotting

IARC 301.5 cells or YT cells (2.5×10^7 cells/immunoprecipitate) were incubated with 12.5 μ M ALLN or 10 μ M MG132 or 200 μ M chloroquine for 3 h. After two washes in cold PBS, the cells were lysed in 0.5% NP-40 (Sigma) complemented with 1% protease inhibitor cocktail (Sigma), 2 mM phenylmethylsulfonyl fluoride, and 5 μ M ALLN for 10 min at 4°C. After a further 30-min incubation at 37°C in the presence of 1% Triton X-100 and 10 passages through a syringe needle, a postnuclear supernatant was collected by centrifugation ($800 \times g$, 4°C). Lysate supernatants were immunoprecipitated with mAb 561 (anti-IL2R β), or with a rabbit anti-ubiquitin antiserum, or with a mouse monoclonal anti-ubiquitin antibody or with an irrelevant antibody. The immune complexes were recovered by the addition of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) and after five washes in lysis buffer, bound proteins eluted into electrophoresis sample buffer were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were probed with an anti-IL2R β mAb (341) and an alkaline phosphatase-conjugated or peroxidase-conjugated secondary antibody was used for immunodetection. After washing, the membranes were incubated with enhanced chemiluminescence or Vistra (enhanced chemifluorescence) reagent for 5–20 min. A quantitative analysis of Western blots was conducted for some experiments by scanning on a Storm Phosphorimager/Fluorimager (Molecular Dynamics, Sunnyvale, CA). Bands were quantitated and density values were in the linear range of the detection method.

RESULTS

An Active Ubiquitination System Is Required for Efficient Down-Regulation of IL2R β Chain

After internalization, the IL2R β chain is sorted to late endosomes (Hémar *et al.*, 1995). To determine whether ubiquitination was involved in this process, we made use of the ts20 cell line carrying a temperature-sensitive ubiquitin-activating enzyme E1. This enzyme is fully functional when cells are incubated at 30°C, but becomes inactivated when cells are incubated at the nonpermissive temperature of 42°C, thereby impairing ubiquitination of proteins (Kulka *et al.*, 1988). Stably transfected ts20 cells expressing IL2R β chain were thus preincubated at permissive or nonpermissive temperature before and during the assays. We have previously demonstrated that the follow up, by flow cytometry analysis, of the disappearance of receptors from the surface of cells incubated with cycloheximide accounts for the analysis of their intracellular degradation (Subtil *et al.*, 1997). Results (Figure 1A) show that the half-life of IL2R β was significantly increased when the cells were incubated at 42°C in comparison to those incubated at 30°C. This difference was not due to a decrease in internalization rate, because IL2R β was internalized more rapidly at nonpermissive temperature (Figure 1B). It was checked that in the parental cell line E36, the IL2R β half-life was not significantly increased at nonpermissive temperature compared with permissive temperature (our unpublished results). These data strongly suggest that an active ubiquitination machinery is required for the efficient degradation of the β chain of the IL2R, but not for its internalization.

Biochemical Study of IL2R β Chain Ubiquitination

It was previously reported that, after internalization, the IL2R β chain is sorted to late endosomes/lysosomes and that

Figure 1. Cell surface half-life (A) and endocytosis (B) of IL2R β on stably transfected ts20 mutant cells. Adherent ts20 β cells were harvested using a 10-min incubation with PBS containing 5 mM EDTA at 33°C and, after one wash, preincubated for 1 h at permissive (30°C) or nonpermissive (42°C) temperature before and during the assays. (A) Cell surface expression of IL2R β on cells treated for different times with of 50 μ M of cycloheximide was assayed by flow cytometry by using mAb 341 as described in MATERIALS AND METHODS. (B) For internalization assays, after 1-h preincubation at 30°C or 42°C, cells were labeled with the antibody at 4°C for 1 h, washed once at 4°C, and incubated for the indicated times at permissive or nonpermissive temperature. Disappearance of anti-IL2R β mAb from the cell surface upon internalization was assessed by cytofluorimetry and the percentage of internalized antibody was calculated. The y-axis is the ratio of the antibody internalized to the amount of antibody bound to the cell surface at each time point. The slope of this plot is the internalization rate constant (Wiley and Cunningham, 1982). The results presented in A and B are the means $\pm$ SE of at least three independent experiments.

its degradation is inhibited in the presence of weak bases (Weissman *et al.*, 1986; Hémar *et al.*, 1995). We therefore treated lymphocytic cells with chloroquine, a weak base that inhibits lysosomal function by increasing the pH of acidic intracellular compartments, and immunoprecipitated cell lysates with an anti-IL2R β . After electrophoresis, the presence of IL2R β chain was revealed and quantitated by immunoblotting with an anti-IL2R β antibody and fluorimager analysis. The intensity of the band corresponding to IL2R β (70 kDa) increased fourfold following chloroquine treatment, compared with control cells (Figure 2, lanes a and b), confirming the involvement of the acidic compartments in the degradation of this receptor. The presence of a band of higher molecular weight (78 kDa) was also observed and this upper band was also more intense when chloroquine was used (lane b). Because ubiquitin is a small protein of $\sim$ 7.5 kDa, the 78-kDa form of the β chain could be attributed to a ubiquitin moiety appended to the IL2R β chain. To test this hypothesis, the lysates of cells incubated with or without chloroquine were immunoprecipitated with a rabbit polyclonal anti-ubiquitin antibody and immunoblotting was performed with an IL2R β -specific antibody. The anti-ubiquitin antibody was able to precipitate the 78-kDa form of the β chain that accumulates when cells are incubated with chloroquine. It indicates that this band corresponds to the ubiquitinated β chain (Figure 2, lanes c and d). No higher molecular weight bands were detectable, suggesting that IL2R β is only monoubiquitinated.

Figure 2. Western blot analysis of lysates of IARC301.5 cells preincubated for 3 h with control medium (lanes a, c, and e), or 200 μ M chloroquine (lanes b and d). Immunoprecipitation (IP) was performed using anti-IL2R β (lanes a and b), a rabbit polyclonal anti-ubiquitin antibody (lanes c and d) or an irrelevant antibody (lane e) and analyzed by immunoblotting (Blot) by using anti-IL2R β (341 mAb). The upper arrow indicates a ubiquitinated form of the IL2R β chain migrating at 78 kDa.

Effect of Mutating Lysine Residues

It is well established that ubiquitin is coupled to proteins on their lysine residues (Hershko and Ciechanover, 1998). To assess the role of the presence of potential ubiquitination sites in the function of the IL2R β chain degradation signal, we made use of a chimeric receptor, $\alpha_Y\beta_{18-27}$, carrying this sorting signal and containing only two cytosolic lysine residues. We had previously shown that a 10 amino-acid motif in this chain, including residues 18 to 27 (β_{18-27} sequence), was sufficient to mediate the efficient sorting of a recycling receptor, α_Y , toward degradation. The α_Y chimera was constructed by inserting the strong tyrosine-based internalization signal of the transferrin receptor YTRF at the carboxyl end of the IL2R α chain cytosolic domain. This chimera is efficiently internalized and recycled back to the cell surface. In contrast, when the degradation motif β_{18-27} was added to the cytosolic domain of the α_Y chimera, the resulting receptor, $\alpha_Y\beta_{18-27}$, was rapidly degraded after endocytosis (Subtil *et al.*, 1997). We mutated the two lysine residues contained in the cytosolic part of the $\alpha_Y\beta_{18-27}$ chimera, which are potential ubiquitination sites. The sorting of this lysine-mutated receptor was analyzed by measuring its internalization rate and its half-life at the cell surface. As shown in Figure 3, the mutated chimeric receptor was internalized with the same kinetics as $\alpha_Y\beta_{18-27}$, but was less efficiently degraded because its half-life was doubled. The chimera carrying only one of the two mutations had the same degradation rate as the nonmutated $\alpha_Y\beta_{18-27}$ chimera (our unpublished results), showing that the absence of both lysine residues was necessary to increase the half-life. These results imply that the sorting signal of the IL2R β chain toward degradation needs the presence of lysine residues to function.

Effect of Proteasome Inhibitors on IL2Rβ Chain Down-Modulation

We then wanted to evaluate the contribution of proteasomes. To this end, we performed immunoblot experiments on lymphocytic cell lysates incubated with or without MG132, a strong inhibitor of proteasome (Lee and Goldberg, 1998). Lysates were immunoprecipitated with mouse monoclonal anti-IL2R β or anti-ubiquitin antibodies and immuno-

$\alpha_V\beta_{18-27}$: TWQRRQRKSEPLSYTRFQASSPDPSKFFS β QL

Figure 3. Effect of mutating the cytosolic lysine residues of the chimeric receptor $\alpha_V\beta_{18-27}$ on its cell surface half-life (A) and endocytosis (B). Experiments were performed as described in Figure 1, except that the incubations were performed at 37°C. The half-life values (A) or the percentage of internalized antibody (B) are shown. The results are the means $\pm$ SE of at least three independent experiments. The sequence of the cytosolic tail of the $\alpha_V\beta_{18-27}$ chimera is shown with its two lysine residues boxed.

blotting was performed with an IL2R β -specific antibody. Treatment with MG132 increased the total amount of IL2R β in the cell lysate, but more particularly the upper band (Figure 4, lanes a and b). This upper band is the ubiquitinated form of the receptor as shown by immunoprecipitation with an anti-ubiquitin mAb (Figure 4, lane c). The same results were obtained when another proteasome inhibitor, ALLN (Lee and Goldberg, 1998), was used (our unpublished results). These results show that proteasome inhibitors lead to the accumulation of the monoubiquitinated form of the β chain.

Effect of Lysosome or Proteasome Inhibitors on Down-Regulation of IL2R β or of a Chimera Carrying Its Degradation Sorting Signal

We next measured the effect of proteasome inhibitor on IL2R β chain half-life and endocytosis. We observed that

Figure 4. Lysates of YT cells preincubated for 3 h with control medium (lanes a and c) or with 10 μ M MG132 (lane b) were immunoprecipitated with an anti-IL2R β antibody (lanes a and b) or with a mouse monoclonal anti-ubiquitin antibody (lane c) and analyzed by immunoblotting with anti-IL2R β antibody. The upper arrow indicates a ubiquitinated form of the IL2R β chain migrating at 78 kDa.

Figure 5. Cell surface half-life (A) and internalization rate (B) of IL2R β on IARC301.5 cells treated with or without the proteasome inhibitor lactacystin. Cells were preincubated for 3 h at 37°C before and during the assays with 12.5 μ M lactacystin or with control medium and the experiments were performed as described in Figure 1. The results presented in A and B are the means $\pm$ SE of at least three independent experiments.

treating human T cells with the specific proteasome inhibitor lactacystin (Lee and Goldberg, 1998) led to a significant decrease in the degradation rate of the IL2R β chain, as shown by the increase of its half-life (300 min instead of 90 min) (Figure 5A). This confirms the observation that the use of proteasome inhibitors leads to an accumulation of IL2R β . In contrast, the internalization rate (Figure 5B) was only slightly affected. Therefore, proteasome inhibitors affect the intracellular routing of IL2R β toward degradation without significantly altering its internalization.

The effects of lactacystin on the degradation and internalization rates of the $\alpha_V\beta_{18-27}$ chimera were also assayed. The degradation rate of the chimera was reduced in the presence of lactacystin but not its uptake (Figure 6). We also studied the effect of the lysosomal protease inhibitor leupeptin and of chloroquine. These compounds strongly impaired the degradation of $\alpha_V\beta_{18-27}$ (Figure 6A) without significantly affecting the internalization rate of the chimera (Figure 6B). These results show that the two different intracellular proteolytic systems, lysosomes and proteasomes, are involved in the turnover of IL2R β chain or of the chimeric receptor containing its degradation signal.

Analysis of Intracellular Localization of Receptors by Confocal Microscopy

The intracellular localization of the chimeric $\alpha_V\beta_{18-27}$ receptor in cells treated with lactacystin and of the ΔLys mutant was analyzed by confocal microscopy. After treatment with lactacystin when indicated, the cells were fixed and permeabilized and the chimeric receptor or ΔLys mutant was stained. In parallel, the transferrin receptor (TfR), a marker of early and recycling endosomes or the late endosome/lysosome marker Lamp-1 were labeled. The cells were observed by confocal microscopy (Figure 7, A–C) and the extent of colocalization of the chimeric $\alpha_V\beta_{18-27}$ or ΔLys mutant receptor with the TfR or Lamp-1 was measured as described in MATERIALS AND METHODS (Figure 7D). In control cells expressing $\alpha_V\beta_{18-27}$, the extent of colocalization

Figure 6. Cell surface half-life (A) and endocytosis (B) of the chimeric receptor $\alpha_v\beta_{18-27}$ in cells preincubated with or without (control) proteasome inhibitor (lactacystin), chloroquine, or the lysosomal proteases inhibitor leupeptin. Cells were incubated with 25 μ M lactacystin, 200 μ M chloroquine, or 100 μ M leupeptin for 3 h at 37°C and experiments performed as described in Figure 1. Cell surface expression of the chimera on cells treated for different times with 50 μ M cycloheximide was assayed by flow cytometry (A) with mAb 2A3A1H. The kinetics of endocytosis was measured and results expressed as internalization rate constants (B). The results presented in A and B are the means $\pm$ SE of at least three independent experiments.

with the Tf receptor was low, $\sim$ 20%, whereas with Lamp-1, it was higher, $\sim$ 60% (Figure 7, A and D). This result was expected because this chimera is first internalized in early endosomes and then sorted to late endosomes/lysosomes (Subtil *et al.*, 1997). In contrast, the Δ lys mutant chimera displayed a different pattern: the mutated chains accumulated in a compartment strongly stained with the transferrin receptor, but poorly with the lysosomal marker (Figure 7, B and D), suggesting some retention in the early/recycling compartment and/or less efficient sorting to late endosomes.

In cells incubated with lactacystin (Figure 7, C and D), the chimera appeared to be colocalized equally with Lamp-1 and the transferrin receptor, $\sim$ 50% suggesting that in the presence of lactacystin it is less efficiently targeted to late endosomes/lysosomes.

In the course of this study, we noticed that in cells treated with lactacystin $\alpha_v\beta_{18-27}$ receptors, in some areas, colocalized with both Lamp-1 and TfR. This can be due to the fact that the proteasome inhibitor might also alter trafficking of Lamp-1. The extent of colocalization between Lamp-1 and TfR was quantified and we found that only 7% of Lamp-1 colocalized with TfR in control cells versus 28% in cells treated with lactacystin (Figure 7E). The increase in the colocalization of these intracellular markers suggests that proteasome inhibitors might cause a global disturbance of protein trafficking in endocytic pathway.

DISCUSSION

Various growth factor receptors are found to be ubiquitinated at the plasma membrane and a direct involvement of the ubiquitin/proteasome system in the down-regulation of some of them has been demonstrated (Bonifacino and Weissman, 1998; Hicke, 1999; Strous and Govers, 1999). In this article, we have investigated its role in the down-mod-

ulation of the IL2R β . We have shown that a fraction of IL2R β can be monoubiquitinated and that indeed, ubiquitination of IL2R β or of the chimera containing its degradation signal, $\alpha_v\beta_{18-27}$, is required for the sorting toward late degradative compartments. Our results also indicate that impairment of proteasome function, by the use of inhibitors, affects the IL2R β degradation. Thus, the IL2R β chain joins the increasing number of membrane receptors shown to be down-regulated, at least in part, by their ubiquitination and/or by proteasomes. However, the mechanism by which the ubiquitin/proteasome machinery acts on the intracellular fate of receptors is still unclear. From our current knowledge of how degradation of cell surface receptors occurs, there are three steps at which ubiquitin/proteasome system can be involved: internalization, intracellular routing, and processing of the receptor by itself.

Ubiquitination-dependent endocytosis has been reported for several yeast membrane proteins: the ABC peptide transporter, the α -factor receptor, the a-factor receptor, uracyl permease Fur4p, and the maltose permease transporter (reviewed by Bonifacino and Weissman, 1998; Hicke, 1999; Strous and Govers, 1999). A role for mono- or diubiquitination of some of these receptors at the internalization step of endocytosis has been shown (Galan and Haguenaer-Tsapis, 1997; Terrell *et al.*, 1998; Lucero *et al.*, 2000). Recently, it has been demonstrated that the monoubiquitin chain appended to the α -factor receptor (Shih *et al.*, 2000) or to a-factor receptor (Roth and Davis, 2000) itself carries the information sufficient for internalization. Similarly, in mammalian cells, a recent report showed that a signal within a single ubiquitin moiety appended to a chimeric receptor is involved in its internalization (Nakatsu *et al.*, 2000). For two other mammalian membrane proteins, ubiquitination was clearly involved in their internalization; the epithelial sodium channel (Staub *et al.*, 1997) and the growth hormone receptor (GHR) (Strous *et al.*, 1996). However, the mechanism by which ubiquitination initiates their uptake is still obscure. The interaction between the ubiquitin/proteasome system and cell surface receptors was further analyzed for the GHR, which belongs to the same cytokine receptor family as the IL2R β chain. For GHR, ubiquitination of the receptor itself does not appear to be necessary (Govers *et al.*, 1999). One possibility is that the GHR needs to bind to a factor, regulated by the ubiquitin/proteasome pathway, before endocytosis can occur (van Kerkhof *et al.*, 2000). From the experiments reported here, different conclusions can be drawn for the IL2R β chain. Impairment of ubiquitination machinery in ts20 cells shows that ubiquitination of receptors is not required for their cellular uptake. Use of proteasome inhibitors also revealed that the proteasomes are not involved in the internalization step of IL2R β or of the chimera $\alpha_v\beta_{18-27}$, carrying its sorting signal. Similarly, the overall turnover, but not the internalization, of the PDGF receptor was shown to be ubiquitin/proteasome-dependent (Mori *et al.*, 1995).

The second step at which ubiquitination or proteasome action may be implicated is after internalization, in routing of the receptor from early endocytic to late degradation compartments. Our studies on the chimera $\alpha_v\beta_{18-27}$, which is degraded after endocytosis, as is the IL2R β receptor, show that mutation of the lysine residues (Δ lys) impairs receptor degradation and causes its accumulation in early/recycling

Figure 7. Intracellular localization of internalized receptors in K562 cells transfected with $\alpha_V\beta_{18-27}$ and incubated for 4 h at 37°C with or without 25 μ M lactacystin or in cells expressing the Δ Lys mutant. Cells were incubated for 30 min with 50 μ M cycloheximide in the presence of 100 nM Tf-Cy5. After fixation and permeabilization, cells were first incubated with anti-chimera antibody (7G7B6) and with anti-Lamp-1 antibody (H4A3). The cells were then incubated with secondary labeled-antibody (anti-IgG2a-Texas Red and anti-IgG1-FITC) and analyzed by confocal microscopy. Merged images are shown where chimeric receptors are stained in green and the immunodetected intracellular markers (TfR or Lamp-1) in red. The yellow color indicates colocalization. (A) Control cells. (B) Cells expressing the Δ Lys mutant. (C) Lactacystin-treated cells. Bar, 5 μ M. (D) colocalization of the chimeric receptors with TfR or Lamp-1 was quantified as described in MATERIALS AND METHODS. (E) Colocalization of Lamp-1 with TfR in K562 $\alpha_V\beta_{18-27}$ cells incubated with or without lactacystin.-32767.

endosomes. This is a strong argument in favor of a direct role of receptor ubiquitination as a signal in sorting from early/recycling to late endocytic compartments. Interestingly, ubiquitination of proteins can be regulated by specific ubiquitin proteases (Wilkinson, 1997). In this respect, it is

worth noting that one of these deubiquitinating enzymes (named *DUB-2*) is induced by IL2 and down-regulated after the initiation of T-cell activation (Zhu *et al.*, 1997).

The role of ubiquitination in IL2R β sorting could be independent of proteasome function. In fact, in agreement with

this we detected only a monoubiquitinated form of IL2R β , whereas at least four residues of ubiquitin appear to be required for recognition by the proteasome (Deveraux *et al.*, 1994; Thrower *et al.*, 2000). However, treatment of cells with proteasome inhibitors also affected IL2R β and $\alpha_V\beta_{18-27}$ degradation. Moreover, we showed that the sorting of this receptor seemed to be altered because, in the presence of lactacystin, the $\alpha_V\beta_{18-27}$, which is normally directed toward degradation (Subtil *et al.*, 1997), colocalizes equally with TfR and the lysosomal marker Lamp-1. These data suggest that the proteasome function is necessary for a proper sorting of receptors carrying IL2R β signal. However, we observed that, in the presence of the proteasome inhibitor, the extent of colocalization of two markers of normally distinct compartments, TfR and Lamp-1, was increased. This indicates that the proteasome inhibitor might have a general effect on intracellular trafficking, compromising several cellular compartments, which may explain the effect on the degradation of IL2R β . Effect of proteasome malfunction in the overall defect of vacuolar integrity was also one of the models proposed for the proteasome-dependent degradation of the a-factor transporter in yeast (Loayza and Michaelis, 1998). Interestingly, a link between ubiquitin system and maintenance of intracellular integrity has also previously been described (Lenk *et al.*, 1992). In this report, it was shown, using ts20 mutant cell line, that a functional ubiquitination machinery is necessary for the maturation of autophagic vacuoles. The role of proteasome in the routing of membrane proteins from the endocytic to degradation compartments is unclear. One possible mechanism is that proteasome function might be involved in the regulation of a protein playing a crucial role in trafficking through the endocytic pathway. One potential candidate might be the recently described sorting nexin SNX15 (Barr *et al.*, 2000).

The third possible action of ubiquitin/proteasome system is on the degradation of the receptor itself. Proteasomal degradation of some mammalian receptors has previously been described. For the platelet-derived growth factor (Mori *et al.*, 1995), the Met Tyrosine kinase receptor (Jeffers *et al.*, 1997), and the GHR (van Kerkhof *et al.*, 2000), it has been proposed that both proteasomes and lysosomes might function to degrade different parts of given receptors. Herein, we have confirmed that lysosomes were involved in IL2R β degradation because degradation was inhibited by chloroquine or leupeptin. Because, as discussed above, proteasome inhibitors affected the trafficking of intracellular markers, we could not question whether proteasomes were also directly involved in the degradation of IL2R β . Again, the fact that only monoubiquitinated form of the β chain was detected argues against this possibility (Deveraux *et al.*, 1994; Thrower *et al.*, 2000).

In conclusion, our data, together with other recent reports, indicate that the ubiquitin/proteasome system, in addition to its involvement in cell cycle and signal transduction, in the elimination of endoplasmic reticulum-retained proteins, is also a key player in the modulation of the expression of cell surface growth factor receptors. Its role in many aspects of cell life (Bonifacino and Weissman, 1998; Ciechanover, 1998; Hershko and Ciechanover, 1998) supports the emerging idea that the proteasome/ubiquitin system might play an essential role in general intracellular protein routing and turnover.

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