Endocytosis of the Common Cytokine Receptor $\gamma_c$ Chain

IDENTIFICATION OF SEQUENCES INVOLVED IN INTERNALIZATION AND DEGRADATION*

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The common cytokine receptor $\gamma_c$, shared by interleukin 2, 4, 7, 9, and 15 receptors, has a major role in lymphocyte proliferation and differentiation, leading, when mutated, to a genetic disease, X-linked severe combined immunodeficiency. In this study, we report that $\gamma_c$ is internalized and degraded in lymphoid cells. To identify $\gamma_c$ regions involved in sorting along the endocytic pathway, we have studied a chimeric protein composed of the extracellular part of interleukin 2-receptor $\alpha$ and transmembrane and intracellular part of $\gamma_c$, $\alpha\gamma_c\omega$. When transfected in Jurkat T cells, $\alpha\gamma_c\omega$ is as efficiently internalized and degraded as $\gamma_c$, demonstrating that the transmembrane and cytosolic tail of $\gamma_c$ carry sequences involved in this process. To identify these motifs, we have analyzed the trafficking of chimeric proteins with serial truncations in their cytosolic tail. Internalization studies showed that the cytosolic tail of $\gamma_c$ contains three regions located between cytosolic amino acids 1-35, 35-40, and 40-65 involved in $\gamma_c$ endocytosis. Successive deletions of these motifs result in reduced endocytosis. One region containing the 5 cytosolic amino acids 36-40 is essential to direct $\gamma_c$ to the degradation pathway. These sorting sequences, by participating in the fine tuning of cell surface $\gamma_c$ expression, might somewhat regulate the cell responsiveness to interleukins whose receptors share this component.

Communication between cells in the immune and hematopoietic systems is mediated by soluble factors, cytokines, which exert their biological activities through specific cell surface receptors. The molecular structure of a growing number of cytokine receptors has been determined and led to the definition of a new family of receptors (1, 2). The hematopoietic cytokine receptor superfamily is defined by structural homology in the extracellular, ligand binding domain and limited similarity in the membrane proximal cytosolic regions (3). This family of receptors includes receptors to many interleukins (IL), in particular, IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL12, and IL15, receptors for hormones such as growth hormone and prolactin, and receptors for growth factors. Another characteristic of this family is that many receptors subfamily members share at least one component, explaining in part the redundant action of different cytokines. One member of this family of receptors was named the common $\gamma$ chain ($\gamma_c$); first described as the third component of the IL2 receptor (IL2R), it participates in the formation of high-affinity forms of IL2, IL4, IL7, IL9, and IL15 receptors (4–9). Because it is shared by these interleukin receptors, $\gamma_c$ has a critical role in lymphocyte differentiation and proliferation. Indeed, patients suffering from X-linked severe combined immunodeficiency have mutations in the gene encoding $\gamma_c$ (10, 11).

One of the early events that follows ligand binding to receptors on the cell surface is the internalization of the ligand-receptor complex. After internalization from the plasma membrane in early endosomes, receptors can either recycle to the cell surface or be degraded within intracellular compartments. Endocytosis and degradation are essential for the rapid down-regulation of surface receptors after ligand binding. Down-regulation results in a decrease in the number of receptors on the cell surface and, together with biosynthesis, controls the cell responsiveness. For several receptors, internalization is driven by the presence of specific sequences in their cytosolic part, recognized by the cellular machinery. These internalization signals have been classified into two groups, one characterized by a tyrosine-based motif and the other by a di-leucine-based motif (12, 13). They allow the recruitment of receptors into clathrin-coated pits and promote high-efficiency endocytosis. Whereas the molecular mechanisms that direct internalization from the plasma membrane to early endosomes are well documented for receptors, much less information is available concerning the sorting from early endosomes to the other intracellular compartments. For receptors to exit from the recycling pathway and to move to other intracellular destinations (e.g., in most cases toward the late endosomes and lysosomes) or to new domains on the cell surface (as in transcytosis) requires selective routing and depends upon the possession of additional, specific sorting signals. Indeed, tyrosine or di-leucine families of signals described for clathrin-coated pit endocytosis are also involved in lysosomal targeting of lysosomal membrane glycoproteins (reviewed in Ref. 12). Sequences necessary for the degradation of nonlysosomal membrane proteins have been reported for two proteins, the P-selectin and the IL2R$\beta$ chain (14, 15). In both cases a short sequence is sufficient for degradation after endocytosis. These two degradation signals are different and do not match the trafficking signals described so far.

The $\gamma_c$ chain is a part of IL2R, IL4R, IL7R, IL9R, and IL15R. Its role in endocytosis of these cytokines and their receptors is not known. We have previously shown that $\gamma_c$ is internalized and degraded when part of the IL2-IL2R complex (16) and that the cytosolic part of $\gamma_c$ is involved in IL2 endocytosis and in the

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1 The abbreviations used are: IL, interleukin; ILR, interleukin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; mAb(s), monoclonal antibody(ies); FITC, fluorescein isothiocyanate.
down-regulation of the IL2Rβ chain (17). These results suggest that γc may carry the necessary information for endocytosis and degradation. In the present study we show that the cytosolic tail of γc contains sequences involved in receptor endocytosis and degradation.

EXPERIMENTAL PROCEDURES

Cells, Monoclonal Antibodies, and Reagents
YT 12881, a subclone from the NK ceil line YT, was obtained from Dr. K. Smith (Dartmouth Medical School, Hanover, NH). The Jurkat 77.31.13 T cell line was a kind gift of Dr. A. Alcover (Institut Pasteur, Paris, France), and was stably transfected in J77 cells from now on (18). All cells were grown in suspension in RPMI 1640, 10% decomplemented fetal calf serum, and 10 mM HEPES, pH 7.2, supplemented with 2 mM L-glutamine. Stably transfected Jurkat cells were grown in the same medium supplemented with 400 μg/ml hygromycin (Boehringer Mannheim, Mannheim, Germany). Monoclonal antibodies (mAb) 2A3A1H (mouse IgG1) or 7G7B6 (mouse IgG2a), directed against the α chain of the IL2 receptor, were obtained from the American Tissue Culture Collection (Manassas, VA). TUGh4 (rat IgG2b), directed against the γ chain of the IL2 receptor, was obtained from PharMingen (San Diego, CA). Monoclonal antibodies FG 1/6 (mouse IgG1), directed against the human transferrin receptor, were a kind gift from Dr. B. Alarcon (Centro de Biologia Molecular Severo Ochoa, Madrid, Spain). The second antibodies used with mAb TUGh4 were FITC-conjugated anti-rat IgG antibodies (Southern Biotechnology Associates, Birmingham, AL), with 2A3A1H F(ab)2', phycoerythrin-conjugated goat anti-mouse IgG (Immunotech, Marseilles, France) and with 7G7B6 F(ab)2', FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). Cycloheximide and saponin were obtained from Sigma.

Plasmids

The constructs were subcloned in the NT expression vector, containing the SRα promoter, a kind gift of Dr. C. Bonnerot (Institut Curie, Paris, France). The plasmid pRCHγc, coding for the γc chain, and the plasmid pREP4, were kindly provided by Dr. J. Di Santo (Hôpital Necker, Paris, France). The plasmid T-CHO, coding for the IL2Rα chain, has already been described (15). The plasmid pME18Sα was kindly provided by Dr. W. Leonard (National Institutes of Health, Bethesda, Md.), pRPea was constructed by subcloning IL2Ra from TXO into the KpnI/XhoI site of the plasmid pREP4. It contains a BgIIII restriction enzyme site just upstream the transmembrane part of IL2Ra. The transmembrane and cytosolic regions of human γc were derived by polymerase chain reactions (PCR) using pRCHγc and primers with terminal BglII and XhoI restriction enzyme sites. pRPeaγγc was obtained by ligating the transmembrane and cytosolic regions of γc, to the pRPea digested with BglII and XhoI.

The truncated forms of αγγc were generated by PCR by insertion of a stop codon after the nα amino acid of the cytoplasmic part of the protein (assuming that Glutamine is the first cytosolic amino acid) and were cloned into the NotI/XhoI sites of NT by standard techniques. They were used for stable transfections in J77 cells. The plasmid TXOγγc and TXOγγc were constructed by inserting amino acids 25–65 or 35–65 from the cytosolic tail of γc, derived by PCR into the HindIII/XhoI cloning cassette, localized at the 3′ end of IL2Ra cDNA (15). These plasmids were stably transfected in J77 cells. Sequences were confirmed when synthetic oligonucleotides or PCR were involved.

Cell Transfection

All endocytosis and half-life analyses described here have been performed in stably transfected J77 cells. To generate stable transfectants, 20 × 10⁶ J77 cells were washed once in culture medium and resuspended in 800 μl of the same medium, with 20 μg of the plasmid of interest. Electroporation was performed using the Easyjet electroporator (Eurogentec, Serain, Belgium) with a simple pulse, 260 V, 900 microfarads. Selection with 400 μg/ml hygromycin was initiated 2 days after transfection, and the cells were cloned in 96-well dishes. Hygromycin-resistant clones were assayed for expression by flow cytometry using anti-α (2A3A1H) antibodies. The expression levels of recombinant product were then assayed by SDS-PAGE and the presence of their normal level in activated lymphocytes. J77 cells stably transfected with the αγγc construct will be called J77αγγc from now on.

Endocytosis of Antibodies

Endocytosis of antibodies directed against the chimeric molecules was quantitated by flow cytometry essentially as described previously. Briefly, 5 × 10⁵ J77αγγc cells were incubated at 4 °C for 60 min with the anti-IL2Ra 2A3A1H mAb (1/2000 ascitic fluid). The cells were washed once in phosphate-buffered saline (PBS) at 4 °C, and after incubation at 37 °C for the indicated times, the cells were rapidly cooled to 4 °C and washed twice in PBS, 2% fetal calf serum. The cells were incubated at 4 °C for 1 h with the IgG1Fab′, then incubated with a biotinylated goat anti-IgG secondary antibody, washed once at 4 °C, and the level of expression of the constructs on the cell surface was assessed by flow cytometry on a FACSscan (Becton Dickinson, San Jose, CA) immediately after labeling. Base line cell fluorescence intensity was determined with cells incubated only with the secondary antibody. For each time point, the distribution of antibodies was determined by immunofluorescence staining and flow cytometry on a FACScan (Becton Dickinson, San Jose, CA) immediately after labeling. All experiments were performed at least four times.

Cell Surface Half-life Measurement

To measure the half-life at the cell surface of γc and of the different αγγc constructs, cells were incubated with cycloheximide to prevent the synthesis of new receptors. After different times of incubation at 37 °C in culture medium with 50 μM cycloheximide, the cells were cooled to 4 °C, washed, and cell surface expression of the constructs was assayed by flow cytometry as described (20). Time 0 on the graph corresponds to a 30-min incubation in cycloheximide, which is the time required for a newly synthesized IL2 receptor to reach the cell surface (21). All experiments were performed at least four times.

Immunofluorescence and Confocal Microscopy

Endocytosis of Anti-γc, mAb—YT 12881 cells were incubated for the indicated times at 37 °C with TUGh4 mAb, before being washed in PBS at 4 °C and fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 60 min at 4 °C. Subsequent steps were performed at room temperature. The cells were washed once in PBS, quenched for 10 min on 50 μM NH4Cl in PBS, and washed once in PBS supplemented with 1 mg/ml bovine serum albumin. After two washes in the permeabilizing buffer (PBS with 1 mg/ml bovine serum albumin and 0.05% saponin), the presence of antibodies was revealed by incubating the cells for 60 min in PBS containing FITC-conjugated anti-rat IgG antibody (1/50).

Direct Observation of γc, IL2Ra, or αγγc Inside the Cells—Either HeLa cells, grown on coverslips and transfected 2 days before with the plasmid of interest, or YT 12881 cells were used. Exponentially growing cells, incubated for 45 min with 50 μM cycloheximide, were washed twice with PBS and permeabilized as described above. The cells were then incubated in permeabilizing buffer for 1 h with TUGh4 mAb (1/50) for YT 12881 cells and with 7G7B6 mAb (1/800 ascites) for HeLa cells. After two washes in permeabilization buffer, cells were labeled with the second FITC-labeled antibody for 1 h in the same buffer. After washes and sample mounting, cells were examined by epifluorescence microscopy (for the YT 12881 cells) or by epifluorescence microscopy (for HeLa cells). Washes, sample mounting, and confocal microscopy were performed as described (16). No immunofluorescence staining was ever observed when second antibodies were used without the first antibody or with an irrelevant first antibody.

Cell Iodination

YT cells (2 × 10⁵) washed in PBS and resuspended in PBS, pH 7.3, 1 mM Ca²⁺, 1 mM Mg²⁺ were surface-labeled using the lactoperoxidase method with 1.5 μCi of Na¹²⁵I (22). After iodination, the cells were washed twice in culture medium and then kept at a 37 °C incubator. After a 10-min incubation, considered as time 0, cells were harvested at 30-min intervals, washed in PBS, pelleted by centrifugation, and kept frozen at −20 °C before analysis by immunoprecipitation.

Immunoprecipitation and Gel Analysis

Cells were lysed for 30 min at 4 °C in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) complemented with 1 mM EDTA, 50 mM NaF, 1 mM NaVO₄, 10 μg/ml leupeptin, 20 μg/ml aprotonin, and 2 mM phenylmethylsulfonyl fluoride). Insoluble material was pelleted at 15,000 × g for 15 min, and the supernatant was then preclared for 120 min at 4 °C with protein A-Sepharose CL-4B (Amersham, Buckinghamshire, UK). Fractionated fractions were immunoprecipitated overnight at 4 °C with relevant first antibodies and protein A-Sepharose coated with anti-rat antibody (Southern Biotechnology Associates). The first antibodies were anti-γc, mAb TUGh4 (1 μg/ml) and anti-transferrin receptor mAb FG1/6 (1/200 ascitic fluid) for the measurement of γc and transferrin receptor half-life after iodination. The Sepharose beads were then washed three times in 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, before being resuspended in 0.5% NP-40 and 150 mM NaCl.
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**FIG. 1.** Internalization of \( \gamma_c \) in YT 12881 cells. \( a \), localization of \( \gamma_c \) in endocytic vesicles. YT 12881 cells were incubated for 45 min in 50 \( \mu \)M cycloheximide to prevent proteins synthesis and then fixed and permeabilized. The localization of \( \gamma_c \) was then revealed by incubating permeabilized cells with TUGh4 mAb, followed by FITC-anti-rat antibodies. \( b \), localization of internalized anti-\( \gamma_c \) antibodies. The cells were incubated for 30 min at 37 °C with TUGh4 and then fixed and permeabilized. The antibody localization was revealed with FITC-labeled second antibodies. Samples were analyzed by confocal microscopy. One representative medial optical section is presented. The cell contour is drawn.

0.5 \( \times \) NaCl, 10 \( \times \) Tris-HCl, pH 8.0, and once in 10 \( \times \) Tris-HCl, pH 8.0. Bound proteins were eluted into electrophoresis sample buffer (60 \( \times \) Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% \( \beta \)-mercaptoethanol) before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were fixed and dried, and radioactivity in the gel bands was quantitated using a PhosphorImager and ImageQuaNT software (Molecular Dynamics, Inc., Sunnyvale, CA). Gels were subsequently exposed to Hyperfilm-MP (Amersham, Pharmacia Biotech, Les Ulis, France) at -80 °C.

**RESULTS**

The \( \gamma_c \) Chain Is Constitutively Internalized in the NK Cell Line YT12881—When receptors are constitutively internalized, some of them are found in endocytic intracellular compartments at any time. To study the endocytic behavior of \( \gamma_c \), we observed its intracellular localization by immunofluorescence. First, the cellular location of \( \gamma_c \) inside the cells was studied in the absence of any ligand. To rule out a potential \( \gamma_c \) staining from the secretory pathway, the cells were treated with cycloheximide to prevent protein synthesis. In the NK cell line YT12881, \( \gamma_c \) staining appears as bright intracellular vesicles dispersed in the cytoplasm (Fig. 1\( a \)). Cell surface receptors are too sparse to be detectable. Second, mAbs were used as ligands to study the capacity of \( \gamma_c \) to be internalized in YT12881 cells. The cells were incubated with anti-\( \gamma_c \) mAB TUGh4 for 30 min at 37 °C and after permeabilization stained with a FITC-labeled secondary antibody. Anti-\( \gamma_c \) antibodies are found in intracellular endocytic vesicles (Fig. 1\( b \)). These two methods show that \( \gamma_c \) is endocytosed in the YT 12881 cell line.

The \( \gamma_c \) Chain Is Degraded after Endocytosis—We have shown previously that the measure of the half-life of a receptor on the cell surface allows for the analysis of both internalization and degradation of the receptor (15, 16, 20). At steady state, the total number of cell surface receptors results from balance between receptor biosynthesis and endocytosis. After endocytosis, most membrane molecules are recycled back to the cell surface or degraded. The level of expression at the cell surface of a protein that is entirely recycled after internalization remains the same, even when protein synthesis is inhibited. On the other hand, the level of expression at the cell surface of a protein that is not recycled after internalization decreases with time when protein synthesis is inhibited.

We have measured the half-life on the cell surface of \( \gamma_c \) in the absence of any ligand in the YT12881 cell line. Cells were incubated with cycloheximide to prevent the synthesis of new receptors. The surface expression of the \( \gamma_c \) chain was then probed with anti-\( \gamma_c \) mAb by flow cytometry measuring the mean fluorescence intensity. The half-life of \( \gamma_c \) in the YT12881 cell line was about 120 min (Fig. 2\( A \)). This short half-life suggests that \( \gamma_c \) is degraded after endocytosis.

To show that \( \gamma_c \) was degraded, its turnover was measured in YT12881 cells after surface labeling. Cells were iodinated and incubated in medium at 37 °C for different times as described under "Experimental Procedures." Proteins were quantitatively immunoprecipitated from cell lysates using antibodies against the \( \gamma_c \) chain and the transferrin receptor, the precipitates resolved by electrophoresis, and the radioactivity in the band corresponding to the transferrin receptor (not shown) or the \( \gamma_c \) chain was quantitated by PhosphorImager analysis (Fig. 2\( B \)). The transferrin receptor, which recycles very efficiently to

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**FIG. 2.** Degradation of \( \gamma_c \) in YT 12881 cells. \( A \), cell surface half-life of \( \gamma_c \). Cell surface expression of \( \gamma_c \) on cells treated for different times with 50 \( \mu \)M of cycloheximide was assessed by flow cytometry using TUGh4 mAb. \( B \), degradation of \( \gamma_c \). The cells were iodinated, washed, and incubated in medium. At the indicated time, the cells were harvested and lysed. Transferrin receptor and \( \gamma_c \) were immunoprecipitated from the lysates with anti-transferrin receptor FG1/6 mAb and anti-\( \gamma_c \) TUGh4 mAb and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radioactivity in the bands was quantitated by PhosphorImager analysis. The ratio of \( \gamma_c \) to transferrin receptor was calculated at each time point. The values in the graph represent the percentage of this ratio at different times relative to that at time 0. Immunoprecipitation of \( \gamma_c \) is shown in the inset. One representative experiment of three is presented.
the plasma membrane after endocytosis, was stable over the 2 h of chase, whereas the intensity of the band corresponding to the γc chain decreased with a half-value of about 120 min. This value is the same as that measured for the half-life of γc chain on the cell surface. Thus, the loss of the γc from the cell surface when protein synthesis is inhibited can be equated with degradation. This was shown previously for the IL2Rβ chain (15, 20). Because measuring the half-life on the cell surface is a simple and quantitative method, it was used to probe for receptor turnover in subsequent studies.

We conclude from these experiments that γc is internalized and degraded in the YT12881 cells.

The Transmembrane and Cytosolic Region of γc Chain Are Sufficient for Endocytosis and Degradation—The γc chain is a component of at least five interleukin receptors. The regulation of its surface expression by endocytosis and degradation may affect the response to the corresponding cytokines. IL2Rβ, an another IL2R component, also expressed in the YT12881 cells, carries its own endocytic and degradation signals (20). To rule out a potential role of IL2Rβ in the endocytic behavior of γc, even though these two proteins are not associated in the absence of ligand (23), and to study the intrinsic molecular mechanisms involved in γc internalization and degradation, we have transfected and studied the intracellular traffic of a chimeric molecule composed of the extracellular region of IL2Ra and the transmembrane and intracellular regions of γc, called αγγc, in the Jurkat cell line J77. We have used this T cell line because it does not express any of the IL2 receptor components on its cell surface, as assessed by cytofluorimetry (not shown). IL2Ra does not carry any signals for its endocytosis and degradation (15, 16) and has been used previously to prepare other chimeric membrane proteins, because good antibodies against its extracellular domain are available (15, 24, 25). It was therefore a good tool to study the potential role of the transmembrane and cytosolic domain of γc in endocytosis and degradation. The endocytosis of IL2Ra and αγγc was quantitated by flow cytometry in stably transfected J77 cells as described under “Experimental Procedures.” The chimeric molecule αγγc was internalized very efficiently in contrast to IL2Ra, which was very poorly internalized (Fig. 3A). Similar results were obtained by studying the endocytosis of both proteins with radiolabeled antibodies (data not shown). Also, HeLa cells were transiently transfected with IL2Ra or the αγγc construct, and 2 days after transfection, the cells were processed for immunofluorescence using anti-IL2Ra mAb. Staining of IL2Ra-transfected cells showed a strong surface labeling (Fig. 3B, left), whereas in αγγc-transfected cells, αγγc was found in intracellular compartments (Fig. 3B, right).

We also measured the half-life of IL2Ra and αγγc at the cell surface, as described above, in stably transfected J77 cells. Their surface expression was probed with anti-IL2Ra mAb 2A3A1H. As shown in Fig. 3C, when protein synthesis was inhibited, IL2Ra had a very long half-life, as described previously in other cell lines (15, 22). In contrast, the chimeric construct αγγc had a short half-life of about 20 min, indicating that it is degraded after endocytosis.

In summary, the chimeric protein αγγc is internalized and degraded and has the same half-life as γc. We conclude that the transmembrane and cytosolic regions of γc carry signals for its internalization and degradation.

The Cytosolic Tail of γc Contains Different Regions Involved in Its Internalization—To further characterize the region(s) involved in the internalization of γc, we constructed truncated forms of the chimeric molecule αγγc, with 1, 25, 35, 40, and 65 cytosolic amino acids (αγγc1, αγγc25, αγγc35, αγγc40, αγγc65, respectively) represented schematically in Fig. 4A. These constructs...
were stably transfected in J77 cells, and their endocytosis was measured by flow cytometry as described above. Four patterns of internalization kinetics were obtained (Fig. 5). The construct αγγ65 was internalized as fast as αγγ65 wt, showing that the last 21 carboxyl amino acids of γc are not necessary for endocytosis. Internalization of the chimera αγγ65 was slow and inefficient, with only 10–15% of the molecules being internalized at 20 min. Thus, most of the internalization signals are located between the first and the 65th cytosolic amino acid. The third pattern of internalization, observed for αγγ25 and αγγ35, and the fourth one, observed for αγγ40, show two intermediate profiles of internalization. The αγγ25 and αγγ35 constructs were endocytosed in a similar fashion, with ~25% of internalization at 20 min, whereas αγγ40 was more efficiently internalized (~40% of endocytosis at 20 min) but not as fast as the αγγ65 wt and the αγγ65 constructs.

These results show that three regions in the cytosolic tail of γc seem to contain sequences involved in its endocytosis. These regions are located within amino acids 1–35, 35–40, and 40–65 in the cytosolic tail. Strikingly, when these motifs are successively deleted, endocytosis of the remaining receptor is progressively diminished.

The Cytosolic Amino Acids 36–40 of γc Are Necessary for Degradation—We have shown above and elsewhere (16, 17) that a receptor that is degraded after its internalization has a short half-life on the cell surface, whereas the half-life of an internalized receptor that recycles to the plasma membrane is long. Therefore, to study the fate of the different chimeric constructs after internalization, and to determine the regions involved in sorting toward degradation, we have measured their half-life on the cell surface in stably transfected cells. The αγγ40 and αγγ65 constructs have a short half-life of about 120 min, similar to that of αγγ65 wt (Fig. 6). These results suggest that the three constructs are efficiently degraded after their internalization, in the same fashion as γc. Thus, the first 40 cytosolic amino acids of γc contain sequences sufficient to promote the degradation of the receptor. In contrast, the half-life of the αγγ25 and αγγ35 constructs was very long (Fig. 6). Thus, it appears that these two chimeric proteins are not degraded. Taken together with the results presented in Fig. 5, these experiments show that the deletion of the last 5 amino acids of αγγ40 leads to a complete loss of degradation and only a partial decrease of internalization. Therefore, these amino acids may belong to a strong degradation and a weak endocytic signal. The αγγ65 wt and truncated forms of γc do not contain another degradation signal. In conclusion, whereas the successive deletion of three different cytosolic sequences leads to a progressive decrease of endocytosis of the constructs, the deletion of the 36–40-amino acid sequence leads to complete loss of degradation.

The Cytosolic Amino Acids 25–65 of γc Contain Transferable Internalization and Degradation Signals—To demonstrate positively that the distal region of γc contains sequences directing internalization and degradation, we constructed chimeric molecules, αγγ25-ag and αγγ35-ag, between the entire IL2Rα chain and amino acids 25–65 or 35–65 from the cytosolic part of the γc chain (Figs. 7C and 8C). The internalization of the chimera in stably transfected J77 cells was measured by flow cytometry as described above. The chimeric molecule αγγ25-ag and αγγ35-ag were efficiently internalized in J77 cells, in contrast to IL2Rα (Figs. 7A and 8A). We also determined their half-lives at the cell surface in stably transfected cells by flow cytometry. As seen in Fig. 7B the
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**DISCUSSION**

Endocytosis and degradation of the cytokine receptors participate in the fine tuning of their expression on the cell surface and control the cell responsiveness to some extent. In this report we show that γc, common to at least five interleukin receptors, is constitutively internalized and degraded in lymphoid cells. We have replaced the extracellular region of γc by the extracellular part of IL2Ra. This allowed us to follow the derived chimeric constructs with available anti-α antibodies. Internalization studies show that three regions in the cytosolic tail of γc are involved in endocytosis. These regions are located between amino acids 1–35, 35–40, and 40–65 (Fig. 5). Interestingly, successive deletions of these regions lead to a decrease in endocytosis, suggesting that normal endocytosis involves several motifs along the cytosolic tail and not only one specific short sequence as described for other receptors (12). The role of various cytosolic regions in endocytosis has been described previously for receptors such as the F-selectin (26), the prolatin receptor (27), and is also found in the IL2Rβ chain (28). The cytosolic domains of many membrane proteins have short sequences, usually including a tyrosine or a di-leucine motif, which mediate their rapid internalization through clathrin-coated pits. The cytosolic tail of γc contains two Tyr and two Leu-Val sequences between the first and the 65th amino acid (Fig. 4B). Further studies are needed to determine whether these amino acids are part of the internalization motifs of IL2Rγc. The αγγc construct, although poorly internalized, is endocyotosed more efficiently than IL2Ra, suggesting that the transmembrane part of γc may also participate in the endocytic process.

Besides the clathrin-mediated endocytic pathway, receptors can also be internalized via an alternative pathway. Indeed, receptor-mediated endocytosis pathways through non-clathrin-coated pits have been reported for ricin (29, 30), epidermal growth factor (31), and cholera and tetanus toxins (32, 33). Interestingly, this pathway has also been described for IL2 (34) whose receptors contain γc. The three regions found in the cytosolic tail of γc may carry signals recognized by the cellular components of this alternative pathway. In this case, the other cytokines whose receptors share the γc chain might also be similarly internalized.

In the absence of clathrin-coated-pit structures, IL2 endocytosis is 2-fold less efficient (34), suggesting that some IL2Rs may also be internalized via the clathrin-coated pit pathway. In this case, the three cytosolic regions of γc may contain sequences recognized with low affinity by the adaptor complexes. Addition of three weak signals may constitute an efficient signal. We have already prepared a chimeric molecule in which the internalization signal of the transferrin receptor YTRF was inserted in the cytosolic region of IL2Ra (15). When transfected in J77 cells, this chimeric molecule is internalized more efficiently than αγγmwt indicating that the YTRF motif forms a stronger internalization signal than the addition of the three regions described above.

The cytosolic domain of γc associates constitutively with the...
tyrosine kinase Jak3 (8, 35). Jak3 has a key role in $\gamma_c$-mediated signal transduction. It is phosphorylated and activated by cytokines such as IL2 and IL4, whose receptors contain $\gamma_c$ (36, 37), and its activation correlates with lymphocyte proliferation (37, 38). The cytosolic region of $\gamma_c$ necessary for binding and activating Jak3 is located between residues 40 and 52 (38). Here we show that the first 40 cytosolic amino acids of $\gamma_c$ are sufficient for internalization and degradation, suggesting that Jak3 is dispensable in endocytic process. The region between residues 40 and 65, which contains the association site with Jak3, increases endocytosis, which might suggest that activation of Jak3 might participate in efficient endocytosis, as do other kinases in endocytosis of epidermal growth factor and insulin receptors (39–41). However, in the case of IL2-dependent Jak3 stimulation, the serine-rich region of the IL2R$\beta$ chain is required to obtain the phosphorylation of Jak3 (37), indicating that stimulation via $\gamma_c$ alone does not allow the activation of Jak3. In our cells, the chimeric constructs were studied independently of other components of cytokine receptors and should not be able to activate Jak3. Therefore, it is unlikely that Jak3 plays a role in the process of endocytosis.

The half-lives of the different chimeric constructs show that a region containing the 36–40 cytosolic amino acids is involved in the degradation of $\gamma_c$. Indeed, whereas the deletion of the last 46 carboxyl amino acids does not modify degradation, deletion of amino acids 36–40 leads to a complete loss of degradation. Although $\alpha Y_{\gamma 5 a}$ is internalized less efficiently than $\alpha Y_{\gamma 40}$ (Fig. 5), this reduced efficiency of endocytosis is unlikely to account for the absence of degradation of $\alpha Y_{\gamma 5 a}$. In this respect, it is worth noting that $\alpha Y_{\gamma 5 a}$ is degraded as fast as $\alpha Y_{\gamma 65}$ and $\alpha Y_{\gamma wt}$, although its internalization is less efficient (Fig. 5). Thus, the 36–40-amino acid ESLQP sequence may belong to a strong degradation signal. Interestingly, these amino acids are a potential target for a protease, calpain, found to associate to $\gamma_c$ in a double hybrid assay and to degrade it in vitro (42). Although many internalization signals have now been described, less is known about the mechanisms by which internalized receptors are directed toward degradation. Sequences necessary for internalization and degradation of plasma membrane proteins have been reported (24, 43–46). However, in these cases, it has not been shown that a short sequence is sufficient to serve as degradation signal. Two cases of a membrane molecule with a short sequence sufficient for lysosomal degradation after endocytosis have been reported for the cell adhesion molecule P-selectin (14) and for the IL2R$\gamma$ chain (15). In both cases, the degradation signal does not match with any of the signals described so far.

Altogether, our results suggest that endocytosis of $\gamma_c$ is mediated by discrete internalization and degradation motifs. This is confirmed by the fact that addition of amino acids 25–65 of $\gamma_c$ confers internalization and degradation to a membrane protein, which is by itself neither internalized nor degraded (IL2R$\alpha$). In conclusion the 25–65-amino acid cytosolic sequence of $\gamma_c$ contains both internalization and degradation signals. Interestingly, when only amino acids 35–65 of $\gamma_c$ were transferred instead of amino acids 25–65, the chimera was similarly internalized, but it was not degraded. This confirms that the internalization and degradation signals are not the same. The positive data shown thus far map the degradation motif to amino acids 25–40 (conferved by the 25–65-amino acid chimera, retained by the truncation mutant). Further experiments will be necessary to precisely define the minimal transferable degradation signal.

The presence of endocytic and degradation signals in $\gamma_c$ suggests that it participates in endocytosis and degradation of cytokines whose receptors share the $\gamma_c$ chain. The role of $\gamma_c$ in cytokine receptors internalization has been essentially reported for IL2Rs. High-affinity IL2Rs are composed of three chains (IL2R$\alpha$, IL2R$\beta$, and $\gamma_c$) that associate noncovalently on the cell surface after ligand binding. The first report concerning the role of $\gamma_c$ in IL2Rs endocytosis showed that IL2 is internalized only if $\gamma_c$ is present (47). However, this does not clarify the molecular mechanisms and the respective roles of IL2R$\beta$ and $\gamma_c$ in IL2 endocytosis, as the IL2 binding also depends on the composition of the receptor. We have shown previously that in a B-cell line derived from a patient suffering from a X-linked severe combined immunodeficiency (ST B-cell line), whose IL2Rs are composed of normal IL2R$\alpha$ and IL2R$\beta$ chains, and truncated $\gamma_c$ (containing only the 6 first residues in its cytosolic tail), IL2 is internalized 2-fold less efficiently than in the normal control B-cell lines (17). This difference between the B-cell lines was not because of a modification in the ability of IL2 to bind to IL2Rs. It is rather likely because of the loss of the $\gamma_c$ endocytic signals that we have characterized in this report. This indicates that in normal IL2Rs, the cytosolic regions of $\gamma_c$ can account for 50% of the ligand internalization. In contrast to IL2R$\alpha$, which does not carry any internalization sequences, IL2R$\beta$ carries also endocytic and degradation signals (15, 20). Therefore, from these and other previously reported data (15–17, 20, 21, 48), it appears that IL2R endocytosis takes place as follows: both IL2R$\beta$ and $\gamma_c$ carry their own endocytic signals. Upon heterodimerization of both chains by IL2, these signals...
are used. Therefore, the rate of entry of high-affinity receptors is higher than that of the separate chains, leading to a decrease in surface receptor expression. The role of $\gamma_c$ in receptor endocytosis, also reported for IL15 (49), is likely to concern the other receptors containing $\gamma_c$. Its behavior after endocytosis induced by one cytokine may allow for a fine balance to be achieved between the expression of the other cytokine receptors containing $\gamma_c$ on the same cells. For instance, in NK cells, where both IL2Rs and IL15Rs are expressed on the cell surface, IL2-induced down-modulation of $\gamma_c$ (50) decreases the number of $\gamma_c$ on the cell surface available for the formation of IL15 receptors and is likely to control the response of both cytokines. It will be worthwhile to determine whether such regulation, previously reported for IL6R (51), can apply to the other $\gamma_c$-containing cytokine receptors and to members of the expanding family of cytokine receptors sharing one component.

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REFERENCES

1. D'Andrea, A. D., Fasman, G. D., and Lodish, H. F. (1989) Cell 58, 1023–1024
2. Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6934–6938
3. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Tago, T., and Kishimoto, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11349–11353
4. Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993) Science 262, 1877–1880
5. Giri, J. G., Ahdieh, M., Eisenman, J., Shanesack, K., Grubstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) EMBO J. 13, 2822–2830
6. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I., and Sugamura, K. (1993) Science 262, 1874–1877
7. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S.-I., and Sugamura, K. (1994) Science 265, 1453–1454
8. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I., and Sugamura, K. (1994) Science 266, 1042–1045
9. Noguchi, M., Yabuuchi, K., Orimo, A., Sato, A., Fujishima, H., and Edery, I. (1994) Science 265, 56–58
10. Nakamura, Y., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I., and Sugamura, K. (1994) Science 266, 1042–1045
11. DiSanto, J. P., Dautry-Varsat, A., Certain, S., Fischer, A., and de Saint Basile, G. (1996) Blood 88, 1708–1717
12. Sandovig, K., Oulnes, S., Petersen, O. W., and van Deurs, B. (1987) J. Cell Biol. 105, 679–689
13. Lund, K. A., Opresko, L. K., Starbuck, C., Walsh, B. J., and Wiley, H. S. (1990) J. Biol. Chem. 265, 15713–15723
14. Green, S. A., Setiadi, H., McEver, R. P., and Kelly, R. B. (1994) J. Biol. Chem. 269, 26818–26826
15. Subtil, A., and Dautry-Varsat, A. (1995) J. Biol. Chem. 270, 525–530
16. He´mar, A., Subtil, A., Di Santo, J. P., and Dautry-Varsat, A. (1994) Eur. J. Immunol. 24, 1851–1855
17. Duprez, V., Cornet, V., and Dautry-Varsat, A. (1988) J. Biol. Chem. 263, 12860–12865
18. Alcover, A., Alberini, C., Acuto, O., Clayton, L. K., Tranzy, C., Spagnoli, G. C., Moingeon, P., Lopez, P., and Reinherz, E. L. (1988) EMBO J. 7, 1973–1977
19. Marks, M. S., Woodruff, L., Ohno, H., and Bonifacino, J. S. (1996) J. Cell Biol. 135, 341–354
20. Hémar, A., Lieb, M., Subtil, A., Di Santo, J. P., and Dautry-Varsat, A. (1994) Eur. J. Immunol. 24, 1851–1855
21. Yamaguchi, M., Michishita, M., Hirayoshi, K., Yasukawa, K., Okuma, M., and Nagata, K. (1992) J. Biol. Chem. 267, 22035–22042