

# Endocytosis of Interleukin 2 Receptors in Human T Lymphocytes: Distinct Intracellular Localization and Fate of the Receptor $\alpha$ , $\beta$ , and $\gamma$ Chains

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**Abstract.** Members of the cytokine receptor family are composed of several noncovalently linked chains with sequence and structure homologies in their extracellular domain. Receptor subfamily members share at least one component: thus the receptors for interleukin (IL) 2 and IL15 have common  $\beta$  and  $\gamma$  chains, while those for IL2, 4, 7, and 9 have a common  $\gamma$  chain. The intracellular pathway followed by IL2 receptors after ligand binding and endocytosis was analyzed by immunofluorescence and confocal microscopy in a human T lymphocytic cell line. Surprisingly, the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains had different intracellular localizations after being endocytosed together. The  $\alpha$  chain was always in transferrin-positive compartments (early/recycling endosomes), both at early and late internalization times, but was never detected in

rab7-positive compartments (late endosomes). On the other hand, at late internalization times, the  $\beta$  and  $\gamma$  chains were excluded from transferrin-positive organelles and did not colocalize with  $\alpha$ . Furthermore,  $\beta$  could be found in rab7-positive vesicles. These differences suggest that the  $\alpha$  chain recycles to the plasma membrane, while the  $\beta$  and  $\gamma$  chains are sorted towards the degradation pathway. The half-lives of these three chains on the cell surface also reflect their different intracellular fates after endocytosis. The  $\beta$  and  $\gamma$  chains are very short-lived polypeptides since their half-life on the surface is only  $\approx 1$  h, whereas  $\alpha$  is a much more stable surface protein. This shows for the first time that components of a multimeric receptor can be sorted separately along the endocytic pathway.

**T**HE cytokine interleukin 2 (IL2)<sup>1</sup> is produced by activated helper T lymphocytes and stimulates proliferation and effector functions of a variety of cells of the immune system (36, 46, 57). High-affinity IL2 receptors ( $K_d \approx 10$ –100 pM) consist of three distinct components, the  $\alpha$  chain (IL2R $\alpha$ , 50–55 kD), the  $\beta$  chain (IL2R $\beta$ , 70–75 kD) and the  $\gamma$  chain (IL2R $\gamma$ , 65 kD), that are associated in a non-covalent manner (36, 57). Both the  $\beta$  and  $\gamma$  chains, but not the  $\alpha$  chain, belong to the cytokine receptor superfamily (1, 5). This hematopoietic cytokine receptor family includes receptors such as IL3R, IL4R, IL5R, IL6R, IL7R, IL9R, IL15R, the erythropoietin receptor, the granulocyte colony-stimulating factor receptor, the granulocyte-macrophage colony-stimulating factor receptor, and the leukemia inhibitory factor receptor. This family also includes receptor pro-

teins for factors that are believed to function normally outside the immune and hematopoietic system, i.e., growth hormone, prolactin and ciliary neurotrophic factor. They have an extracellular  $\approx 200$ -amino acid region of structural homology that is characterized by four conserved cysteine residues in the amino terminal half of this region and a Trp-Ser-X-Trp-Ser motif (where X is a non-conserved amino acid) at its carboxy-terminal end. Recently the IL2R $\gamma$  chain was found to also participate in the formation of high-affinity forms of IL4, IL7, IL9, and IL15 receptors (19, 32, 33, 39, 43, 44). IL2R $\beta$  is also a component of the IL15 receptor (19). IL2R $\gamma$  plays a critical role in thymic maturation of precursor human T lymphocytes since patients suffering from X-linked severe combined immunodeficiency have a mutation in the gene encoding this IL2R $\gamma$  chain (9, 10, 40).

IL2R $\alpha$  is not expressed on resting cells but is strongly and transiently induced following T cell activation (36, 48). The expression of high-affinity receptors (IL2R $\alpha\beta\gamma$ ) is transient and is preceded by IL2R $\alpha$  expression. In addition to high-affinity receptors, activated T lymphocytes express low-affinity receptors (IL2R $\alpha$ ,  $K_d \approx 10$  nM) and natural killer

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1. *Abbreviations used in this paper:* IL, interleukin; IL2R, IL2 receptor.

cells express intermediate-affinity receptors (IL2R $\beta\gamma$ ,  $K_d \approx 1$  nM) (46, 56). The high and intermediate-affinity receptors are capable of IL2 signaling. None of the IL2 receptor chains has tyrosine kinase activity, but protein tyrosine kinases such as p56<sup>lck</sup>, p59<sup>lyn</sup>, p53/56<sup>lyn</sup>, and JAKs are associated with IL2 receptors and some have already been shown to be implicated in signal transduction (2, 6, 28, 29, 31, 37, 43, 50, 51, 58). IL2 receptor stimulation is coupled to ras signaling via Shc and Grb2 (41, 45, 60).

One of the early events that follows IL2 binding to intermediate and high-affinity receptors on the cell surface is the internalization of IL2 receptor complexes (12, 46, 47). As a consequence, high-affinity receptors have a very short half-life on the cell surface, about 30 min in the presence of IL2 (11). This short half-life could have been accounted for by the internalization of only one component of the IL2 receptor. This does not appear to be the case, however, since the  $\alpha$  and  $\beta$  chains of high-affinity receptors are internalized together with IL2. Furthermore, after 10 min endocytosis, the multimolecular complex formed between IL2 and the receptor chains is found in endocytic compartments (13, 16, 18).

We have analyzed the intracellular fate of IL2R chains after their internalization. The endocytic compartments were identified using known markers such as transferrin and rab 7. The intracellular endocytic pathway of transferrin and its receptors has been extensively studied in numerous cell types. Transferrin accompanies its receptor through the recycling pathway (7, 30) and thus defines early and recycling endocytic organelles. Rab 7, a member of the rab family of small GTP-binding proteins, has been localized to late endocytic organelles and is a marker of such late compartments (3).

We have made use of a human tumor cell line, IARC 301.5, which constitutively expresses about 3,000 high-affinity IL2 receptors, internalizes IL2, and proliferates in response to this growth factor. Similarly to normal activated T lymphocytes, they also express low-affinity receptors (IL2R $\alpha$ ) to 5- to 10-fold higher levels than high-affinity (IL2R $\alpha\beta\gamma$ ) receptors (8, 11). Here we show that in the absence of protein synthesis, IL2R $\beta$  and  $\gamma$  are found in organelles distinct from those containing IL2R $\alpha$ . IL2R $\alpha$  is colocalized with transferrin after internalization as part of the IL2-receptor quaternary complex, and both IL2R $\alpha$  and transferrin can be found in a pericentriolar recycling compartment (34, 59). Also, IL2R $\alpha$  is never found in rab 7-positive compartments. On the other hand, IL2R $\beta$  can be found in late endocytic compartments containing rab 7, and IL2R $\beta$  and  $\gamma$  colocalize only partly with transferrin. Therefore, it appears that IL2R $\alpha$  is only found in early and recycling compartments while IL2R $\beta$  and  $\gamma$  are also detected in late endosomes. In agreement with these differences in morphology, we have shown that IL2R $\beta$ ,  $\gamma$ , and  $\alpha$  have different half-lives on the cell surface: IL2R $\beta$  and  $\gamma$  have a short half-life, about 1 h, whereas IL2R $\alpha$  is very stable, with a half-life of about 48 h (24). Thus the components of this multimolecular receptor have a different intracellular fate after internalization: one of the chains, IL2R $\alpha$ , recycles to the plasma membrane, while the others, IL2R $\beta$  and  $\gamma$ , are destined for degradation.

## Materials and Methods

### Cells, Monoclonal Antibodies, and Reagents

IARC 301.5 is a subclone from a cell line derived from a human T lympho-

phoma, that expresses high- and low-affinity IL2 receptors (11, 14). Cells were grown in RPMI 1640, 10% fetal calf serum, 10 mM HEPES, pH 7.2, supplemented with glutamine.

Monoclonal antibodies 7G7B6, directed against the  $\alpha$  chain of IL2 receptors (42) were obtained from the American Type Culture Collection (Rockville, MD). The anti-IL2 receptor  $\beta$  chain monoclonal antibodies 341 and 561 were kind gifts from Dr. R. Robb (Dupont Merck Pharmaceutical Co., Wilmington, DE) (54), and the rat monoclonal antibody against human IL2R $\gamma$ , TUGh4, was kindly provided by Dr. K. Sugamura (27). The anti-rab 7 purified rabbit antiserum (3) was kindly provided by Dr. M. Zerial (EMBL, Heidelberg, Germany).

To prepare TRITC-transferrin, human transferrin (Sigma Chem. Co., St. Louis, MO) was iron loaded (4) and labeled with lissamine rhodamine (Eastman Kodak), as described (59).

### Immunofluorescence and Confocal Microscopy

Exponentially growing cells, incubated with 50  $\mu$ M cycloheximide (Sigma Chem. Co.) and/or 200 pM IL2 (Roussel Uclaf, Romainville, France) and/or TRITC-transferrin and/or antibodies, were washed twice in cold PBS. For all labelings, cells were fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 15 min at 4°C. Subsequent steps were performed at room temperature. After quenching for 10 min in 50 mM NH<sub>4</sub>Cl in PBS, the cells were washed once in PBS supplemented with 1 mg/ml bovine serum albumin, and permeabilized for 15 min at 37°C in 0.05% saponin in the buffer used for washing. Cells were then incubated, when indicated, with the first antibody(ies) in the permeabilizing buffer for 1 h. After two washes in this permeabilizing buffer, the presence of antibodies was revealed by incubating the cells for 1 h in permeabilizing buffer containing labeled second antibodies. After three washes in permeabilizing buffer, and one wash in PBS, the cells were mounted in 25 mg/ml 1,4 diacylbicyclo (2.2.2) octane (Dabco; Sigma Chem. Co.), 100 mg/ml moviol (Calbiochem-Behring Corp., San Diego, CA) 25% (vol/vol) glycerol, 100 mM Tris-HCl, pH 8.5.

For 7G7B6, an IgG<sub>2a</sub>, the second antibodies were either FITC-labeled anti-murine Ig (1/50; Amersham Corp., Arlington Heights, IL) or Texas red-labeled anti-IgG<sub>2a</sub> (1/250; Southern Biotechnology, Birmingham, AL). For 341, an IgG<sub>1</sub>, the second antibodies were either the same FITC-labeled antibodies, as above, or FITC-labeled anti-IgG<sub>1</sub> (1/100; Southern Biotechnology) in double labeling experiments with 7G7B6. For TUGh4 monoclonal antibody, the second antibody was FITC-labeled anti-rat antibody (1/100; Southern Biotechnology). For anti-rab7 rabbit antiserum, the second antibody was TRITC-labeled anti-rabbit Ig (1/100; Amersham Corp.).

The samples were examined under a confocal microscope attached to a diaphan microscope equipped with a double laser, Argon-Krypton (Leica). Bleed-through from the FITC channel to the TRITC/Texas red channel was negligible in all experiments. Optical sections were recorded with a 63 $\times$  lens and a pinhole aperture such that the thickness of the sections was about 0.5  $\mu$ m. Photographs were taken on Ilford FP4 and Kodak Ektachrome 100 ASA.

No immunofluorescence staining was ever observed when second antibodies were used without the first antibody or with an irrelevant first antibody.

### Endocytosis of Radiolabeled IL2

Pure recombinant IL2 was radiolabeled with <sup>125</sup>Iodine by the chloramine T method to specific activities of 360  $\mu$ Ci/ $\mu$ g. For labeling, three successive additions of chloramine T were performed within 5 min at room temperature to a final concentration of 25  $\mu$ g/ml. The reaction was stopped after another 5 min, and labeled ligand was separated from free <sup>125</sup>I by passage through an Excellulose GF-5 column (Pierce Chemical Co., Piscataway, NJ).

For endocytosis experiments, 2–3  $\times 10^6$  cells were incubated in 100  $\mu$ l RPMI-HEPES, pH 7.2, 10% FCS, with 200 pM <sup>125</sup>I-IL2 at 37°C for various times. At the end of the incubation, the cells were washed twice at 4°C to remove unbound ligand. They were then subjected to two successive acid pH treatments (pH 2.8) to separate cell surface associated from intracellular <sup>125</sup>I-IL2 (12). This treatment removed more than 98% of surface associated <sup>125</sup>I-IL2. Nonspecific binding, measured by adding a 100-fold excess of the same unlabeled ligand, was <5% in every case. The data presented here show specific binding and internalization.

The amounts of degraded and undegraded material in medium were determined by precipitation of undegraded material with 10% TCA (12).

### Half-Life Measurements

Cell surface expression of IL2R $\beta$  or  $\gamma$  on cells treated for different times

with 50  $\mu$ M of the protein synthesis inhibitor cycloheximide in the presence of 200 pM IL2 was assessed by cytofluorimetry. The indicated times are after a 1 h preincubation in cycloheximide.

Surface expression of  $\beta$  and  $\gamma$  chains was measured as previously described by indirect immunofluorescence (24, 25) using the murine monoclonal antibody 341 directed against IL2R $\beta$  followed by phycoerythrin anti-Ig Fab2 (Immunotech, Luminy, France) or the rat monoclonal antibody TUGh4 directed against IL2R $\gamma$  followed by FITC-labeled anti-rat antibody (Southern Biotechnology). Cells were analyzed by flow cytofluorimetry on a FACScan (Becton Dickinson & Co., Mountain View, CA) immediately after labeling, and the mean fluorescence intensity was calculated from the recorded data.

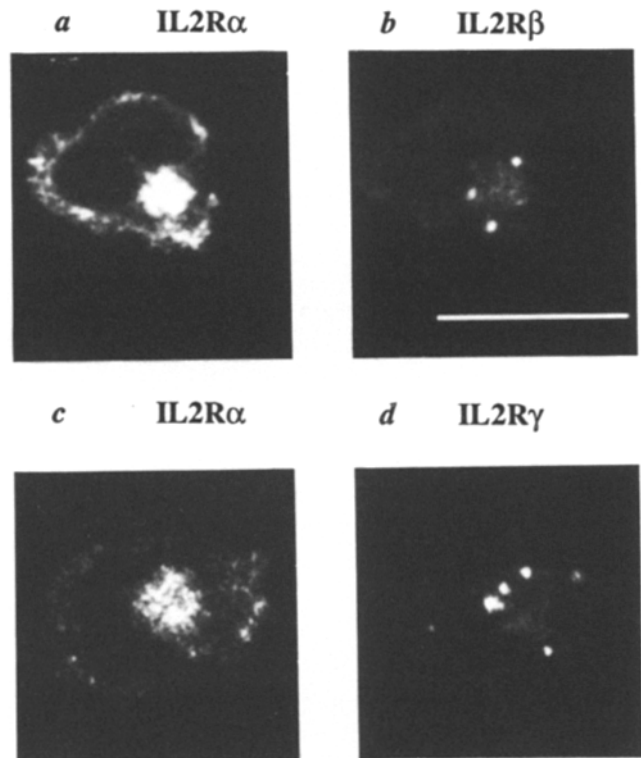
## Results

### *The $\alpha$ Chain of IL2 Receptors Is Not in the Same Endocytic Compartments in T Lymphocytes as the $\beta$ and $\gamma$ Chains*

Previous experiments demonstrated that both the  $\alpha$  and  $\beta$  chains of high-affinity IL2 receptors are internalized together with the ligand (13, 16). To observe the compartments reached by IL2 receptors by endocytosis, and not IL2 receptors along the biosynthetic pathway, the T lymphocytic human cell line IARC 301.5 was incubated with IL2 for 1 h with the protein synthesis inhibitor cycloheximide. We have previously shown that this cycloheximide incubation is sufficient to chase out the newly synthesized IL2 receptor chains en route to the cell surface (12, 25). The concentration of IL2 used is sufficient to saturate high-affinity receptors only, and cycloheximide does not inhibit their internalization although it slows it down by a factor two (12, 16, 25). After the one hour incubation with cycloheximide and IL2, the cells were fixed and permeabilized, and the  $\alpha$  and  $\beta$  chains were stained with monoclonal antibodies (IgG2a and IgG1) followed by subclass specific anti-Ig labeled with Texas red and FITC, respectively. It was checked that these second antibodies were indeed subclass specific and did not recognize the irrelevant first antibody. The samples were analyzed by confocal microscopy. Fig. 1 shows a representative medial optical section of IL2R $\alpha$  staining (a) and IL2R $\beta$  staining (b). When the same experiment was performed in the absence of cycloheximide, the same images were obtained showing that staining of the biosynthetic pathway is very weak (not shown).

This double staining experiment showed that IL2R $\alpha$  and  $\beta$  were not colocalized. The intracellular staining was different for IL2R $\alpha$  and  $\beta$ : IL2R $\alpha$  appeared as a densely stained compartment, while IL2R $\beta$  staining was weak in this compartment and appeared mostly in vesicles surrounding the compartment defined by IL2R $\alpha$ .

It is worth noting that IL2R $\beta$  was detected only in intracellular compartments, while IL2R $\alpha$  was found both inside the cells and at the plasma membrane. Detection of surface IL2R $\alpha$  is not surprising since these cells express 5–10-fold more IL2R $\alpha$  than IL2R $\beta$ . These IL2R $\alpha$  by themselves constitute the so called low-affinity receptors which are not internalized (13, 16, 17, 22, 38). Also, IL2R $\alpha$  could not be found in intracellular vesicles in the absence of IL2, in cells in which protein synthesis had been blocked by cycloheximide (not shown). IL2R $\alpha$  is thus internalized only when it is part of high-affinity receptors (IL2R $\alpha$ ,  $\beta$ , and  $\gamma$ ) and not when it forms low-affinity receptors by itself, whether or not IL2 is present. In conclusion, IL2R $\alpha$  observed intracellularly was internalized as a component of the quaternary com-



**Figure 1.** Localization of IL2R $\alpha$ , IL2R $\beta$ , and IL2R $\gamma$  in endocytic vesicles after IL2 endocytosis. The cells were incubated for 1 h at 37°C in the presence of 50  $\mu$ M cycloheximide and 200 pM IL2, and then fixed and permeabilized. IL2R $\alpha$  and  $\beta$  or  $\gamma$  localizations were then revealed by incubating the cells with anti-IL2R $\alpha$  antibody, 7G7B6 (IgG<sub>2a</sub>), together with anti-IL2R $\beta$  antibody, 341 (IgG<sub>1</sub>) or with anti-IL2R $\gamma$  antibody, TUGh4 (from rat) followed by Texas red anti-murine IgG<sub>2a</sub> and FITC anti-murine IgG<sub>1</sub> or FITC anti-rat immunoglobulins. Representative medial optical cuts show (a) IL2R $\alpha$  and (b) IL2R $\beta$ , or (c) IL2R $\alpha$  and (d) IL2R $\gamma$ . Bar, 10  $\mu$ m.

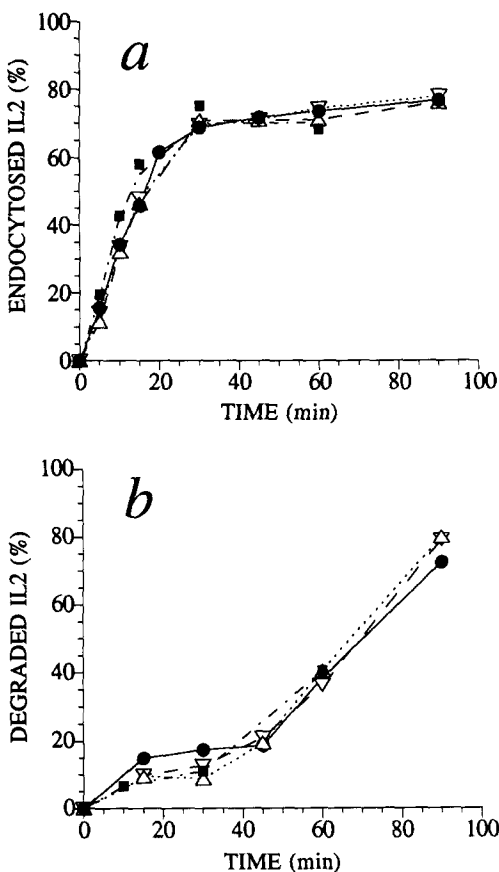
plex comprised of the high-affinity receptors harboring bound IL2.

Until now nothing was known concerning the internalization of IL2R $\gamma$ , since antibodies have become available only recently (27). We used the same approach to localize IL2R $\gamma$  in endocytic compartments as described above for IL2R $\beta$  and  $\alpha$ . Cells in which protein synthesis had been inhibited by cycloheximide were allowed to internalize IL2. The cells were then fixed and permeabilized, and stained for IL2R $\alpha$  and  $\gamma$  and analyzed by confocal microscopy. Representative medial optical sections of IL2R $\alpha$  staining (c) and IL2R $\gamma$  staining (d) are presented in Fig. 1. IL2R $\gamma$  can be observed in intracellular vesicles, and therefore is endocytosed. It is not found in the same compartments as IL2R $\alpha$  and its intracellular distribution resembles that of IL2R $\beta$ .

### *The $\alpha$ Chain of IL2R Is Restricted to Early, Sorting and Recycling Endosomes*

During receptor-mediated endocytosis, receptors and ligands are internalized in transport vesicles that deliver their cargo to the endosomal compartment. Some proteins then recycle back to the cell surface while others are sorted to a variety of destinations. The characterization and nomenclature of the endosomal compartments relies on either morphologi-

cal, kinetic, or functional considerations, in various cell types. They are at the moment difficult to reconcile (53). Transferrin remains bound to its receptor throughout its intracellular journey and recycles with a very high efficiency to the plasma membrane. We used TRITC-transferrin to stain the organelles through which it transits, i.e., the early, sorting, and recycling endosomes. Ligands and receptors destined to degradation are sorted from the transferrin-containing compartments towards late endosomes/prelysosomes and lysosomes. These late endosomes/prelysosomes are reached by fluid phase tracers after about 30 min, and the small GTP binding protein rab 7 is a specific marker for these compartments (3). To define the organelles containing the  $\alpha$  chain of IL2 receptors and to get some insight in its intracellular fate, we have compared its intracellular location with that of these two markers, transferrin and rab 7. Endocytosis of IL2R $\alpha$  was probed by internalizing IL2 together with a monoclonal antibody to IL2R $\alpha$ , 7G7B6. We first



**Figure 2.** Endocytosis and degradation of  $^{125}\text{I}$ -IL2 in the presence of anti-IL2R $\alpha$ ,  $\beta$ , and  $\gamma$  monoclonal antibodies. The cells were incubated at 37°C with 200 pM  $^{125}\text{I}$ -IL2 in the absence of antibody (●) or in the presence of anti-IL2R $\alpha$  antibody 7G7B6 (Δ) or anti-IL2R $\beta$  antibody 341 (▼) or anti-IL2R $\gamma$  antibody TUG4h (■). (a) Endocytosis of  $^{125}\text{I}$ -IL2: after the indicated times at 37°C, intracellular  $^{125}\text{I}$ -IL2 was measured as the radioactivity remaining cell associated after acid pH wash, as described in Materials and Methods. The ratio of intracellular to total cell associated radioactivity is presented. (b) Degradation: the amounts of degraded radioactive material in the medium was measured as non TCA precipitable. The ratio of degraded to total radioactivity in the extracellular medium is presented.

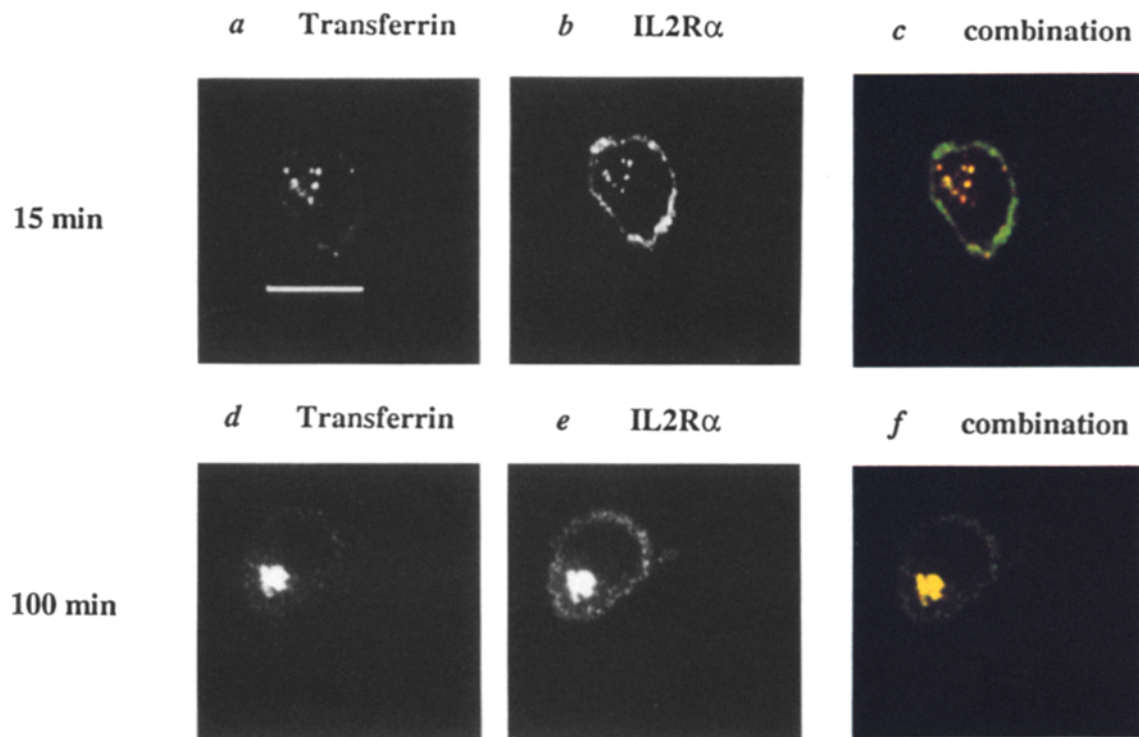
showed that this antibody does not modify IL2 binding (42) and does not affect either IL2 internalization or its degradation. For this purpose  $^{125}\text{I}$ -IL2 was incubated for different times at 37°C with or without the anti-IL2R $\alpha$  antibody, under the conditions to be used for confocal microscopy experiments. As shown in Fig. 2, the presence of anti-IL2R $\alpha$  antibody does not modify the kinetics of IL2 endocytosis and degradation. To visualize internalized transferrin and IL2R $\alpha$ , the cells were incubated at 37°C with 150 nM TRITC-transferrin, together with IL2 and 7G7B6 antibody. The cells were then washed at 4°C, fixed, permeabilized, and incubated with a second FITC-labeled anti-murine Ig antibody. The samples were analyzed by confocal microscopy. Representative medial optical sections, after 15 and 100 min incubation with the ligands at 37°C, are shown in Fig. 3. Intracellular vesicles stained with anti-IL2R $\alpha$  antibody were always co-stained with TRITC-transferrin, after 15 min as well as after a long time of internalization, 100 min. After 15 min endocytosis, transferrin and IL2R $\alpha$  staining appears as small vesicles throughout the cytoplasm. After longer incubations, e.g., 100 min, the appearance of the transferrin and IL2R $\alpha$  containing compartment had changed and it appeared as a juxta-nuclear compartment. Double staining for IL2R $\alpha$  or the transferrin receptor after 100 min ligand endocytosis, and for centrosomes with anti-centrosomal monoclonal antibody, revealed that both IL2R $\alpha$  and the transferrin receptor were in the pericentrosomal region (not shown). This peri-centrosomal compartment, which contains IL2R $\alpha$  and transferrin receptors after 100 min endocytosis, is probably the peri-centriolar recycling compartment (34, 59).

The location of IL2R $\alpha$  was also compared to that of the late endocytic compartment marker, rab 7. After 100 min internalization of IL2 in the presence of 7G7B6 monoclonal antibody, the cells were fixed, permeabilized, and stained with anti-rab 7 antibody and then with anti-murine and anti-rabbit Ig labeled with FITC and TRITC, respectively. No colocalization of IL2R $\alpha$  and rab 7 could ever be detected by confocal microscopy, as seen in Fig. 4, which shows a representative medial optical section.

In conclusion, IL2R $\alpha$  was at all times colocalized with transferrin, at early times of internalization in vesicles and at late times in a pericentriolar recycling compartment, and could not be detected together with the late endocytic marker, rab 7. Therefore, IL2R $\alpha$  has the characteristics of a recycling receptor.

### *The $\beta$ Chain of IL2R Is Found in Late/Prelysosomal Compartments*

The same approach was used to study the location of IL2R $\beta$  as that described above for IL2R $\alpha$ . Endocytosis of IL2R $\beta$  was probed by internalizing IL2 together with monoclonal antibody 341 directed against IL2R $\beta$ . This antibody does not inhibit IL2 binding to high-affinity receptors (54), nor does it modify IL2 internalization or degradation (Fig. 2). After 15 min IL2 and TRITC-transferrin internalization at 37°C, IL2R $\beta$  was mainly colocalized with transferrin as shown in Fig. 5 by confocal microscopy. Some vesicles containing IL2R $\beta$ , but not transferrin, could already be observed. After 100 min IL2 and transferrin internalization, transferrin was in the pericentriolar compartment, while IL2R $\beta$  was found

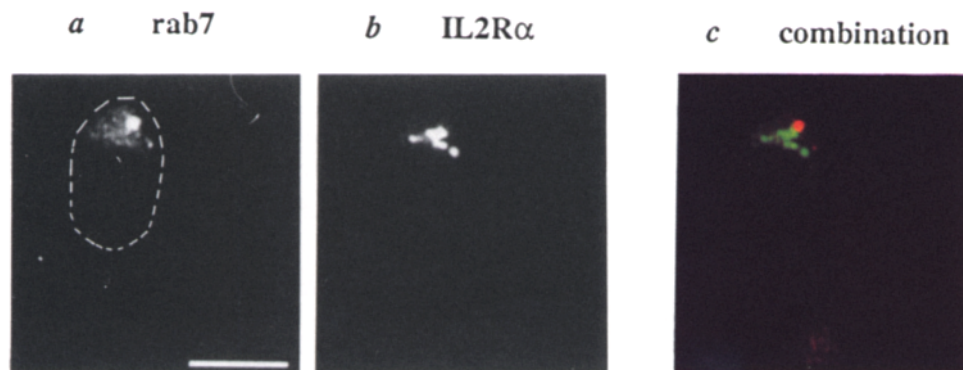


**Figure 3.** Localization after endocytosis of IL2R $\alpha$  relative to transferrin. The cells were incubated at 37°C with 150 nM TRITC-transferrin, IL2, and anti-IL2R $\alpha$  antibody 7G7B6. After 15 min (a-c) or 100 min (d-f), the cells were washed at 4°C to stop endocytosis, fixed, permeabilized, and incubated with FITC-labeled anti-murine Ig antibody. One representative medial optical section is presented at 15 min endocytosis (a-c) and at 100 min endocytosis (d-f). (a and d) Transferrin staining; (b and e) IL2R $\alpha$  staining; (c and f) combinations of these two stainings. Areas of colocalization appear yellow in the computer-generated composite image. Bar, 10  $\mu$ m.

in vesicles which did not contain transferrin (Fig. 5). The location of IL2R $\beta$  was also compared to that of rab 7 at late internalization times (100 min at 37°C) since rab 7 is a marker of late endocytic vesicles. IL2R $\beta$  was partly colocalized with rab 7 (Fig. 5). In conclusion, at early endocytosis times, IL2R $\beta$  was mainly colocalized with transferrin, although a few vesicles containing only IL2R $\beta$  were detectable, and at late endocytosis times the location of IL2R $\beta$  was distinct from that of transferrin, and in part similar to that of rab 7. IL2R $\beta$ , thus, has the characteristics of a receptor routed towards degradation.

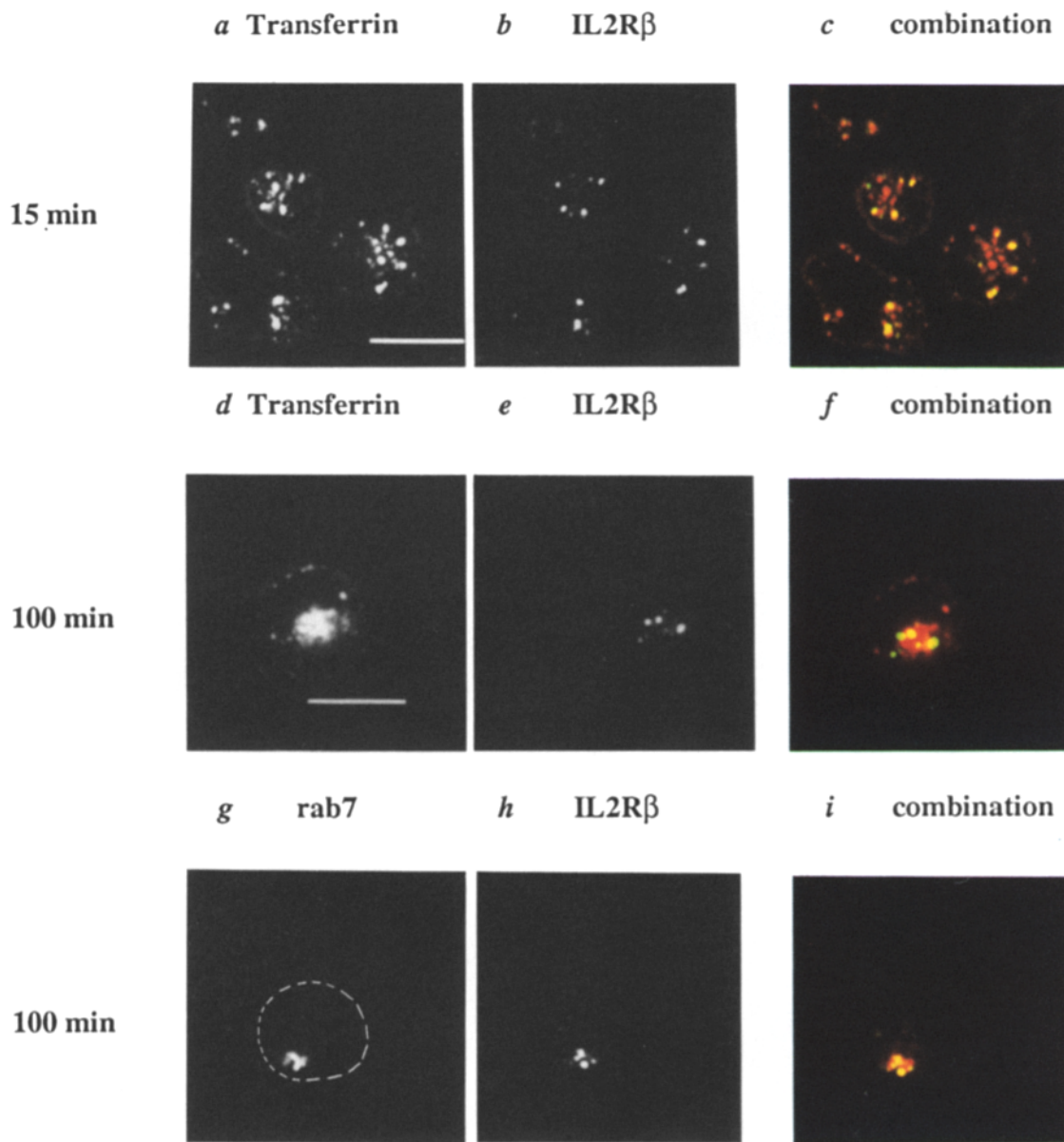
#### **The $\gamma$ Chain of IL2R Is Not Colocalized with Transferrin**

Endocytosis of IL2R $\gamma$  following IL2 binding was probed with monoclonal antibody TUGh4. It was verified that this antibody, at the concentration used for immunofluorescence, does not inhibit IL2 binding or modify IL2 internalization and degradation (Fig. 2). The cells were allowed to internalize TRITC-transferrin together with IL2 and TUGh4 for 15 or for 100 min at 37°C. The cells were then fixed and permeabilized, and IL2R $\gamma$  was revealed by FITC-labeled second



**Figure 4.** Localization of IL2R $\alpha$  relative to rab 7. Cells were incubated for 100 min at 37°C with IL2 and 7G7B6 monoclonal antibody, washed at 4°C, fixed, permeabilized, and then stained with purified anti-rab 7 rabbit serum. The cells were then incubated with FITC anti-murine Ig and TRITC anti-rabbit Ig. A representative optical section showing rab 7 (a), IL2R $\alpha$  (b), and the combination of these two images (c) is presented. The cell contour is shown (dotted line). Bar, 10  $\mu$ m.





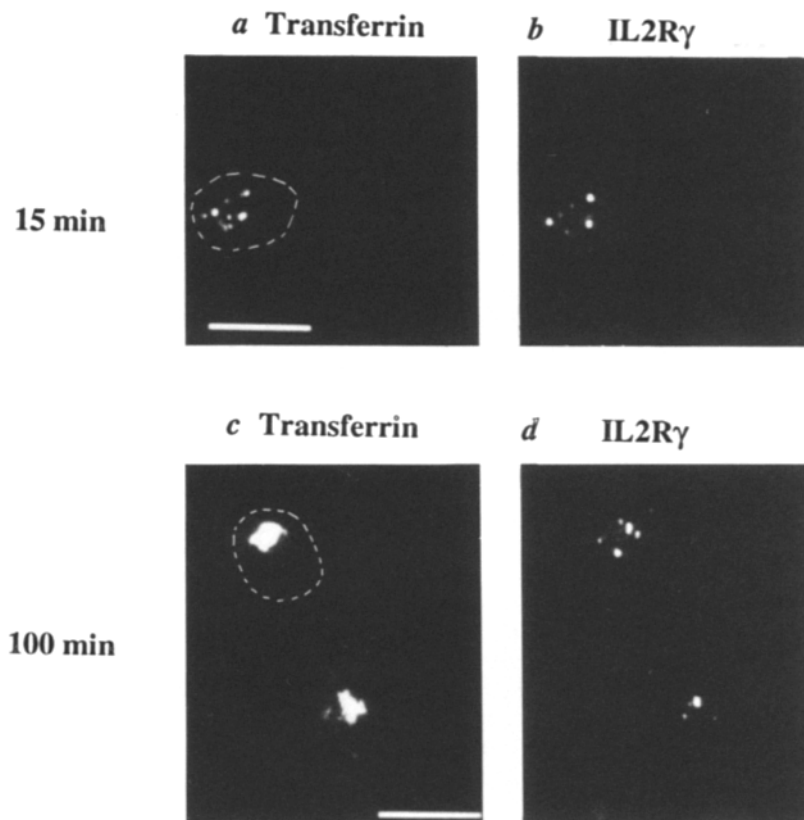
**Figure 5.** Localization after endocytosis of IL2R $\beta$  relative to transferrin and rab 7. Cells were incubated at 37°C for 15 min (*a-c*) or 100 min (*d-f*) with 150 nM TRITC-transferrin, IL2, and anti-IL2R $\beta$  antibody 341. The cells were then washed at 4°C to stop endocytosis, fixed, permeabilized, and incubated with FITC-labeled anti-murine Ig antibody. One representative medial optical section is presented. (*a* and *d*) transferrin staining; (*b* and *e*) IL2R $\beta$  staining; (*c* and *f*) combination of these two stainings. Areas of colocalization appear yellow in the computer-generated composite image. (*g-i*): Cells were incubated for 100 min at 37°C with IL2 and anti-IL2R $\beta$  monoclonal antibody 341, washed at 4°C, fixed, permeabilized, and then stained with purified anti-rab 7 rabbit serum. The cells were then incubated with FITC anti-murine Ig and TRITC anti-rabbit Ig. A representative medial optical section showing rab 7 (*g*), IL2R $\beta$  (*h*), and the combination of these two images (*i*) is presented. The cell contour is drawn (*dotted line*). Bar, 10  $\mu$ m.

antibodies. Fig. 6 shows that after 15 min internalization, transferrin and IL2R $\gamma$  were mainly colocalized, and after 100 min they were located in different compartments. Therefore IL2R $\gamma$  is detected in endocytic organelles other than early and recycling endosomes, most likely in late/prelysosomal compartments.

#### Half-Life of IL2 Receptors

The number of cell surface receptors results from the balance between their rates of synthesis, internalization and recycling. In the presence of cycloheximide, synthesis is in-

hibited, and therefore the number of surface receptors is the net result of degradation and recycling after internalization. We had previously reported that IL2R $\alpha$  has a very long half-life (24), in agreement with IL2R $\alpha$  recycling after internalization. We have now measured the half-life on the cell surface of IL2R $\beta$  on IARC 301.5 cells in the presence of IL2 and cycloheximide, after a 1 h preincubation with cycloheximide. Using the same method as described for high-affinity receptors (12), we have verified that 1 h cycloheximide pretreatment was sufficient to chase out the internal pool of  $\beta$  chain en route to the cell surface (not shown). The surface



**Figure 6.** Localization after endocytosis of IL2R $\gamma$  relative to transferrin. Cells were incubated at 37°C with 150 nM TRITC-transferrin, IL2, and anti-IL2R $\gamma$  antibody TUGh4. After 15 min (*a* and *b*) or 100 min (*c* and *d*), the cells were washed at 4°C to stop endocytosis, fixed, permeabilized, and incubated with FITC-labeled anti-rat Ig antibody. One representative medial optical section is presented at 15 min endocytosis (*a* and *b*) and at 100 min endocytosis (*c* and *d*). (*a* and *c*) Transferrin staining and (*b* and *d*) IL2R $\gamma$  staining. The cell contour is drawn (dotted line). Bar, 10  $\mu$ m.

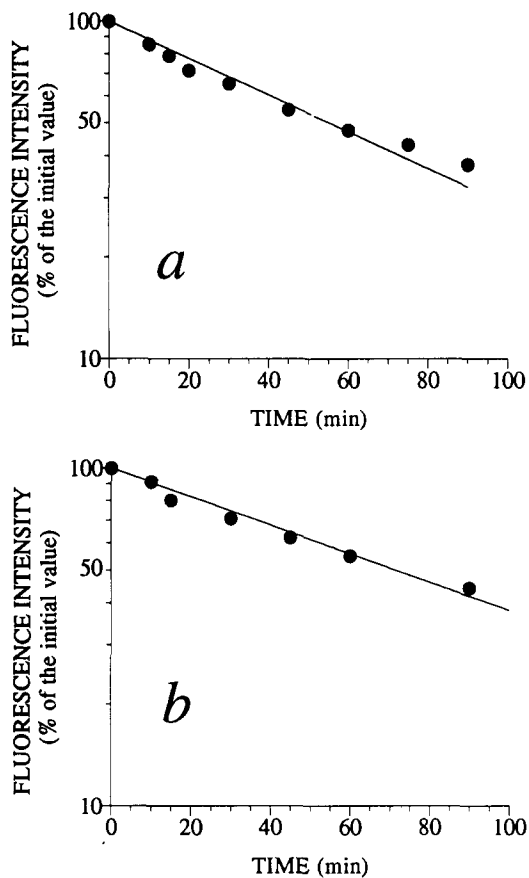
expression of the  $\beta$  chain was then probed with anti- $\beta$ -monoclonal antibodies by flow cytometry measuring the mean fluorescence intensity. The half-life of IL2R $\beta$  in the presence of IL2 was about 55 min (Fig. 7 *a*). The same experiment was performed to measure the half-life of IL2R $\gamma$ , which was about 70 min (Fig. 7 *b*). Both half-lives are very short, among the shortest for membrane proteins, and are very similar to the value previously found for high-affinity IL2 receptors (11). The rapid decrease of the number of surface receptors directly reflects their degradation. This biochemical result confirms the data obtained by confocal microscopy, showing that IL2R $\beta$  and IL2R $\gamma$  have characteristics of proteins going through the degradation pathway.

### Discussion

Communication between cells in the immune and hematopoietic systems is mediated by soluble factors, the 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. Cytokines function in a redundant manner, i.e., the same biological effect can be fulfilled by different cytokines. For instance IL2, IL4, or IL15 may function on the same cell type as long as their receptors are expressed and trigger at least in part the same biological responses (20, 33, 39, 44). This redundancy can be explained by the composition of cytokine receptors. They usually consist of ligand specific chain(s) and of one or more chains common to several cytokine receptors, which may be involved in signal transduction. The

IL2 receptor is a prototype of a cytokine receptor and consists of three IL2 binding proteins,  $\alpha$ ,  $\beta$ , and  $\gamma$ . IL2R $\beta$  and  $\gamma$  belong to the cytokine family of receptors; IL2R $\beta$  is shared by the IL15 receptor, and IL2R $\gamma$  is shared at least by the IL4, IL7, IL9, and IL15 receptors.

Expression of cytokine receptors, because of their function, is usually tightly regulated during development and growth, both at the gene level and by their time of residence at the cell surface. Because of the participation of some of the chains in several receptors, and because cytokine receptors form noncovalent multimers, it can be expected that the different chains may behave separately along the secretory and endocytic routes. This would allow for the fine regulation of expression of the different receptors. Although the regulation of transcription of IL2 and other cytokine receptors has been studied in detail, very little is known about their endocytosis and intracellular fate after cell entry. We have addressed this question in the case of IL2 receptors. We have observed that the IL2 receptor  $\alpha$ ,  $\beta$ , and  $\gamma$  chains have different intracellular destinations despite having been internalized together with IL2. IL2R $\alpha$  is found only in transferrin-containing compartments at all times of IL2 endocytosis, and cannot be detected in compartments stained by anti-rab 7 antibodies, as observed by confocal microscopy. On the other hand, IL2R $\beta$  and IL2R $\gamma$  can be found in transferrin containing vesicles at early endocytic times. After prolonged endocytosis, IL2R $\beta$  is also detected in rab 7 positive organelles. However the proportion of IL2R $\beta$  in transferrin positive organelles is higher than that in rab 7 positive compartments up to 1 h endocytosis of IL2R $\beta$  antibodies. This observation may be related to what was observed by



**Figure 7.** Half-lives of IL2R $\beta$  and IL2R $\gamma$ . Cell surface expression of IL2R $\beta$  (a) or IL2R $\gamma$  (b) on cells treated for different times with 50  $\mu$ M cycloheximide and 200 pM IL2 was assessed by cytofluorimetry. Times indicated are after a 1 h preincubation in cycloheximide. One representative experiment out of three is shown.

subcellular fractionation, which showed that internalized  $^{125}$ I-IL2 is mostly found in transferrin-containing endosomes and not in late/prelysosomal compartments (15), although it is known that IL2 is degraded after endocytosis. This suggests that IL2 and IL2R $\beta$  might not accumulate for a long time in these rab 7 positive late/prelysosomal compartments. The same observation has been reported for epidermal growth factor receptors (26). At long times (100 min) of IL2 and transferrin endocytosis, transferrin and IL2R $\alpha$  are still colocalized in a compartment with a morphology different from that observed at early internalization times. We observed this pericentriolar transferrin-containing organelle in T lymphocytes, and it appears very similar to that previously described in other cell types (34, 59). Almost no IL2R $\beta$  or IL2R $\gamma$  has ever been observed in this compartment, which may thus be a specialized recycling compartment.

Taken together, these results suggest that the three chains of IL2R are internalized together as a quaternary complex with IL2, but are then sorted separately within the cell. However this conclusion holds only if the chains are not internalized independently of each other. We had previously reported that IL2 internalized in endocytic compartments could be cross-linked both to IL2R $\alpha$  and IL2R $\beta$ , showing

that these two chains were internalized forming a complex with IL2 (13). In this work, we demonstrate that IL2R $\gamma$  is also internalized. This is in agreement with the fact that, in cross-linking experiments, there was an additional band of the expected molecular weight for IL2 cross-linked to IL2R $\gamma$  (13). The following observations further strengthen the view that the chains cannot be internalized separately in the experiments reported here. (a) The IL2R $\alpha$  chain is present in excess over the other two chains, raising the possibility that it could be internalized by itself. This is not the case for the following reasons: IL2R $\alpha$  alone, without  $\beta$  and  $\gamma$ , can bind IL2 with a  $K_d \approx 10$  nM, but then it cannot internalize IL2 and is not internalized itself (13, 16, 17, 22, 38). Since the IL2 concentration used in the experiments shown here (200 pM) is much less than the  $K_d$ , a large majority of IL2R $\alpha$  on the cell surface have no bound IL2. Therefore, we also checked the localization of IL2R $\alpha$  in the absence of IL2: IL2R $\alpha$  was found only on the plasma membrane, and was never detectable in intracellular vesicles when protein synthesis was prevented by cycloheximide (not shown). IL2R $\alpha$  is thus internalized only when part of high-affinity receptors formed when IL2 is present. Finally, a similar localization of IL2R $\alpha$  has been observed in a human natural killer cell line YT 12881 (not shown). This cell line bears receptors formed of IL2R $\beta$  and IL2R $\gamma$ , and can be induced by IL1 to also express IL2R $\alpha$  in quantities not exceeding those of IL2R $\beta$  (52). (b) Concerning IL2R $\beta$  and  $\gamma$ : it is highly unlikely that, in the presence of IL2, some of these chains could associate without IL2R $\alpha$  because of the large excess of this third chain. However, if they did, their association should have been detectable since IL2R $\beta$  and IL2R $\gamma$  associate in the presence of IL2 to form receptors of intermediate affinity,  $K_d \approx 1$  nM (49, 55). We have never been able to detect such receptors on IARC 301.5, although we are capable of measuring as little as 100 receptors per cell (10). Therefore there is not an excess of IL2R $\beta$  and  $\gamma$  that would be internalized independently of  $\alpha$  in IARC 301.5. Likewise, the same results as reported here for IARC 301.5 were obtained with YT #12881 cells induced to express similar amounts of the three chains (not shown).

The different intracellular localizations of the three IL2 receptors chains therefore suggest that they have different intracellular fates, IL2R $\alpha$  recycling to the plasma membrane while  $\beta$  and  $\gamma$  are degraded, and also that their differential sorting occurs in endocytic organelles. The acidic pH of endosomes may play a role in this sorting, dissociating IL2 and the receptor's components.

An expected consequence of sorting is that IL2R $\alpha$  and IL2R $\beta$  and IL2R $\gamma$  should have very different half-lives. Indeed, IL2R $\beta$  and IL2R $\gamma$  have short half-lives on the cell surface, 55 and 70 min, respectively; while we had previously shown that the half-life of IL2R $\alpha$  is much longer, 48 h in IARC 301.5 cells (24). In this lymphocytic cell line, as well as in activated T lymphocytes, only about 10% of surface  $\alpha$  chains are part of high-affinity receptors, while the rest of the  $\alpha$  chains form low-affinity receptors which are not endocytosed (13, 16, 17, 22, 38; and unpublished data). We have previously reported that the very long half-life of IL2R $\alpha$  in IARC 301.5 indicates that, following endocytosis, IL2R $\alpha$  is recycled to the surface (24). In cells that do not express more IL2R $\alpha$  than the other receptor components, one would expect a half-life of about 5 h for IL2R $\alpha$ , assuming that most



IL2R $\alpha$  returns to the plasma membrane at each internalization cycle (with a recycling probability of 0.9). We have measured the half-life of IL2R $\alpha$ , in the presence of IL2, on the plasma membrane of the natural killer human cell line YT 12881, induced by IL1 to express the same amount of IL2R $\alpha$  as IL2R $\beta$ . This half-life has the expected value, 5 h (not shown).

In conclusion, the different recycling or degradation behavior of chains of the same receptor that are internalized together allows their cell surface expression to be regulated separately, and adds another level to the modulation due to regulation of gene expression. This allows the expression of high-affinity IL2 receptors to last for a few days, although the transient activation of IL2R $\alpha$  gene expression following antigen stimulation is short (IL2R $\alpha$  mRNA peaks at 6 h after antigen stimulation of T lymphocytes, while IL2R $\beta$  is constantly transcribed). Furthermore, some of the chains of IL2 receptors are also part of other cytokine receptors and their different behavior after endocytosis may allow for a fine balance to be achieved between the expression of several cytokine receptors on the same cells.

Sorting of proteins takes place in different compartments: the endoplasmic reticulum, Golgi cisternae, *trans*-Golgi network, endosomes, and plasma membrane. The cell machinery involved depends on the compartment considered, and is in some cases well understood, while in other cases it remains completely unknown. All sorting processes presumably require sorting signals carried by some of the trafficking molecules. These signals are recognized by the cell machinery and allow the "tagged" molecules to escape from a default pathway and be directed to specific compartments. For molecules leaving the endosome, it is usually accepted that recycling occurs by default while a signal is required for them to be directed towards degradation compartments. No such degradation signal has yet been defined. A sequence in P-selectin has recently provided the first example of a sequence able to direct a protein towards degradation (21). No homology has been found between this sequence and cytoplasmic tails either of other degraded receptors or of IL2R $\beta$  or  $\gamma$ . This sequence might cause rapid lysosomal degradation. One of the evoked mechanisms is an aggregation of receptors routing them out of the recycling membrane (35).

In the case of IL2 receptors, we have shown that the  $\alpha$  chain follows the default recycling pathway, as does the transferrin receptor. On the contrary, we assume that a degradation signal is associated with the  $\beta$  and/or  $\gamma$  chain. We have previously shown that IL2R $\beta$ , in the absence of IL2R $\gamma$ , is internalized and degraded and also has a short half-life, as when IL2R $\beta$  is part of high-affinity receptors (25). This indicates that IL2R $\beta$  itself may carry a degradation signal independently of its association with IL2R $\gamma$ . Concerning IL2R $\gamma$ , it may either carry a degradation signal itself, or remain associated with IL2R $\beta$  throughout the sorting process.

Finally, the IL2 receptor is now the first multichain receptor composed of non-covalently linked subunits which has been shown to be sorted differentially along the endocytic pathway. It will be worthwhile to determine whether this is a general property of the expanding family of cytokine receptors.

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