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An α-Helical Signal in the Cytosolic Domain of the Interleukin 2 Receptor β Chain Mediates Sorting Towards Degradation after Endocytosis

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Abstract. High-affinity IL2 receptors consist of three components, the α, β, and γ chains that are associated in a noncovalent manner. Both the β and γ chains belong to the cytokine receptor superfamily. Interleukin 2 (IL2) binds to high-affinity receptors on the cell surface and IL2-receptor complexes are internalized. After endocytosis, the components of this multimolecular receptor have different intracellular fates: one of the chains, α, recycles to the plasma membrane, while the others, β and γ, are routed towards late endocytic compartments and are degraded. We show here that the cytosolic domain of the β chain contains a 10–amino acid sequence which codes for a sorting signal. When transferred to a normally recycling receptor, this sequence diverts it from recycling. The structure of a 17–amino acid segment of the β chain including this sequence has been studied by nuclear magnetic resonance and circular dichroism spectroscopy, which revealed that the 10 amino acids corresponding to the sorting signal form an amphipathic α helix. This work thus describes a novel, highly structured signal, which is sufficient for sorting towards degradation compartments after endocytosis.

During receptor-mediated endocytosis, receptors are transported from one membrane compartment to another. From each compartment, they can be routed to different destinations, and intracellular transport therefore requires sorting (reviewed in references 18, 54). The best documented sorting is the one which functions at the plasma membrane to concentrate receptors in clathrin-coated pits. It involves internalization signals, short peptides in the cytosolic part of receptors which have been classified in two groups, a tyrosine based motif and a di-leucine based motif (reviewed in reference 49).

After internalization from the plasma membrane, receptors rapidly reach early/recycling endosomes. From there, some receptors recycle back to the cell surface while others are sorted to different destinations (46). Recycling to the plasma membrane is generally considered as the default pathway (34, 54). For receptors to exit from the recycling pathway and move to other intracellular destinations (e.g., in most cases towards late endosomes and lysosomes, or to the trans-Golgi network or major histocompatibility complex class II compartments) or to new domains on the cell surface (as in transcytosis) requires selective routing and depends upon the possession of additional, specific sorting signals.

Although many internalization signals have now been described, much less is known about sorting steps from intracellular endocytic organelles. Receptors which transport nutrients to the cells, such as transferrin or low density lipoprotein receptors, recycle to the plasma membrane, while receptors for hormones and growth factors are often degraded after internalization. Degradation causes receptor down modulation and is important for arresting the cell response to growth factors and hormones. The mechanism by which these receptors are directed towards degradation is unknown.

Sequences which are necessary for diverting lysosomal membrane glycoproteins to lysosomes have been described by mutation analysis (reviewed in reference 49). They belong to the tyrosine or di-leucine families of signals described for clathrin-coated pit endocytosis (20, 24, 33, 43, 59). After synthesis, lysosomal proteins are sorted from the trans-Golgi network to endosomes and lysosomes (20, 24). Alternatively, some lysosomal proteins are exocyted and then internalized by receptor-mediated endocytosis (33, 43, 59); they are then sorted from endosomes to lysosomes. In these cases, the same mutations usually affect both endocytosis and lysosomal targeting, and the distance between the signal and the plasma membrane may also be important for sorting (47). By mutational analysis, sequences necessary for internalization and degradation of nonlysosomal membrane proteins have been reported (2, 29, 32, 41, 55). However, in all of these cases, it has not been shown that a short sequence is sufficient to
serve as a degradation signal by transferring it to a normally recycling receptor, which would then become degraded as a result. Only one case of a membrane molecule with a short sequence sufficient for lysosomal degradation after endocytosis has been reported, that of P-selectin. The cytosolic tail of this cell adhesion molecule contains a degradation signal which does not match any of the signals described so far (15).

The cytokine interleukin 2 (IL2) 1 is produced by activated helper T lymphocytes and stimulates proliferation and effector functions of a variety of cells of the immune system (36). High-affinity IL2 receptors (KD = 10 – 100 pM) consist of three distinct components, the α, β, and γ chains, that are associated in a noncovalent manner (36). Both the β and γ chains, but not the α chain, belong to the cytokine receptor superfamily (5). This hematopoietic cytokine receptor family includes receptors such as the IL3, IL4, IL5, IL6, IL7, IL9, and IL15 receptors, 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 proteins for factors that are believed to function normally outside the immune and hematopoietic system, i.e., growth hormone, prolactin, and ciliary neurotrophic factor. Many receptor subfamily members share at least one component: thus, the α, β, and γ chains, and the receptors for IL2 and IL15 share the β chain (22). In this paper, we show that one of the early events following IL2 binding to high-affinity IL2 receptors, the phosphorylation of the α chain, is internalized and not recycled when expressed with a short sequence sufficient for lysosomal degradation. In this sequence, a 10–amino acid peptide encodes a signal for sorting towards degradation compartments.

Materials and Methods

Cells, Monoclonal Antibodies, and Reagents

K562, a human erythroleukemia cell line, was grown in suspension in RPMI 1640, 10% decomplemented FCS, supplemented with 2 mM L-glutamine. Stably transfected K562 cells were grown in the same medium supplemented with 1.5 mg/ml G418. HeLa cells were grown in DMEM, 10% decomplemented FCS, supplemented with 2 mM L-glutamine.

Monoclonal antibodies 7G7B6 (IgG2a) and OKT9 (IgG1), directed against the α chain of the IL2 receptor and the transferrin receptor, respectively, were obtained from the Amer. Tissue Culture Collection (Rockville, MD) (48, 53). Monoclonal antibodies 341 and 561 were kind gifts from Drs. K. Robb (Dupont Merck Pharmaceutical Co., Wilmington, DE) (56). FITC-conjugated anti-murine IgG1 antibodies and Texas red-conjugated anti-murine IgG2a antibodies were obtained by Southern Biotechnology (Birmingham, AL).

Saponin and cycloheximide were obtained from Sigma Chem. Co. (St. Louis, MO). 2,2,2-Trifluoroethyl alcohol-d2,OH (99 atom %D) and deuterium oxide (99.9 atom %) were purchased from Solvants Documenta Synthes (S.D.S., France).

Plasmids

All constructs were prepared in the NT vector, a kind gift of Dr. C. Bonnerot (Institut Curie, Paris, France). The plasmid pdKCRβ, coding for the IL2R β chain was kindly provided by Dr. T. Kono (Osaka University, Japan) (37). The truncated forms of β6 were generated by PCR by insertion of a stop codon after the 10th amino acid of the cytosolic part of the protein (assuming that Asparagine is the first cytosolic amino acid), and were cloned into the NotI/SacI sites of NT by standard techniques. The plasmid T-XO, a kind gift of Dr. P. Cosson (Basel, Switzerland), is a modified version of IL2Ra cDNA in the pCDM8 vector. In this plasmid, the 3’ side of the sequence is modified to create a HindIII/XbaI cloning cassette (ACAATCTAG to ACAATTCAGCTCTCTGAGTAGCGTCTAGA). This modification adds four amino acids (QASS) to the wild-type IL2Ra chain, but its cellular behavior is not modified, and therefore will refer to this protein as "α." This plasmid was further modified by inserting a sequence coding for the transferrin receptor YTRF internalization signal, in the cytosolic part of α. In this T-XO construct, the 3’ side of the sequence AAAGTAGAAGAAATCCAAGCTCTCTCTGTGA was modified to AAGATGGAACATGCTATACCCCGGTCCACAGCTCTCTTG by PCR. The COOH terminus of the T-XOα protein is KSEPL-SYTROQASS, instead of KSRRTOQASS for the T-XOαβ6 construct. All T-XOαβ6 and T-XOαβ6 constructs were subcloned in the NotI/SacI cloning sites of NT. The corresponding constructs were named αβ6 and αβ6, and were used for stable transfections in K562 cells.

Cell Transfection

All kinetics and half-life analyses described here have been performed in stably transfected K562 cells. To generate stable transfectants, 7 × 104 K562 cells were washed once in DMEM, 4.5 g/l glucose 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, Seraing, Belgium) with simple pulse, 240 V, 1,500 µF. Selection with 1.5 mg/ml G418 (Geneticin, Gibco/BRL, Gaithersburg, MD) was initiated 2 d after transfection, and the cells were cloned in 96-well dishes. The truncated forms of β6 were generated by PCR by insertion of a stop codon after the 10th amino acid of the cytosolic part of the protein (assuming that Asparagine is the first cytosolic amino acid), and were cloned into the NotI/SacI sites of NT by standard techniques.

This plasmid was further modified by inserting a sequence coding for the transferrin receptor YTRF internalization signal, in the cytosolic part of α. In this T-XO construct, the 3’ side of the sequence AAAGTAGAAGAAATCCAAGCTCTCTCTGTGA was modified to AAGATGGAACATGCTATACCCCGGTCCACAGCTCTCTTG by PCR. The COOH terminus of the T-XOα protein is KSEPL-SYTROQASS, instead of KSRRTOQASS for the T-XOαβ6 construct. All T-XOαβ6 and T-XOαβ6 constructs were subcloned in the NotI/SacI cloning sites of NT. The corresponding constructs were named αβ6 and αβ6, and were used for stable transfections in K562 cells.

Endocytosis of Radiolabeled Antibodies

7G7B6 and 561 antibodies were radiolabeled with 125I by the chloramine T method to a specific activity of 2–10 µCi/µg. For endocytosis experiments, 2 × 105 cells were incubated in 100 µl RPMI-Hepes, pH 7.2, 1 mg/ml BSA, at 37 ºC, and 1–5 × 106 125I-labeled antibody was added. After incubation at 37 ºC for the indicated times, the cells were rapidly cooled to 0 ºC and washed twice. Cell surface–associated radioactive ligand was then removed by two successive acid pH treatments (2 min at pH 2.0) at 4 ºC as previously described (11). Nonspecific binding, measured for each ligand by adding a 100-fold excess of the same unlabeled ligand, was <5% and was subtracted. The efficiency of removal of cell surface–associated ligands by acid pH washes was measured for each ligand and was >95%.

In all figures, the ratio of intracellular to total associated ligand is expressed as

1. Abbreviations used in this paper: CD, circular dichroism; IL2, interleukin 2; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; NMR, nuclear magnetic resonance; ROESY, rotating frame NOE; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy.

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antibody is represented. All experiments were done with different clones expressing the same construct. One representative experiment is shown.

**Cell Surface Half-life Measurement**

To measure the half-life at the cell surface of the different β, βα1, or γδβα1 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, and cell surface expression of the constructs was assayed by flow cytometry as described (21). Time zero 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 (11). All experiments were done in triplicate with different clones expressing the same construct. One representative experiment is shown.

**Immunofluorescence and Confocal Microscopy**

Either stably transfected K562 cells, or HEK cells grown on coverslips and transfected 2 d before with the plasmid of interest, were used. The cells were incubated for the indicated times at 37°C with 7G7B6 mAb, before being washed in PBS at 4°C and fixed in 3.7% parafomaldehyde and 0.03 M sucrose for 30 min at 4°C. Subsequent steps were performed at room temperature. The cells were washed once in PBS, and after quenching for 10 min in 50 mM NH4Cl in PBS, the cells were washed once in PBS supplemented with 1 mg/ml BSA. Cells were then incubated, when indicated, with OKT9 mAb in permeabilization buffer (PBS with 1 mg/ml BSA and 0.05% saponin) for 20 min at 37°C. After two washes in the permeabilization buffer, the presence of antibodies was revealed by incubating the cells for 30 min at 37°C in permeabilization buffer containing labeled second antibodies. For 7G7B6 and OKT9, the second antibodies were Texas red–labeled anti-IgG2a (1/250) and FITC-labeled anti-IgG1 (1/100), respectively. Washes, sample mounting, and confocal microscopy were performed as described in reference 23.

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

**Metabolic Labeling**

For pulse metabolic labeling with 35S-amino acids, 35 × 106 cells were washed with PBS, then incubated for 1 h in DMEM lacking methionine and cysteine and containing 2% dialyzed FCS. Cells were labeled for 2 h in the same medium containing 200 μCi/ml 35S-amino acid mixture (Promix, Amersham, England), then washed in PBS. After 75 min incubation at 37°C in complete medium, cells were harvested at 30-min intervals, washed in PBS, pelleted by centrifugation, and kept frozen at −20°C before analysis by immunoprecipitation.

**Cell Iodination**

YT cells (2 × 106) washed in PBS and resuspended in PBS, pH 7.3, 1 mM CaCl2, 1 mM MgCl2, were surface labeled using the lactoperoxidase method with 1 μCi Na125I (21). After iodination, the cells were washed twice in culture medium and then kept in a 37°C incubator. After 30 min incubation, 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 (1% NP-40, 150 mM NaCl, 10 mM Tris HCl, pH 8.0) complemented with 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 10 mM leupeptin, 20 μg/ml aprotinin, and 2 mM PMSF. Insoluble material was pelletted at 15,000 rpm for 30 min, and the supernatant was then precleared for 30 min at 4°C with protein A Sepharose CL-4B (Pharmacia, Sweden) before being immunoprecipitated overnight at 4°C with relevant first antibodies, and protein A–Sepharose coupled with anti–mouse antibody (Biosys, France). First antibodies were anti-IL2Rα mAb and 7G7B6 mAb in the absence of IL2 or anti-IL2Rα mAb and 7G7B6 mAb and anti-transferrin receptor mAb OKT9 for the measurement of γδβα1 half-life after iodination. The Sepharose beads were then washed three times in 1% NP-40, 0.5 M NaCl, 10 mM Tris HCl, pH 8.0, and once in 10 mM Tris HCl, pH 8.0. Bound proteins were eluted into electrophoresis sample buffer (60 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) before analysis by SDS-PAGE. Gels were 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, England) at −80°C.

**Peptide Synthesis**

The peptides were synthesized by the Merrifield solid-phase method (35) using a 430 A synthesizer and a Pam (Boc, t-butoxycarbonyl; Pam, acetimidomethyl) resin (Applied Biosystems, Foster City, CA). The final purity (99.5%) of the peptides was checked by analytical reverse-phase HPLC.

**Circular Dichroism Experiments**

Circular dichroism (CD) spectra of the wild-type peptide were recorded at 25°C on a Jobin Yvon CD6 dichrograph with a 0.2-mm path-length cell. The peptide was first dissolved in water at pH 5.3. The solution was then diluted in trifluoroethanol (TFE)/water mixtures varying from 0 to 95% TFE (V/V). Peptide concentration was 0.32 mg/ml (0.2 mM) as determined from amino acid analysis.

**Proton Nuclear Magnetic Resonance Experiments**

The samples were prepared as 6 mM peptide solutions in water at pH 3.4 and 5.3 for BETApep and 5.3 for P20Lpep, respectively. The solutions were then lyophilized and the samples solubilized in either 90%H2O:10%D2O or in 25% and 40%T2F-D2O.

All nuclear magnetic resonance (NMR) measurements were obtained on a Varian Unity 500 spectrometer operating at a proton frequency of 500 MHz and interfaced to a Sun Sparc+1+ station. The sweep width was 5,000 Hz. Spectra in water were recorded at 0°C and 25°C, while spectra in TFE:water mixtures were recorded at 25°C. Spectra in water are referenced to external trimethylsilyl-3-proponic acid-d4 2.23.3 sodium salt (TMS) and in TFE to the internal methylene resonance of the solvent at 3.8 ppm relative to tetramethylsilane (TMS). The OH resonance of water or of TFE-d2 were suppressed by selective irradiation during the relaxation delay and, in the case of NOESY and ROESY spectra, during the mixing time as well. All 2D-data were collected in the phase-sensitive mode using the States-Haberkorn method (50). A total of 512 FIDs of 2K complex data points were collected in 12 with 32 scans per increment and zero-filling was applied in both dimensions before Fourier transformation to form a matrix of 4K × 2K. These data were then processed with shifted sine-bell window functions in both dimensions.

The phase-sensitive two-dimensional double-quantum-filtered correlated spectroscopy (DOQ-COSY) (44) and total correlated spectroscopy (clean-TOCSY) (16) with 80-ms mixing time were used for spin system assignment. Nuclear Overhauser two-dimensional experiments (NOESY) (30) and rotating frame nuclear Overhauser two-dimensional experiments (ROESY) (6) were recorded for sequential assignment and structure determination using several mixing times (100, 200, 300 ms) for the NOESY and 400 ms for the ROESY.

1H-1H NOESY spectra were measured from one dimensional spectra recorded with a digital resolution of 0.15 Hz/pixel at 0°C and 25°C.

**Results**

**The IL2Rβ Chain Is Degraded after Endocytosis**

We have previously shown that the β chain of IL2 receptor is internalized and has a half-life at the cell surface of ~110 min, in the absence of IL2, in three different lymphocytic cell lines (YT, IARC 301.5, CIAC) (22). At steady state, the total number of cell surface receptors results from the 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 syn-
thesis is inhibited. Therefore, the short half-life of IL2Rβ shows that it is not recycled after endocytosis. Its localization in late endocytic compartments suggests that it is degraded (23).

We studied the turnover of IL2Rβ in the YT cell line, which has been extensively used to study IL2 receptors. The rate of turnover of IL2Rβ was measured in these cells by pulse-chase metabolic labeling and immunoprecipitation. Cells were labeled with 35S-amino acids and incubated in chase medium at 37°C for different times as described in the Materials and Methods section. After a 75-min incubation to allow export to the plasma membrane, cells were harvested at 30-min intervals. Proteins were quantitatively immunoprecipitated from cell lysates, the precipitates resolved on SDS-polyacrylamide gels, and the radioactivity in the IL2Rβ band quantitated by phosphorimager analysis. An autoradiograph of a typical experiment is shown in Fig. 1. Analysis of the turnover of IL2Rβ showed that it was degraded with a half-life of 2 h. Immunoprecipitation of the transferrin receptor in these experiments showed that it was stable, as expected for this recycling receptor (not shown). The half-life of IL2Rβ measured here is the same as its half-life on the cell surface previously measured by flow cytometry (22). Therefore the loss of β chain from the cell surface can be equated with degradation. Since measuring the loss of receptors from the cell surface is a very sensitive, simple, and quantitative method, it was used to probe for receptor turnover in subsequent studies.

The First 27 Cytosolic Amino Acids of the IL2Rβ Chain Are Sufficient for Its Endocytosis and for Its Short Half-Life on the Cell Surface

The IL2Rβ chain is a type I transmembrane protein, with 286 amino acids in its cytosolic part. We had previously shown that the β chain by itself, in the absence of the α and γ chains of the high-affinity IL2 receptor, is internalized and has a short half-life on the cell surface (22). To understand the molecular basis for the degradation of this protein, we constructed a truncated form of β, with only the 27 amino acids adjacent to the cytosolic region (Fig. 2). This β27 construct was transfected in K562 cells, and its behavior was analyzed in several stably transfected clones.

First, we measured the rate of endocytosis of β27 and βwt using radiolabeled anti-β mAb 561 (Fig. 3a). We had previously shown that this antibody does not modify the kinetics of entry and degradation of the IL2 receptor (51). Both forms of the protein were internalized efficiently and β27 was even internalized faster than βwt. This difference may be due to the presence of negative signals for endocytosis in the βwt chain, as has already been described in other receptors (25). It is also possible that an internalization signal is better presented in the 27-amino acid tail than in the wild-type form of β. We concluded that the first 27 amino acids of β are sufficient to promote endocytosis.

We also measured the half-life of β27 at the cell surface. Stably transfected β27 or βwt cells were incubated with the protein synthesis inhibitor cycloheximide for various times. The surface expression of the chain was then probed with anti-IL2Rβ mAb by flow cytometry, as described (23). The half-lives of both forms of β were the same, ~150 min (Fig. 3b). This represents a short half-life for a membrane receptor, and suggests that these proteins are degraded after endocytosis.

The 27th Amino Acid of β Is Necessary for its Short Half-Life on the Cell Surface, but Not for Its Endocytosis

To further characterize the sequence responsible for β27
A peptide encompassing amino acids 16 to 32 of $\beta_2^\text{b}$ was synthesized and will be called BETApep. NMR analysis of this peptide in solution in water at pH 5.3 and 3.4 was performed. At the two pH values, the presence of NH$_i$-NH$_{i+1}$, $\alpha$CH$_i$-NH$_i+2$, and $\alpha$CH$_i$-NH$_i+3$ dipolar connectivities indicates turn-like structures in the 20–27 fragment (Fig. 5). The Pro$_{26}$H$_α$-Lys$_{22}$NH and the Pro$_{20}$H$_α$-Se$_{21}$NH NOE connectivities as well as the fact that the trans conformation was the major one for both prolines, suggest that the Asp$_{19}$-Lys$_{22}$ fragment forms a type I or type I$_{\text{a}}$ turn (13). The small value observed for the $\text{Se}_{21}$NH-$\text{Se}_{21}$H$_α$ would be in favor of a type I$_{\text{a}}$ turn rather than a type I$\beta$ turn (Fig. 5b). The possibility that a secondary structure might arise through aggregation was ruled out by running a 10-fold diluted sample: identical chemical shifts were observed.

The presence of molecules in an ordered conformation is confirmed by other NMR parameters, such as chemical shift and temperature coefficients. Alpha protons displayed slight upfield shifts throughout the sequence Pro$_{26}$H$_α$ to Se$_{25}$ with the exception of the Phe$_{25}$H$_α$ proton, probably due to a Phe$_{25}$ ring current effect (Fig. 5c). The chemical shifts of all amide protons varied linearly with the temperature between 0°C and 40°C, implying no conformational changes. The temperature coefficients ($\Delta\delta/\delta$T) obtained in aqueous solution vary between 4 and $8 \times 10^{-3}$ ppm°C. The lowest value was observed for Glu$_{30}$ (4 $\times 10^{-3}$ ppm°C), while intermediate values (4.8 to 6 $\times 10^{-3}$ ppm°C) were obtained.
for the amide protons of fragment Ser21 to Gln26, indicative of a partial shielding of these protons from the solvent, probably through the formation of a hydrogen bond. Finally, these NMR studies in water strongly suggest that, at 25°C, BETApep adopts a turn between residues Asp19 and Lys22 and a nascent helix from Phe23 to Leu27.

To better define the structure, spectra were obtained in a TFE/water mixture, a mixed solvent known to increase the population of existing folded conformations (39). Indeed, the CD spectrum of BETApep in water displayed weak negative ellipticities at 220 nm and 199 nm, together with a stronger positive ellipticity at 193 nm, suggesting that a small population of BETApep molecules are in a helical conformation, in agreement with the NMR measurements. As the percentage of TFE increased, these spectra exhibited characteristic features of an α-helical structure.

NMR spectra were obtained in 20% and 40% TFE/water solutions. Addition of TFE induced substantial shifts of the proton resonances. In fragment 20 to 28, the peptide Hα resonances shifted upfield as compared to their position in water (Fig. 5 c). The induced shifts of Hα protons are given in water as compared to random coil values, and in TFE as compared to values observed in water.
stable α-helical structure. In conclusion, in the presence of TFE, residues 19 to 29 of BETAppe form an α helix.

Disruption in the Turn at the Beginning of the Sorting Signal Does Not Affect Receptor Trafficking

In aqueous solution at pH 5.4 two structural elements, a type I β turn for fragment Asp19 to Lys22 and a nascent helix for fragment Phe23 to Leu27 were observed for the peptide. To elucidate the relative importance of these two structural elements in the biological function, a peptide called P20Lpep was synthetized, in which Pro20 was replaced by a leucine to remove the turn. The structural analysis was done in the same conditions with P20Lpep as with BETAppe, and similar results were obtained, except that the turn characterized in BETAppe was absent from P20Lpep. In water, a nascent helix was observed throughout fragment Leu20 to Ser28. This helix was stabilized in the presence of TFE (Fig. 5, d, e, and f).

A mutation replacing Pro20 by Leu was introduced in the β27 construct and the behavior of this mutant was analyzed after transfection in K562 cells. The kinetics of endocytosis and the half-life on the cell surface of this mutant were the same as those of β27 (not shown). Therefore, disruption of the β turn does not modify the sorting of β27.

Amino Acids 18–27 of β Divert from Recycling a Receptor Internalized Via Clathrin-coated Pits

If this nascent helix is responsible for the sorting of β27, and if it can act more or less independently of its location in the protein, then it might act as a sorting signal that could be transferred into another internalized membrane protein and remain active. The best characterized mechanism for gaining entry into the cell is the clathrin-coated vesicle one (reviewed in reference 45). Membrane receptors which carry an internalization signal in their cytosolic domain are concentrated in the clathrin-coated regions of the membrane. Eventually, clathrin-coated pits invaginate until a closed clathrin-coated vesicle is formed. This mechanism of internalization is very efficient and is used by many membrane proteins, such as the transferrin receptor. The internalization signal of the transferrin receptor has been well characterized. It consists of four amino acids,
YTRF, which promote the internalization of the receptor independently of their location in the cytosolic tail, provided that at least 7 residues separate the tetrapeptide from the transmembrane region (8, 27).

We prepared chimeric receptors using the α chain of the IL2 receptor. This α chain recycles to the plasma membrane when internalized as part of high-affinity IL2 receptors (23). It has been used previously to prepare other chimeric membrane proteins, because good antibodies against its extracellular domain are available (29). When the α chain is expressed alone, without β and γ, it is internalized inefficiently. We therefore designed a construct, αY, in which the transferrin receptor coated-pit internalization signal was inserted in the cytosolic part of α. In αY, the EPLSYTRF sequence from the transferrin receptor was inserted at the COOH terminus of α (Fig. 2). HeLa cells were transfected with α or αY constructs, and 2 d after transfection, the cells were processed for immunofluorescence using anti-α mAb. Staining of α-transfected cells showed mostly a strong surface labeling, while in αY-transfected cells, αY was found in intracellular compartments (Fig. 6 a). We measured the internalization of αY using radiolabeled anti-α mAb 7G7B6. We had previously shown that 7G7B6 mAb does not modify the kinetics of entry and degradation of the IL2 receptor (51). The endocytosis of αY in stably transfected K562 cells was very efficient (Fig. 6 b). We next inserted in this construct the putative sorting signal by adding amino acids 17 to 27 or 18 to 27 of β to the COOH-terminal extremity of αY. The chimeras were named αYβ17–27 and αYβ18–27, respectively (Fig. 2). These chimeras were also rapidly internalized with the same kinetics as αY (Fig. 7 a). When protein synthesis was inhibited, αY expression was stable, indicating that after internalization, αY is efficiently recycled to the plasma membrane, as is the case for the transferrin receptor. Conversely, the chimeras containing the putative sorting signal, αYβ17–27 and αYβ18–27, had a half-life of ~200 min (Fig. 7 b). This indicates that these chimeras were degraded after internalization via the clathrin-coated pits.

The αYβ18–27 Chimera Is Found in Transferrin Negative Compartments

Transferrin and its receptor recycle very efficiently to the plasma membrane after endocytosis and are widely used as markers of early/recycling endosomes. If αY is in fact internalized and recycles as the transferrin receptor, both receptors should be localized in the same intracellular compartments; whereas if the αYβ18–27 chimeras are sorted to a degradation pathway, they should not colocalize entirely with the transferrin receptor.

We had previously shown that 7G7B6 mAb was a suitable marker for following the endocytosis of α, as it accompanies α along its recycling pathway (23). To study the intracellular distribution of αY or αYβ18–27 after endocytosis, cells stably transfected with these chimeras were incubated for 120 min at 37°C with 7G7B6 mAb before fixation and permeabilization. Early/recycling endosomal compartments were then labeled using anti-transferrin receptor mAb OKT9. Subclass-specific antibodies were chosen to reveal each marker. In αY and αYβ18–27 transfected cells, there was strong intracellular labeling with both antibodies. Most of the compartments were double-labeled, as expected because the transferrin receptor and the αY or αYβ18–27 constructs have a clathrin-coated pit internalization signal and are found in the same endosomal compartments. In αY-transfected cells, almost every compartment labeled with 7G7B6 was also labeled with anti-transferrin receptor mAb (Fig. 8 c). On the other hand, in αYβ18–27 transfected cells, we could detect a significant number of 7G7B6 positive compartments that were negative for transferrin receptor labeling (Fig. 8 f). Therefore, when the 18–27 sequence is added to the cytosolic tail of a recycling receptor, the resulting chimera can exit from early/recycling endosomes.

The Sorting Signal of the β Chain Does Not Function as a Transferable Internalization Signal

In most of the cases previously described, sequences necessary to orient proteins towards lysosomes are very similar to internalization signals and function as such. We therefore determined if this is also the case for the 18–27 β chain sequence. The α chain of the IL2 receptor, without β and γ, is internalized very inefficiently when transfected in K562 cells. However, this internalization is measurable (Fig. 9 a). We constructed a chimera between the IL2Rα chain and amino acids 18–27 from the cytosolic part of the

Figure 7. Endocytosis and cell surface half-life of α, αYβ17–27, and αYβ18–27 in K562 cells. (a) Kinetics of 125I-labeled 7G7B6 mAb internalization in cells stably transfected with αY, αYβ17–27, or αYβ18–27. Experiments were performed as described in Fig. 6 b. (b) Half-life of these chimeras. Cell surface expression of α on cells treated for different times with 50 μM of cycloheximide was assessed by flow cytometry using 7G7B6 mAb. Times indicated are after a 30-min preincubation in cycloheximide. In each case, one representative experiment out of three is shown. Error bars indicate standard deviations for averages of three or more experiments.
β chain, named αβ18–27 (Fig. 2). We measured the internalization of the chimera using radiolabeled anti-α mAb 7G7B6. Internalization of the chimera αβ18–27 was slow and inefficient, identical to that of α, with only 10% of the molecules being internalized at steady state (Fig. 9 a). This result was surprising because amino acids 18–27 contain part of the information for the endocytosis of β27, as β27 is internalized faster than β18 (Fig. 3 a). One could therefore expect that amino acids 18–27 of β may contribute a positive signal for the internalization of α in the αβ18–27 chimera. As this does not appear to be the case, it seems that the internalization of β27 requires other parts of the protein.

We measured the half-lives of α and αβ18–27 at the cell surface, as described above, in stably transfected K562 cells. Their surface expression was probed with anti-α mAb 7G7B6. As seen in Fig. 9 b, the α chain has a very long half-life, while the half-life of αβ18–27 was ~300 min.

To show that this half-life of αβ18–27 was due to degradation, the rate of turnover of αβ18–27 was measured in these cells by a pulse-chase experiment. Cells were iodinated and incubated in chase medium at 37°C for different times as described in the Materials and Methods section. Proteins were quantitatively immunoprecipitated from cell lysates using antibodies against the IL2Rα chain and the transferrin receptor, the precipitates resolved on SDS-polyacrylamide gels, and the radioactivity in the band corresponding to the αβ18–27 chimera or to the transferrin receptor was quantitated by phosphorimager analysis (Fig. 10). The transferrin receptor was stable over the 2 h of chase, while the intensity of the band corresponding to the αβ18–27 chimera decreased with a half-life of ~300 min. This value is the same as that measured for the half-life of αβ18–27 on the cell surface (Fig. 9) and represents a 3–5-fold increase in the rate of turnover of αβ18–27 compared to α. In conclusion, the transfer of amino acids 18–27 from β to the α chain induces degradation of this membrane protein but does not promote its endocytosis.

**Discussion**

The high-affinity IL2 receptor is continuously internalized, and after receptor endocytosis, the β chain is found in late endocytic compartments (23). Here we show by a pulse chase experiment that it is degraded. Most growth factor and cytokine receptors are degraded after internalization, which is important for the regulation of their expression and function, but the mechanism for their sorting towards degradation is not understood. Based on the hypothesis that short sequences act as tags for intracellular routing of receptors, we looked for a signal in the cytosolic tail of the β chain that would direct it towards degradation compartments. We observed that the 27 amino acids adjacent to the membrane of the cytosolic tail were sufficient for this function. We prepared a modified IL2 receptor α chain by adding to its cytosolic tail an efficient coated-pit internalization signal, the transferrin receptor signal YTRF. This construct αγ was rapidly internalized and recycled. By confocal microscopy, it was colocalized with the transferrin receptor which labels early/recycling endocytic compartments. Conversely, αβγ18–27, a chimera containing, in addition to YTRF, amino acids 18–27 of the β chain, was internalized with the same kinetics as αγ, but its half-life was shortened to ~3 h. This represents a 4–6-fold increase in the rate of turnover of αβγ18–27 compared to αγ. By confocal microscopy, αβγ18–27 was not always colocalized with the transferrin receptor, indicating that it was sorted from the recycling pathway. Therefore the P18DPSKFSQ27 sequence of the cytosolic tail of β is sufficient to provide a determinant to divert a normally rapidly internalized recycling receptor from recycling compartments. Since we have shown that the β chain as well as the αβ18–27 construct are degraded, it is most likely that this signal targets proteins towards late endocytic compartments where they are degraded.

A peptide, named BETApep, including the sorting sig-
nal and corresponding to amino acids 16–32 of the β chain, was synthesized and its structure was studied by CD and NMR. This peptide formed a nascent helix involving Phe_{23} to Leu_{27}, preceded by a type I β turn formed by amino acids Asp_{19} to Lys_{22}. It is noteworthy that this α helix is amphipathic with hydrophobic residues Phe_{23}, Phe_{24}, and Leu_{27}, on one side, and hydrophilic residues Lys_{22}, Ser_{25}, and Gln_{26}, on the other side. Strikingly, the fragment that is highly structured in the peptide containing amino acids 16–32 of β corresponds to the sorting signal defined in this work by its biological properties, i.e., amino acids 18–27. However, it cannot be ruled out that NMR data might not reflect the structural requirements in vivo. Additional experiments are needed to illustrate the structure-function relationship in this signal.

To assess the potential role of the helix distortion at the NH_{2}-terminal extremity due to Pro_{20}, we also studied the structure of a synthetic peptide identical to the previous one, except that Pro_{20} had been replaced by a leucine, a helix favoring residue (42). In aqueous solution at pH 5.4, this peptide, P20Lpep, exhibited NOE characteristics of a nascent helix from Leu_{20} to Ser_{20}, stabilized in the presence of TFE. A schematic view of this amphipathic α helix is presented in Fig. 11. In parallel with this NMR analysis, we have studied the routing after endocytosis of a mutated β_{27} receptor in which Pro_{20} had been replaced by a leucine. This receptor was internalized with the same kinetics as β_{27} and its half-life was identical to that of β_{27}. Therefore when Pro_{20} is replaced by a leucine, receptor routing is not affected. The corresponding structure is α-helical, having lost the NH_{2}-terminal kink due to the proline in the wild-type sequence. These results indicate that the kink is not important for the signal. It is interesting to compare this signal with the internalization signal of the invariant chain: both have the same kind of structure, but in the latter case, the kink of the invariant chain seems to be required for internalization (38). Other regular structures have already been reported for signals involved in the endocytic pathway, for coated pit localization of a few receptors (4, 14, 38, 58), targeting to synaptic vesicles of VAMP (17), and ER retention of CD3ε (31). The known structures fall into two categories, those which form a tight β-turn (4, 14) and those which form an α helix (17, 38, 58). This α helix is often preceded or followed by a turn (17, 31, 38, 58). Here we show that the turn at the beginning of the α helix can be removed without altering the signal function.

Most of the signals which sort membrane glycoproteins at different steps of their traffic along the endocytic pathway fall into two categories, the tyrosine-containing and di-leucine-containing signals. The targeting sequences found in lysosomal glycoproteins belong to these two categories (20, 24, 33, 43, 59). It is also the case for proteins which are targeted to late endocytic compartments such as the CD3γ and CD3ε chains of the T cell surface antigen receptor complex (29), the invariant chain (41), CD4 in Nef expressing cells (2), a CD8/gp75 chimera in fibroblasts (55), and the β chain of HLA-DM (32). The sorting signal that we found in the IL2Rβ chain is not related to the tyrosine or di-leucine based motives. It also shares no clear se-
The efficiency of sorting may account for the more efficient sorting. Alternatively, the chain, might function more efficiently in the presence of ligand when present. In the latter case, it did not enhance its low internalization rate.

In this study, we have found that the half-life of the wild-type $\beta$ chain at the cell surface of K562 cells is 150 min. This value is similar to that of P-selectin, between 75 and 150 min, and faster than that of lysosomal acid phosphatase, $\sim 3-4$ h, or of the $\beta$ chain of HLA-DM in HeLa cells, $\sim 3$ h (7, 15, 32). The half-life of $\beta_{27}$ is the same as that of the whole $\beta$ chain, while its internalization is about three times faster. This observation is in agreement with a recent study showing that there was no simple correlation between the internalization and degradation rates (61). Also, there may be another sequence, further downstream in the $\beta$ chain, that is also involved in degradation.

Under physiological conditions, $\beta$ is not expressed by itself: in lymphocytes or natural killer cells, the $\gamma$ chain is also present. The ligand in the surface of K562 cells is 150 min. The expression of each of the chains on the cell surface may be rapidly modulated by the efficiency of its degradation after endocytosis, which allows for subtle control of receptor expression and function.

Over the years, the term “receptor-mediated endocytosis” has become synonymous with internalization via clathrin-coated pits. However, other receptor-mediated endocytosis pathways have also been reported (reviewed in reference 28). When clathrin-coated pit endocytosis is inhibited, IL2 receptors are still internalized (51). When this classical pathway functions, the proportion of IL2 receptors entering via either pathway is not known. Even if a significant proportion of IL2 receptors entered cells by a clathrin-independent mechanism, since both pathways seem to rejoin in early endosomes (19), where sorting probably occurs (34), one would expect the signal to function regardless of the entry pathway used. The sorting signal of the $\beta$ chain was functional in a chimera having the transferrin receptor coated-pit localization signal, $\alpha_\beta_{21-27}$. Therefore this signal directs receptors towards degradation compartments when they are internalized via coated-pits.

The way sorting towards degradation compartments occurs is not known. In some cases, aggregation of recycling receptors has been shown to drive them out of the recycling pathway and towards lysosomes. We do not know if the sorting signal we describe in this paper can cause aggregation. However this seems unlikely, since point mutations in the signal disrupt its function and the resulting receptors have an increased half-life (manuscript in preparation). Alternatively, the degradation signal might be recognized by a component of a cytoplasmic coat, such as COP proteins, which seem to be involved in endosome function (3, 57).

It is striking that sorting motifs which function at different sites in cells and which target membrane glycoproteins to various organelles share common features in their sequence or in their structure. Although the nature of the specific molecules that are able to recognize these motifs is not known, the similarity of the signals suggest that the machineries that decipher them at different cell sites may also be related. Further studies will be needed to understand how these various signals function to specifically target receptors to different organelles.

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References

1. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1993. Current Protocols in Molecular Biology. K.

Figure 11. Helical wheel representation of amino acids 20 to 27 of P20Lpep. Hydrophobic amino acids are boxed.
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10. DiSanto, J.P., F. Rieux-Laucat, A. Dautry-Varsat, A. Fischer, and G. de Griesinger, C., G. Otting, K. Wüthrich, and R.R. Ernst. 1988. Clean TOCSY for 1H spin system identification in macromolecules.

12. Duprez, V., V. Cornet, and A. Dautry-Varsat. 1988. Down regulation of the human transferrin receptor cytoplasmic domain in endocytosis: location of a specific signal sequence for internalization.

15. Green, S.A., H. Setiadi, R.P. McEver, and R.B. Kelly. 1994. The cytoplasmic tail of the mlgp120 (lamp-1) in MDCK cells.

21. Hémar, A., and A. Dautry-Varsat. 1990. Cyclosporin A inhibits the intercellular processing compartment.

23. Höning, S., and W. Hunziker. 1995. Cytoplasmic determinants involved in endocytic processing compartment.

26. Hunziker, W., and H.J. Geuze. 1996. Intracellular trafficking of lysosomal membrane proteins.

28. Lamaze, C., and S.L. Schmid. 1995. The emergence of clathrin-independent pinocytic pathways.

24. Höning, S., and W. Hunziker. 1995. Cytoplasmic determinants involved in intracellular trafficking of lysosomal membrane glycoprotein LEPI010 to lysosomes. J. Biol. Chem. 268:1027–1040.

30. Mota, A., B. Bremnes, M.A. Castiglione Morelli, R.W. Frank, G. Saviano, and O. Bakke. 1995. Structure-activity relationship of the leucine-based sorting motifs in the cytoplasmic tail of the major histocompatibility complex-associated invariant chain. J. Biol. Chem. 270:27165–27171.

32. Nelson, J.W., and N.R. Kallenbach. 1986. Stabilization of the ribonuclease tetrapeptide α-helix by trifluoroethanol. Proteins. 1:211–217.

34. Noguchi, M., H. Yi, H.M. Rosenblatt, A.H. Filipovich, S. Adelstein, W.S. Modi, O.W. McBride, and W.J. Leonard. 1993. Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. Cell. 73:147–157.

36. Odorizzi, C.G., I.S. Trowbridge, L. Xue, C. Hopkins, C.D. Davis, and J.F. Collawn. 1994. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic compartment. J. Cell Biol. 126:317–330.

38. Padmanabhan, S., S. Marguès, T. Robenek, T.M. Luse, and R.L. Baldwin. 1990. Relative helix-forming tendencies of nonpolar amino acids. Nature (Lond.). 344:268–270.

40. Rance, M., O.W. Sorensen, G. Bodenhausen, and G. Wagner. 1983. Improved spectral resolution in COSY/H NMR spectra of proteins via double quantum filtering. Biochem. Biophys. Res. Commun. 117:479–485.

42. Robinson, M.S. 1994. The role of clathrin, adaptors and dynamin in endocytosis.

44. Robinson, M.S., C. Watts, and M. Zerial. 1996. Membrane dynamics in endocytosis. Cell. 84:13–21.

46. Ruprecht, J.A., A. Schweitz, D. Russell, and S. Kornfeld. 1996. The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. J. Cell Biol. 132:565–576.

48. Rubin, L.A., C.C. Kutman, M.E. Fritz, W.E. Biddison, B. Boutin, R. Yarrow, and D.L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. J. Immunol. 135:3172–3177.

50. States, D.J., R.A. Haberkorn, and D.J. Ruben. 1982. A two-dimensional nuclear overhauser experiment with pure absorption phase in four quadrants. J. Magn. Reson. 48:286–292.

52. Sugamura, K., H. Asao, M. Kondo, N. Tanaka, N. Ishii, K. Ohbo, M. Nakamura, and T. Takehisa. 1996. The interleukin 2 receptor α chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. Ann. Rev. Immunol. 14:179–205.

54. Trowbridge, I.S., and C.R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. 9:1–31.

56. Vijayasunder, S., Y.A. Xu, B. Bouchard, and A.N. Houghton. 1995. Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, GP75. J. Cell Biol. 130:807–820.

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56. Voss, S.D., T.P. Leary, P.M. Sondel, and R.J. Robb. 1993. Identification of a direct interaction between interleukin 2 and the p64 interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA.* 90:2428–2432.

57. Whitney, J.A., M. Gomez, D. Sheff, T.E. Kreis, and I. Mellman. 1995. Cytoplasmic coat proteins involved in endosome function. *Cell.* 83:703–715.

58. Wilde, A., C. Dempsey, and G. Banting. 1994. The tyrosine-containing internalization motif in the cytoplasmic domain of TGN38/41 lies within a nascent helix. *J. Biol. Chem.* 269:7131–7136.

59. Williams, M.A., and M. Fukuda. 1990. Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* 111:955–966.

60. Wishart, D.S., B.D. Sykes, and F.M. Richards. 1991. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J. Mol. Biol.* 222:311–333.

61. Zwart, D.E., C.B. Brower, J. Lazarovits, Y.I. Henis, and M.G. Roth. 1996. Degradation of mutant influenza virus hemagglutinins is influenced by cytoplasmic sequences independent of internalization signals. *J. Biol. Chem.* 271:907–917.