Identification of Distinct Roles for a Di-leucine and a Tyrosine Internalization Motif in the Interleukin-13 Binding Component IL-13 Receptor α2 Chain

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The abbreviations used are: IL-13Rα2, interleukin-13 receptor α2 chain; DMEM, Dulbecco's modified Eagles medium; IL13-PE38QQR, a recombinant fusion protein composed of IL-13 and a truncated form of *Pseudomonas* exotoxin A.
SUMMARY

Interleukin-13 Receptor α2 (IL-13Rα2) chain is an essential binding component for IL-13 mediated ligand binding. Recently, we have demonstrated this receptor chain also plays an important role in the internalization of IL-13. To study the mechanism of IL-13 internalization, we generated mutated IL-13Rα2 chains which targeted tri-leucine residues (L335, L336, and L337) in the transmembrane domain and a tyrosine motif (Y343) in the intracellular domain, and transfected these cDNAs in COS-7 cells. Cells that expressed a C-terminally truncated IL-13Rα2 chain (Δ335) did not bind IL-13 suggesting that the tri-leucine region modulates IL-13 binding. Truncation of IL-13Rα2 chain with a mutation in the tri-leucine region resulted in significantly decreased internalization levels compared to wild type IL-13Rα2 chain transfected cells. COS-7 cells transfected with tyrosine motif mutants exhibited a similar internalization level compared to wild type IL-13Rα2 chain transfected cells, however, dissociation of cell surface IL-13 was faster compared to wild type IL-13Rα2 transfectants. These results were further confirmed by determining the cytotoxicity of a chimeric protein composed of IL-13 and a mutated form of *Pseudomonas* exotoxin (IL13-PE38QQR) to cells that expressed IL-13Rα2 chain mutants. We further demonstrate that the IL-13Rα2 chain is not ubiquitinated and internalization of IL-13Rα2 did not depend on ubiquitination. Together, our findings suggest that the di-leucine motif in the tri-leucine region and tyrosine motif participate in IL-13Rα2 internalization in distinct manners.
INTRODUCTION

Unlike receptors for the related cytokine IL-4, the receptors for IL-13 (IL-13R) have not been well characterized. We have been studying the structure of IL-13R in various cell types (1-6). We reported that IL-13 binds to two isoforms of 65-kDa proteins in human renal cell carcinoma cells, and one of these proteins also binds IL-4 (1). On the basis of binding characteristics, cross-linking, and displacement of radiolabeled IL-4 and IL-13 in various cell types, we hypothesized that similar to the IL-4R system, IL-13R may also exist as three different types (1-6). Two different chains of the IL-13 receptor, IL-13Rα1 and IL-13Rα2 (also known as IL-13Rα and IL-13Rα, respectively), have been cloned, and correspond to the two 65-kDa isoforms as we originally proposed (1). The murine and human IL-13Rα1 chains were cloned first (7, 8). This chain binds IL-13 at low levels but when coupled with the IL-4Rα chain (also known as IL-4Rβ) binds IL-13 with higher affinity and mediates IL-13-induced signaling (9, 10). The second chain of IL-13R, termed IL-13Rα2 has been cloned from a human renal cell carcinoma cell line (Caki-1). This chain has 50% identity to the IL-5R at the DNA level, has a short intracellular domain, and binds IL-13 with high affinity (11).

Cells selectively internalize specific surface ligand-receptor complexes through receptor-mediated endocytosis. This process of endocytosis begins when receptors are selectively sequestered into specialized structures on the plasma membrane, termed clathrin-coated pits. These pits are able to recognize receptors through short structures of amino acids in the cytoplasmic domains (12-15). These domains contain specific targeting
information. The most common internalization signals described are: the tyrosine-based motif and the di-leucine motif. The tyrosine-based motif contains a tyrosine residue usually comprised of 4-6 amino acids and is generally formed of NPXY or YXXØ (where X is any amino acid and Ø is a hydrophobic residue; Ref. 16-19). There are various examples that utilize NPXY or YXXØ motifs for endocytosis. Although the precise mechanism for the sequestration of surface receptors in coated pits is unknown, LDL receptors are shown to be endocytosed via their NPXY motif (20). Similarly, numerous other cell surface proteins including epidermal growth factor receptor (EGFR), insulin receptor family, the β-subunits of three integrin receptors, and the amyloid A4 precursor protein utilize the NPXY motif for internalization (20). On the other hand, the transferrin receptor and the asialoglycoprotein receptor endocytose via a YXXØ motif (16, 21). It has been demonstrated that a di-leucine motif in the intracellular domain of various receptor systems, e.g., interleukin-6 receptor (IL-6R) gp130, granulocyte colony-stimulating factor receptor (G-CSFR), epidermal growth factor receptor (EGFR), growth hormone receptor (GHR), human insulin receptor (HIR), β2-adrenergic receptor, lutropin/choriogonadotropin receptor (rLHR), and erythropoietin receptor (EPO-R) plays an essential role in the internalization of ligand (22-30).

Recently, we have demonstrated that the IL-13Rα2 chain plays a critical role in ligand binding and internalization (10, 31). After binding to its receptor, IL-13 can signal through the JAK/STAT signal transduction pathway (4-6, 32-35). Although we and others have reported that IL-13Rα2 does not participate in the signal transduction pathway, it can bind and rapidly internalize IL-13 (10, 31, 36). However, the mechanism how the IL-
13Rα2 chain mediates internalization is unknown (10). To address this issue, we generated IL-
13Rα2 chain mutants which were transfected in COS-7 cells. The roles of the tri-leucine
motif (position L335, L336, and L337) in the C-terminus of the transmembrane domain
and the tyrosine motif (position Y343) in the intracellular domain of the IL-13Rα2 chain
were studied in internalization assays using 125I-IL-13. Internalization assays were also
performed by determining the cytotoxicity of a chimeric protein composed of IL-13 and a
mutated form of *Pseudomonas* exotoxin (IL13-PE38QQR) (Ref. 31, 37, 38) to cells that
were transfected with IL-13Rα2 chain mutants. IL13-PE38QQR binds to IL-13R and is
internalized by endocytosis subsequently causing cell death through the inhibition of new
protein synthesis. Thus, cytotoxicity observed in transfected cells indicates receptor
internalization. Here we demonstrate that the di-leucine motif in a tri-leucine region is
critical for ligand binding and internalization while the tyrosine motif is not responsible for
internalization. Instead, the tyrosine motif appears to be responsible for cell-surface IL-13
binding characteristics. We further demonstrate that the IL-13Rα2 chain is not
ubiquitinated.
EXPERIMENTAL PROCEDURES

Recombinant Cytokine, Toxin, and Cell Culture - Recombinant human IL-13 was produced and purified in our laboratory (39). Recombinant IL-13-PE38QQR was also produced and purified in our laboratory (Joshi et al., unpublished results). Monkey kidney fibroblast (COS-7) cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM containing 10% fetal bovine serum (Biowhittaker, Walkersville, MD), 1 mM HEPES, 1 mM L-glutamine, 100 µg/mL penicillin, and 100 mg/mL streptomycin (Biowhittaker).

Mutagenesis and Transient Transfection of DNA - cDNAs of the human IL-13Rα2 chain (WT; Ref. 11) were cloned into a pCI-neo mammalian expression vector (Promega, Madison, WI). The IL-13Rα2 deletion mutants Δ335, Δ338, Δ343, or Y343F were constructed by polymerase chain reactions (PCRs) using Taq Gold DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) and pME18s-IL13Rα2 as a template (Ref. 31) with the primer 5-CCGCTCGAGATGGCTTTCGTTTGCTTGGCTATCGG-3 and 3'-GCTCTAGATCAACCGGTTACAAATATAACTAATATTAAG-5' (Δ335) or 3'-GCTCTAGATCAACAAAGCAGACCGGTTACAAATATAAC-5' (Δ338) or 3'-GCTCTAGATCAACAAAGCAGACCGGTTACAAATATAAC-5' (Δ343) or 3'-GCTCTAGATCAACAAAGCAGACCGGTTACAAATATAAC-5' (Y343F) each containing an in-frame stop codon. For the other IL-13Rα2 mutants, PCR was performed using the primer 5-

CCGCTCGAGATGGCTTTCGTTTGCTTGGCTATCGG-3 and 3-

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GCACCGGTGTTTACAAATATAACTAAATAAGATGAAACC-5 containing a AgeI restriction site, and 5'-primers containing a AgeI restriction site and 3'-
GAGCTCGGTACCCGGGGATCCAGAC-5. Other 5’-primers with a AgeI restriction sites were: 5’-
GTAACCGGTGCCTTTTGCGTAAGCCAAACACCTACCCTACCCAAAAATG-3’ (L335A),
5’-GTAACCGGTCTTGCTTGCAGCAGCAACACCTACCCTACCCAAAAATG-3’
(L336A), 5’-GTAACCGGTCTTGCTTGCAGCAGCAACCTACCCTACCCAAAAATG-3’ (L337A), 5’-
GTAACCGGTGCCTTGCGTAAGCCAAACACCTACCCTACCCAAAAATG-3’
(L335A/L337A), 5’-
GTAACCGGTATTATATTATCGTAAGCCAAACACCTACCCTACCCAAAAATG-3’
(L335I/L336I/L337I). These PCR products were digested with AgeI restriction enzyme and ligated with the DNA Ligation Kit ver.1 (TAKARA Shuzo, Shiga, Japan). Using these ligation reaction cocktails as template, PCR reactions were performed with the primer 5’-
CCGCTCGAGATGGCTTTCGTTTGCTTGGCTATCGG-3’ and 3’-
GCTCTAGATCATGTATCACAGAAAAATTCTGG-5’ or 3’-
GCTCTAGATCATGTATCACAGAAAAATTCTGGGAATCATTTTTGGGAAGGTG-5’
(L335A/L336A/L337A/Y343F) containing an in-frame stop codon. Finally, the mutant cDNAs for the IL-13Rα2 were subcloned into the expression vector pCI-neo using the XhoI and XbaI sites. All constructs were verified for sequence by ABI Prism 310 (PE Applied
Biosystems, Foster city, CA).

Plasmid DNAs (12 µg/100-mm culture dish) were transfected into semiconfluent cells using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s instructions. Briefly, cells (2 x 10^6/100-mm dish) were incubated with the DNA-GenePORTER mixture for 5 h in DMEM (Biowhittaker). Then DMEM containing 20% FBS was added and incubation was continued. Twenty four hours after transfection, the medium was changed to DMEM with 10% FBS and the cells were incubated for an additional 24 h.

**RT-PCR** - To detect the mRNA expression of the IL-13Rα2 chain in DNAs-transfected COS-7 cells, total RNA was isolated using TRIZOL reagent (Life Technologies, Grand Island, NY), then RT-PCR analysis was performed. Two µg of total RNA was incubated for 30 min at 42°C in 20 µl reaction buffer containing 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 50 mM KCl, 1 mM each of dNTPs, 1 unit/µl RNase inhibitor, 2.5 µM random hexamer, and 2.5 unit/µl of MMLV RT (Perkin-Elmer, Norwalk, CT). A 10 µl aliquot of RT reaction was amplified in 100 µl final volume of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 50 mM KCl, 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer), and 0.1 µg of specific primer (5’-AATGGCTTTCCGTTTGGCTTG-3’ and 5’-ACGCAATCCATATCCTGAAC-3’) (40). The PCR product (20 µl) was run on a 2% agarose gel for UV analysis.

**Radioreceptor Binding Assay** - Recombinant human IL-13 was labeled with ^125^I (Amersham Corp., Arlington Heights, IL) using IODO-GEN reagent (Pierce, Rockford, IL)
as previously described (1). The specific activity of the radiolabeled IL-13 was estimated to be 6.0 µCi/µg of protein. For binding experiments, 5 x 10^5 cells in 100 µl binding buffer (RPMI 1640 containing 0.2% human serum albumin and 10 mM HEPES) were incubated with 200 pM \(^{125}\text{I}-\text{IL-13}\) with or without 40 nM unlabeled IL-13 at 4°C for 2 h. Cell-bound \(^{125}\text{I}-\text{IL-13}\) was separated from unbound by centrifugation through a phthalate oil gradient and radioactivity was determined with a gamma counter (Wallac, Gaithersburg, MD).

**Internalization Assay** - Internalization assays were performed as described before (10, 41). COS-7 cells transfected with the IL-13R\(\alpha_2\) chain were incubated in binding buffer containing 0.2 nM chloroquine at 37°C for 5 min to prevent degradation of internalized \(^{125}\text{I}-\text{IL-13}\). The cells were then washed, and 2 x 10^7 cells were incubated with 0.5 nM \(^{125}\text{I}-\text{IL-13}\) at 4°C for 2 h. After removing free \(^{125}\text{I}-\text{IL-13}\), cell pellets were resuspended in 2 ml of binding buffer and incubated at 37°C. At various time intervals, two duplicate sets of 50 µl aliquots were taken. One set was incubated with glycine buffer (final pH = 2.0) for 10 min on ice. The suspension was then centrifuged through a mixture of phthalate oils, and the radioactivity in the cell pellet (acid-resistant or internalized) and in the supernatant (surface-bound plus dissociated) was determined. The other set of 50 µl aliquots was directly centrifuged through phthalate oils, and the radioactivity observed in the supernatants was used for dissociated \(^{125}\text{I}-\text{IL-13}\) values. Surface-bound \(^{125}\text{I}-\text{IL-13}\) was determined by subtracting dissociated \(^{125}\text{I}-\text{IL-13}\) values from surface-bound plus...
dissociated values.

To determine if proteasome-mediated proteolysis is involved in the expression and internalization of IL-13Rα2 chain, we pretreated COS-7 cells with 0.1% Me₂SO (DMSO) or 50 µM proteasome inhibitor, MG132 (Sigma) for 30 min at 37°C. During binding and internalization assays, DMSO or MG132 continued to be present in the binding buffer.

Protein Synthesis Inhibition Assay - The cytotoxic activity of IL13-toxin was tested as previously described (38). Typically, 10⁴ cells per well were cultured in leucine-free medium with or without various concentrations of IL13-PE38QQR for 20-22 h at 37°C. Then 1 µCi of [³H]leucine (NEN Research Products, Boston, MA) was added to each well and incubated for an additional 4 h. Cells were harvested and radioactivity incorporated into cells was measured by a beta plate counter (Wallac).
RESULTS

Construction of IL-13Rα2 mutants - Single, double, or triple amino acid substitutions in the tri-leucine region of IL-13Rα2 chain were performed by site-directed mutagenesis. As shown in Fig. 1, tri-leucine residues resided in the transmembrane region of the IL-13Rα2 chain. Leucine residues were either changed to alanine or to isoleucine without any other modifications or combined with the substitution of tyrosine at position 343 by phenylalanine of the IL-13Rα2 chain. However, in some cases leucine residues were unchanged while the intracellular domain of IL-13Rα2 chain was either completely or partly deleted or only one tyrosine residue was changed to phenylalanine at position 343. Finally, one mutant lacked all three leucine residues and the complete intracellular domain.

125I-IL-13 Binding to IL-13Rα2 mutants - To confirm the successful transfection of plasmid DNAs for IL-13Rα2 mutants in COS-7 cells, total RNA was extracted from the transfectants and RT-PCR analysis was performed using primers which can detect part of the extracellular domain of the IL-13Rα2 chain (40). As shown in Fig. 2A, wild type IL-13Rα2 and all the mutants showed high expression of mRNA for the extracellular domain of the IL-13Rα2 chain. In naive and vector only (mock) transfected COS-7 cells, very faint expression of this chain was observed as we previously demonstrated (40). These data suggest that all IL-13Rα2 mutants were successfully transfected even though COS-7 cells seemed to express very faint IL-13Rα2 mRNA. To determine the amount of protein for each expressed plasmid, 125I-IL-13 binding assays were performed on various...
transfectants as specific antibody to IL-13Rα2 chain is not commercially available for Western blot analysis. Mutated IL-13Rα2 transfected cells were incubated with $^{125}$I-IL-13 in the absence or presence of 200-fold molar excess of IL-13. As shown in Fig. 2B, $^{125}$I-IL-13 bound to all receptor mutant transfected COS-7 cells at similar levels with the exception of naive cells, mock transfected cells, or cells transfected with the Δ335 construct. Excess unlabeled IL-13 displaced the binding of $^{125}$I-IL-13, indicating specific IL-13 binding. Interestingly, in Δ335 transfected COS-7 cells, $^{125}$I-IL-13 did not bind even though these cells expressed mRNA for this chain.

**Di-leucine Motif Mediated Internalization of IL-13Rα2 Chain** - To investigate the role of the di-leucine motif in the tri-leucine region in the C-terminus of the transmembrane domain of the IL-13Rα2 chain, mutated IL-13Rα2 genes, Δ338, L335A, L336A, L337A, L335/L337A, L335A/L336A/L337A, or L335I/L336I/L337I were transfected in COS-7 cells, and internalization assays were performed. We also performed internalization assays using COS-7 cells transfected with vector only that served as a mock control, however, its binding to radiolabeled IL-13 was too low to detect significant internalization (data not shown). As shown in Fig. 3, di-leucine motif conserved (two of three leucines unchanged) mutants, Δ338, L335A, and L337A showed similar internalization levels (up to 80% at 120 min) as IL-13Rα2 when transfected in COS-7 cells. However, COS-7 cells transfected with L336A or L335A/L337A which has no continuous leucine residues, the internalization level was decreased to 65% (L336A) or 49% (L335A/L337A) at 120 min. Furthermore, COS-7 cells transfected with
L335A/L336A/L337A in which all three leucine residues were converted into alanine, the internalization level decreased to 40% even though the maximum plateau internalization level was observed between 90 min and 120 min. Whereas L335I/L336I/L337I transfectants showed the same internalization level (up to 79% at 120 min) as IL-13Rα2. These results demonstrate that the di-leucine motif in the tri-leucine region of the IL-13Rα2 chain is necessary for efficient IL-13 internalization.

Tyrosine Motif Mediated Internalization of IL-13Rα2 Chain - In the intracellular domain of IL-13Rα2 chain, there is one tyrosine residue at amino acid position 343. To determine whether this tyrosine plays a role in internalization, mutated cDNAs of IL-13Rα2, Δ343, and Y343F were transfected in COS-7 cells and internalization assays were performed. As shown in Fig. 4, IL-13Rα2 transfected COS-7 cells internalized 125I-IL-13 in a time-dependent manner, and internalization level increased up to 81% in 120 min. In Δ343 or Y343F transfected COS-7 cells, the 125I-IL-13 internalization level was found to be similar to IL-13Rα2 transfectants (80% in 120 min). However, dissociation of surface bound 125I-IL-13 in Δ338, Δ343, and Y343F transfectants was faster compared to IL-13Rα2. Half lives (T₁/₂) of the dissociation of cell surface 125I-IL-13 binding in IL-13Rα2 transfectants was estimated to be 31 ± 2 min compared to 11 to 13 min in tyrosine mutated IL-13Rα2 chain transfectants (Table I). These results suggest that although Y343 does not participate directly in the internalization process, it plays an important role in maintaining cell surface IL-13 binding to its receptor.

To further study this phenomenon and determine whether both di-leucine motif and
tyrosine motif mutation in IL-13Rα2 chain modulated endocytosis, internalization assays were performed using L335A/L336A/L337A/Y343F transfected cells. As shown in Fig. 4, the 125I-IL-13 internalization level was similar (32% in 120 min) to L335A/L336A/L337A mutant (Fig. 3). However, this internalization level was lower than that seen in Y343F transfectants. On the other hand, the ligand dissociation was faster in L335A/L336A/L337A/Y343F compared to L335A/L336A/L337A transfectants. These results suggest that L335A/L336A/L337A phenotype dominates over Y343F phenotype as far as endocytosis is concerned, however, Y343F phenotype dominates over L335A/L336A/L337A phenotype for ligand dissociation. These data also suggest that Y343 in the intracellular domain of IL-13Rα2 chain is required to maintain cell surface 125I-IL-13 binding at physiological temperature.

**Effect of Co-expression of Wild Type IL-13Rα2 Chain with Di-leucine Targeted Mutants on 125I-IL-13 Internalization in COS-7 Cells** To further study how leucines affect receptor internalization, we co-transfected COS-7 cells with equal amounts of DNA (6 µg/each) for the IL-13Rα2 chain and either L335A/L337A or L335A/L336A/L337A receptor mutants and internalization assays were performed. As shown in Fig. 5, the maximum internalization level was lower in both type of transfectants (50% in 120 min) compared to wild type IL-13Rα2 transfectants (80% in 120 min; Fig. 3). However, IL-13Rα2 + L335A/L337A or IL-13Rα2 + L335A/L336A/L337A transfectants still showed slightly better internalization compared to L335A/L337A or L335A/L336A/L337A alone transfected cells (Fig. 3; 50% vs 49% in IL-13Rα2 + L335A/L337A and L335A/L337A alone transfectants.
transfectants respectively and 51% vs 40% in IL-13Rα2 + L335A/L336A/L337A and L335A/L336A/L337A transfectants respectively). In contrast, the dissociation rate of surface bound 125I-IL-13 appeared to be similar to wild type IL-13Rα2 transfectants. These results further confirmed our findings that the IL-13Rα2 chain utilizes the di-leucine motif for ligand internalization.

**Cytotoxicity of IL13-PE38QQR to IL-13Rα2 Mutants Transfected COS-7 Cells**

To further confirm the results obtained by internalization assays, the cytotoxicity of recombinant IL13-PE38QQR which targets IL-13R was assessed. IL13-PE38QQR binds to IL-13R and is internalized by endocytosis subsequently causing cell death through the inhibition of new protein synthesis. Thus, cytotoxicity observed in transfected cells indicates receptor internalization (10, 31, 37, 38). COS-7 cells were transfected with the IL-13Rα2 chain or its mutants and sensitivity to IL13-PE38QQR was determined (Fig. 6). The IC50s (IL-13 toxin concentration causing 50% inhibition of protein synthesis) was calculated from the cytotoxicity data (Table II). When COS-7 cells were transfected with IL-13Rα2, the cytotoxicity of IL13-PE38QQR increased in these cells. The IC50 in IL-13Rα2 transfected cells was 10-fold lower compared to vector only transfected cells (from 200 ng/ml to 20 ng/ml). In COS-7 cells transfected with Y343F or L335I/L336I/L337I, sensitivity to IL-13 toxin was similar to that seen in IL-13Rα2 transfectants (Fig. 6C and F). On the other hand, when COS-7 cells were transfected with di-leucine motif deleted or substitution mutants (Δ335, L335A/L337A, or L335A/L336A/L337A), sensitivity to IL-13 toxin did not change compared to vector only transfected cells (Fig. 6B, D, and E). These
data further confirm the results that the di-leucine motif mediates internalization and the tyrosine motif does not play a direct role in this process.

Proteasome Inhibitor MG132 does not alter Internalization or Stabilize the Ligand Dissociation from IL-13Rα2 chain

To assess whether proteasome-mediated proteolysis is involved in the expression and endocytosis of IL-13Rα2 chain, COS-7 cells were transfected with cDNA for the IL-13Rα2 chain and then incubated with a proteasome inhibitor (MG132) and internalization assays were performed. MG132 did not affect on the binding of 125I-IL-13 in IL-13Rα2 transfectants (data not shown). Similarly, as shown in Fig. 7, both internalization and surface bound 125I-IL-13 levels in IL-13Rα2 transfectants were identical in both control (incubated with DMSO) and MG132 treatment groups. In addition, there was no significant difference in the dissociation rate in both groups. The concentration of MG132 used (50 µM) has been shown to stabilize IL-2 induced STAT5 activation in CTLL-2 cells (49). These results suggest that the IL-13Rα2 chain is not ubiquitinated and the internalization process does not depend on ubiquitination.
DISCUSSION

In this study, we have characterized the molecular basis for IL-13 mediated internalization of the IL-13Rα2 chain. By generating IL-13Rα2 mutants targeted to the di-leucine motif in a tri-leucine region in the C-terminus of transmembrane domain and a tyrosine motif in the intracellular domain of the IL-13Rα2 chain, we performed binding assays and internalization assays to investigate the mechanism of IL-13 internalization by the IL-13Rα2 chain.

Although mRNA expression was confirmed by RT-PCR, a truncation mutation Δ335 which deletes three leucine residues in the transmembrane domain and the whole intracellular domain did not demonstrate a great deal of IL-13R on the cell surface of COS-7 cells as assessed by radiolabeled binding assays. This is because naive COS-7 cells express IL-13Rα1 and IL-4Rα chains along with very faint mRNA for the IL-13Rα2 chain by RT-PCR as we have previously reported (40). These cells expressed IL-13R at a levels similar to naive cells and vector only transfected cells. The lack of IL-13 binding in Δ335 transfected cells may be due to the deletion of hydrophobic amino acid residues L335/L336/L337 in the C-terminus of the transmembrane domain and/or deletion of the intracellular domain. This modification in the IL-13Rα2 chain may provide no anchor for cell surface receptor expression and/or proper folding of extracellular domain necessary for IL-13 binding. These results suggest that the tri-leucine residues and/or intracellular domain of the IL-13Rα2 chain may be essential for IL-13 binding.

To characterize the role of the di-leucine motif in a tri-leucine region in the
internalization process, eight IL-13Rα2 mutants were generated in which one, two, or all three leucine residues were changed to alanine or isoleucine and cDNAs were transiently transfected in COS-7 cells. Conversion of all three leucine residues (L338/L336/L337) to alanine decreased the internalization level to half of the wild type IL-13Rα2 transfectants, however, when only one leucine at position 335 (L335A) or 337 (L337A) was converted to alanine, no diminution in internalization was observed. When leucine 336 was converted to alanine (L336A) or two discontinuous leucines 335 and 337 (L335A/L337A) were converted to alanine, a decrease in the internalization rate was observed. These results suggest that the di-leucine motif is required for IL-13-IL-13Rα2 chain complex internalization. Thus, our results confirm previous observations that the di-leucine motif is necessary for internalization of various cytokine-receptor complexes (22-30).

When all three leucine residues were converted to isoleucine (L335I/L336I/L337I), no diminution of internalization was observed. This is in contrast to IL-6R gp130 in which mutation of first leucine (Leu-145) to isoleucine resulted into diminished internalization function (23). However, in the wild type leukemia inhibitory factor receptor (LIFR) a leucine-isoleucine internalization motif exists naturally (43). Furthermore, in IL-6R, GLUT4 and CD4, the di-leucine motif acts in cooperation with an upstream serine for internalization (23, 44, 45). Similarly, CD3γ and invariant chains are internalized by a di-leucine motif and an upstream aspartic acid (46, 47). These results suggest that the di-leucine motif may not be solely responsible for receptor internalization. Although the di-leucine motif in the tri-leucine region of the IL-13Rα2 chain does not have upstream serine or aspartic acid, our findings suggest that the tri-leucine residue or the di-leucine
motif by itself plays an essential role in IL-13 internalization.

It is of interest to note that when double or triple leucine mutants (L335A/L337A and L335A/L336A/L337A) were transfected with the wild type IL-13Rα2 chain, a significant inhibition of internalization was observed compared to IL-13Rα2 transfectants. However, the internalization level was slightly higher compared to that caused by L335A/L337A or L335A/L336A/L337A transfectants. These results suggest that mutant IL-13Rα2 chains may form a complex with the wild type IL-13Rα2 chain resulting into diminished internalization without affecting the dissociation of surface bound 125I-IL-13.

Because IL-13Rα2 chain has a YPKM motif at amino acid 343-346 and this motif is equivalent to the YXXØ [where X is any amino acid and Ø is a hydrophobic motif (11, 16-19)], we generated two mutants targeting this motif and its role in the internalization process was investigated. Interestingly, Δ343 or Y343F did not change internalization level compared to wild type IL-13Rα2 when mutant cDNAs were transfected in COS-7. However, the dissociation of surface bound 125I-IL-13 was faster in Δ343 and Y343F transfectants compared to IL-13Rα2 transfectants. This mechanism of faster dissociation of IL-13 in Δ343 or Y343F mutant is not clear. It is possible that the tyrosine residue at position 343 forms a tight β-turn in the secondary structure of the IL-13Rα2 chain that would retain ligand for a longer period of time on the cell surface (16-19).

Several studies have suggested that covalent modification of proteins such as ubiquitination can modulate receptor internalization and ligand induced signal transduction (48-50). Generally, target proteins are tagged with multiple small protein ubiquitin which
are then destroyed by the proteosome complex. Ubiquitination has been shown to regulate
IL-2 induced signal transduction through stabilization of STAT5 activation (49), and IFN-
γ induced STAT1 activation (51). We examined whether the IL-13Rα2 chain was
ubiquitinated and whether the internalization process was modulated by ubiquitination. We
found that the IL-13Rα2 chain was not ubiquitinated, and that the internalization process
did not depend on ubiquitination. However, whether ubiquitination modulated IL-13-
induced signal transduction pathway is unknown and is subject of investigations in our
laboratory.

In summary, we have characterized the internalization motifs in the IL-13Rα2
chain. Although the interaction between a di-leucine motif and a tyrosine-motif is not the
same in different receptor types, in case of the IL-13Rα2 chain the di-leucine motif in the
tri-leucine region was found to play an essential role in internalization, and the tyrosine
motif was found to play an indirect role in ligand binding and internalization.
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FIGURE LEGENDS

FIG. 1. Schematic representation of the wild type and mutant IL-13Rα2 chains. EC, extracellular domain; TM, transmembrane domain; and IC, intracellular domain of the IL-13Rα2 chain. Leucine-335-337, Alanine-335-337, Tyrosine 343, and Phenylalanine 343 are indicated.

FIG. 2. Expression of wild type and mutant IL-13α2 chains in COS-7 cells. A, two days after transfection, total RNA was extracted from COS-7 cells which were transfected with vector only, wild type, or mutant IL-13Rα2 chains and was examined for IL-13Rα2 chain extracellular domain expression by RT-PCR analysis. B, binding of 125I-labeled IL-13 was performed as described in Experimental Procedures. Cell (5 x 10^5) were incubated at 4°C for 2 h with 200 pM 125I-IL-13 with or without 40 nM unlabeled IL-13. Data represent the mean of duplicate determinations and the experiment was repeated three times with similar results; bars, SD.

FIG. 3. Internalization of 125I-IL-13 by the tri-leucine region targeted mutants. Two days after transfection, COS-7 cells were preincubated in binding buffer containing 0.2 nM chloroquine at 37°C, followed by incubation with 0.5 nM 125I-IL-13 at 4°C for 2 h. Then the temperature was raised to 37°C and internalization assays were performed. Data are expressed as a percentage of total IL-13 bound at time 0. Open squares, surface IL-13
bound on the cells; *closed diamonds*, internalization in the cells. Values are the mean of two independent experiments. When not shown standard deviations are smaller than the symbol.

**FIG. 4.** Internalization of $^{125}$I-IL-13 by the tyrosine motif targeted mutants. Two days after transfection, COS-7 cells were harvested and internalization assays were performed as described in Fig. 3 legend. Data are expressed as a percentage of total IL-13 bound at time 0. *Open squares*, surface IL-13 bound on the cells; *closed diamonds*, internalization in the cells. Values are mean of two independent experiments. When not shown standard deviations are smaller than the symbol.

**FIG. 5.** Internalization of $^{125}$I-IL-13 in the co-existence of wild type IL-13R$\alpha_2$ and the tri-leucine region targeted mutants. Two days after co-transfection with IL-13R$\alpha_2$ and either L335A/L337A or L335A/L336A/L337A mutants, COS-7 cells were harvested and internalization assays were performed as described in Fig. 3 legend. Data are expressed as a percentage of total IL-13 bound at time 0. *Open squares*, surface IL-13 bound on the cells; *closed diamonds*, internalization in the cells. Values are mean of two independent experiments. When not shown standard deviations are smaller than the symbol.

**FIG. 6.** Cytotoxicity of IL-13 toxin to COS-7 cells transfected with IL-13R$\alpha_2$ chain mutants. COS-7 cells were transfected with wild type or mutant IL-13R$\alpha_2$ chains and then IL13-PE38QQR mediated cytotoxicity was determined by a protein synthesis inhibition
assay. COS-7 cells transfected with vector only (open circles) or the wild type or mutant IL-13Rα2 chains (closed squares). The results are represented as means ± SD of quadruplicate determinations.

FIG. 7. **Internalization and dissociation of 125I-IL-13 is not prolonged by the proteasome inhibitor MG132.** Two days after transfection with cDNA for the IL-13Rα2 chain, COS-7 cells were harvested and pretreated with 0.1% Me2SO (DMSO) or 50 µM MG132 for 30 min at 37°C. These cells were then utilized in internalization and dissociation assays as described in Fig. 3 legend. Data are expressed as a percentage of total IL-13 bound at time 0. *Open squares*, surface IL-13 bound on the cells; *closed diamonds*, internalization in the cells. Values are mean of two independent experiments. When not shown standard deviations are smaller than the symbol.
TABLE I

*Ligand-induced dissociation of cell surface $^{125}$I-IL-13 binding capacity*

Half lives ($t_{1/2}$) of the dissociation of $^{125}$I-IL-13 from the cell surface were determined from the internalization data in Fig. 3. and Fig. 4. $t_{1/2}$ and maximum internalization rate represent mean ± SD of 4 determinations.

| Transfected chain | $t_{1/2}$ (min) | Maximum internalization (%) |
|-------------------|-----------------|-----------------------------|
| IL-13Rα2          | 31 ± 2          | 81 ± 5                      |
| Δ338              | 13 ± 2          | 80 ± 7                      |
| Δ343              | 11 ± 3          | 79 ± 9                      |
| Y343F             | 11 ± 1          | 80 ± 7                      |
| Transfected chain                        | IC50$^a$ |
|----------------------------------------|----------|
| Untransfected (naive COS-7 cells)      | 310      |
| IL-13Rα2                                | 27       |
| Δ335                                   | 290      |
| Y343F                                  | 29       |
| L335A/L337A                            | 200      |
| L335A/L336A/L337A                      | 270      |
| L335I/L336I/L337I                      | 24       |

$^a$ IC50, the concentration of IL-13 toxin at which 50% inhibition of protein synthesis is observed compared with untreated cells.

Cells (1 x 10⁴) were cultured with IL-13 toxin for 20-22 h at 37°C, pulsed with 1 µCi of [³H] leucine, and further incubated for 4 h. Cells were harvested and counted as described in Materials and Methods.
