Université de Sherbrooke

Interaction of CysLT1 Receptor with Importin α Proteins

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Abbreviations

cysLTs: Cysteiny1 leukotrienes
CysLT1: Cysteiny1 leukotriene 1 receptor
CysLT2: Cysteiny1 leukotriene 2 receptor
GDP: Guanine diphosphate
GTP: Guanine triphosphate
GPCRs: G-protein coupled receptors
GRK: G-protein coupled receptor kinase
GST: Glutathione S-transferase
LT: Leukotriene
NE: Nuclear envelope
NLS: Nuclear localization signal
NPC: Nuclear pore complex
PKA: cAMP-dependent protein kinase
PKC: Protein kinase C
CK2: Casein kinase 2
Ran: Ras-related nuclear protein
SRS-A: Slow Reacting Substance of Anaphylaxis
SV40: Simian virus 40
Resumé

Le récepteur du leucotriène D4 (CysLt1) fait partie de la grande famille des récepteurs couplés aux protéines G (GPCR). Le CysLT1 comporte un signal potentiel de localisation nucléaire (NLS) de type bipartite dans sa partie C-terminale. Les protéines contenant un NLS sont capables d'interagir avec les importines α, faisant partie de la famille des récepteurs nucléaires (composée d'importines α et β). Les importines α et β jouent une rôle important dans le mécanisme de transport nucléaire, en reconnaissant et en transportant les protéines dans le noyau.

Dans cette étude, nous avons démontré par des essais de type Pull-Down la capacité du queue C-terminale du CysLT1 d'interagir in vitro avec les importines α1, α4 et α5. Cette interaction est comparable à celle du NLS de l'antigène grand T du SV40 et du NLS de la nucléoplasmine de Xenopus laevis avec les mêmes importines. Nos études démontrent aussi que les déterminants structuraux de l'interaction ne sont pas limités seulement au NLS: des mutations dirigées contre les résidus-clé qui forment le NLS n'ont pas empêché l'interaction entre la queue C-terminale et les importines. Nos résultats suggèrent que d'autres résidus que ceux qui forment le NLS potentiel sont impliqués dans la liaison avec les importines.
Nous avons démontré pour la première fois la phosphorylation *in vitro* de la queue C-terminale du CysLT1 par la PKA, et que cette phosphorylation peut moduler la capacité d'interaction avec l'importine α4.

Nos travaux visaient aussi l'étude de l'expression cellulaire du récepteur CysLT1 et de son comportement suite à une stimulation au LTD₄. Les résultats obtenus par microscopie à fluorescence et microscopie confocale démontrent l'expression du récepteur au niveau de la membrane plasrnique et dans des compartiments intracellulaires des cellules COS-7 transfectées avec l'ADNc du CysLT1. La stimulation avec l'agoniste LTD₄ induit l'internalisation du récepteur, un phénomène caractéristique des GPCR. En plus, nous avons aussi démontré qu'au niveau des cellules COS-7 exprimant de façon transitoire le récepteur CysLT1 et l'arrestine3, la stimulation avec LTD₄ induit non seulement l'internalisation du récepteur mais aussi le recrutement d'arrestine et sa colocalisation avec CysLT1. Comme les arrestines sont connues pour leur rôle de molécules adaptatrices dans une des voies principales d'internalisation adoptées par les GPCR, nos résultats suggèrent fortement leur implication dans l'internalisation du CysLT1.

En conclusion, nos résultats suggèrent un possible interaction entre le récepteur CysLT1 et les importines α. Des études additionnelles seront nécessaires pour définir les déterminants structuraux et sites de phosphorylation impliqués dans la modulation et l'interaction avec les importines α. L'importance et l'implication des importines dans la biologie du CysLT1 reste encore à définir.
**Key words**

- CysLT1
- Importin
- Nuclear localisation signal (NLS)
- Phosphorylation
- Trafficking
INTRODUCTION

Part 1 Cysteinyi leukotrienes and asthma

1.1 Definition of asthma

Asthma is a chronic lung disease characterized by airway obstruction and inflammation in response to a variety of nonspecific stimuli, and can be divided in extrinsic (also called allergic or atopic), intrinsic (late onset or non-atopic) and occupational forms. Inflammation in asthma is characterized by several symptoms like bronchospasm, airway hyperresponsiveness and airway smooth muscle hypertrophy, denudation of airway epithelium, subepithelial fibrosis, mucus hypersecretion and activation of sensory nerves (Bousquet et al., 2000).

In allergic bronchial asthma, inhalation of allergens results in acute broncho-obstruction, defined as the allergic early-phase reaction that occurs within minutes after allergen challenge and is short lasting (Cieslewicz et al., 1999). The early-phase reaction is IgE dependent and mainly caused by activation of lung mast cells via allergen cross-linking of IgE antibodies bound to the high-affinity IgE receptor FcεRI (Turner and Kinet, 1999).
Activated cells rapidly degranulate and release proinflammatory mediators such as histamine, eicosanoids (prostaglandins and leukotrienes), cytokines, and reactive oxygen species (Jarjour et al., 1997). Once secreted, these substances induce airway smooth muscle constriction, mucus secretion, and vasodilatation.

The acute asthmatic reaction may be followed by a late-phase reaction that commences several hours later (Durham, 1991). The late-phase reaction is dependent on the recruitment and activation of immune cells, particularly eosinophils and T cells. In this respect, it has been demonstrated in a mouse model that treatment with anti-interleukin (IL)-5 antibodies prevented the influx of eosinophils into the airways and abolished the late-phase but not the early-phase reaction (Cieslewicz et al., 1999).

1.2 Cysteinyl-leukotrienes

Cysteinyl leukotrienes, leukotriene (LT) C₄, D₄ and E₄, are inflammatory mediators belonging to the group of eicosanoids. The name leukotriene originates from the fact that they were first isolated from leukocytes and that their molecular structure includes three conjugated double bonds, a triene (Samuelsson et al., 1987). Leukotriene C₄, D₄ and E₄ all contain cysteines and are therefore referred to as cysteinyl leukotrienes (cysLTs) in order to
distinguish them from LTB$_4$, which is a non-cysteine containing dihydroxy-leukotriene.

A Slow Reacting Substance of Anaphylaxis (SRS-A) (Brocklehurst, 1960) was shown to be released from animal lungs after challenge with a snake venom. This substance induced slow contractile responses of guinea-pig intestinal preparations and was later related to anaphylactic reactions and bronchoconstriction (Brocklehurst, 1960). The identification of the cysteiny1 leukotrienes led to the conclusion that what had been referred to as SRS-A was actually a mixture of LTC$_4$, LTD$_4$ and LTE$_4$ (Samuelsson et al., 1987).

1.2.1 Implication of cysteiny1-leukotrienes in asthma

The biological activities of cysLTs suggest a prominent role in the pathology of asthma, as well as in aspirin and exercise-induced asthma (Szczeklik et al., 2001; Sun et al., 2002). The direct and potent contractile response induced by cysLTs in isolated human bronchi was first demonstrated by Dahlen et al. (1980). In addition, inhaled cysLTs were shown to induce bronchoconstriction (Holroyde et al., 1981), being up to 10,000 times more potent than histamine (Weiss et al., 1982). Furthermore, clinically introduced leukotriene synthesis inhibitors and CysLT1 receptor antagonists have been shown to significantly reduce bronchoconstriction in asthmatics (Dahlen et al., 1994; Drazen et al., 1999).
CysLTs are also important mediators of vascular permeability, vasoconstriction, increased mucus production (Buccellati et al., 2002) and bronchial hyperresponsiveness (Leff, 2000).

A study conducted by Espinosa et al. (2003) supports the role for cysLTs in the airway remodeling observed in asthmatic patients, by demonstrating a synergy between certain cytokines (TGF-β and IL-13) and cysLTs, which is mediated by the augmented expression of the CysLT1 receptor and subsequent LTD₄-triggered bronchial smooth muscle cell proliferation. Fregonese (2002) demonstrated a dose-dependent cysteiny1 leukotriene-induced up-regulation of Mac-1 (integrin α M) and subsequent chemotaxis in eosinophils from atopic asthmatic donors. Moreover, when activated epithelial cells interact with activated eosinophils, significant increase of cysLTs biosynthesis occurs, thus supporting the fact that eosinophil-derived cysLT production represents one of the key elements of the inflammatory response seen in asthma (Jawien et al., 2002).

Bronchial hyperresponsiveness in chronic asthma may also be caused by epithelial denudation. Mitogenic effects of cysLTs at concentrations as low as 10 fM observed with airway epithelial cells strongly suggest implication of chronically released cysLTs in airway epithelium integrity (Leikauf et al., 1990). Moreover, studies using 5-LO inhibitors on isolated guinea-pig airways revealed a diminished contractile response to tachkinins, thus suggesting the exacerbated sensitizing effects of CysLTs on airway denuded epithelium (McAlexander et al., 1998)(Figure 1).
Figure 1. Effects of cysteinyi leukotrienes on different cell types and tissues implicated or affected in asthma pathology (adapted from Hay et al., 1995)

1.2.2 Synthesis of cysteinyi-leukotrienes

Cysteinyi leukotrienes are derived from arachidonic acid that is liberated from cell membrane phospholipids by the action of phospholipase A₂ (PLA₂). Once released from the membrane, arachidonic acid can act as a substrate for cyclooxygenase to form prostaglandins and/or as a substrate for 5-lipooxygenase (5-LO) giving rise to leukotriene A₄ (LTA₄). LTA₄ is transformed into LTB₄ by LTA₄-hydrolase, while LTC₄ synthase converts it into LTC₄. LTC₄ can be exported from the cell and converted by γ-glutamyl-transferase to
LTD₄, which in turn can be metabolized to LTE₄ under action of a dipeptidase (reviewed by Samuelsson et al., 1987) (Figure 2).

\[ \text{Figure 2. CysteinyI leukotriene metabolism} \text{ (adapted from Samuelsson et al., 1987)} \]
Cysteinyl leukotriene metabolism takes place in a number of tissues including human lung (Kumlin and Dahlen, 1990), and cysLTs can be formed by eosinophils, basophils, mast cells and alveolar macrophages. In the lung, they induce airway smooth muscle contraction and proliferation, plasma leak and edema, vasoconstriction or endothelium-dependent relaxation, eosinophil recruitment, mucus secretion, airway hyperresponsiveness, C-fiber depolarization, substance P release, and airway remodeling.

1.3 Cysteinyl-leukotriene receptors

The notion of specific receptors mediating the effects of the cysteinyl leukotrienes originates with the acetophenon, FPL 55712, which in the beginning was described as an antagonist of SRS-A (Augstein et al., 1973). After the structural elucidation of the cysLTs, it was found that FPL 55712 inhibited the contractions of human bronchi (Buckner et al., 1986) and guinea-pig trachea (Jones et al., 1983) induced by cysLTs. Although FPL 55712 apparently antagonizes the receptors for cysLTs, this effect has been shown not to be highly specific (Krell et al., 1981). However, a number of specific and potent cysLT receptor antagonists were subsequently developed, some of which are listed in Table 1.

All these antagonists were shown to be potent inhibitors of LTD4- and LTE4-induced contractions in guinea-pig trachea and ileum, but they were not able
or only marginally, to inhibit the LTC₄-induced contractions (Back et al., 1996; Back et al., 2001).

<table>
<thead>
<tr>
<th>Code name</th>
<th>Generic name</th>
<th>Trade name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ICI 198,615</td>
<td>Zafirlukast</td>
<td>Accolate™</td>
<td>(Snyder et al., 1987)</td>
</tr>
<tr>
<td>2 ICI 204,219</td>
<td></td>
<td></td>
<td>(Krell et al., 1990)</td>
</tr>
<tr>
<td>3 L 649,923</td>
<td></td>
<td></td>
<td>(Jones et al., 1986)</td>
</tr>
<tr>
<td>4 MK 571/L 660,711</td>
<td>Montelukast</td>
<td>Singulair™</td>
<td>(Jones et al., 1989)</td>
</tr>
<tr>
<td>5 MK 0476/L 706,631</td>
<td></td>
<td></td>
<td>(Jones et al., 1995)</td>
</tr>
<tr>
<td>6 LY 171,883</td>
<td>Tomelukast</td>
<td></td>
<td>(Fleisch et al., 1985)</td>
</tr>
<tr>
<td>7 SKF 104,353</td>
<td>Pobilukast</td>
<td></td>
<td>(Hay et al., 1987)</td>
</tr>
<tr>
<td>8 ONO 1078</td>
<td>Pranlukast</td>
<td>Onon™</td>
<td>(Obata et al., 1992)</td>
</tr>
<tr>
<td>9 BAY u9773*</td>
<td></td>
<td></td>
<td>(Cuthbert et al., 1991)</td>
</tr>
</tbody>
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Table 1. Antagonists for cysteinyi leukotriene receptors (adapted from Nicosia et al., 2000 and Back, 2002) (* - dual antagonist for both CysLT receptors)

Based on these findings, the names CysLT1 and CysLT2 were introduced for the receptors being sensitive (CysLT1) or resistant (CysLT2) to the class of antagonists being developed (Table 1, 1-8), which are consequently referred to as specific CysLT1 receptor antagonists.

At this time, two cysteinyi leukotriene receptors have been cloned: they both belong to the G-protein coupled receptor family and are designated as CysLT1 (Lynch et al., 1999; Sarau et al., 1999) and CysLT2 (Heise et al., 2000; Takasaki et al., 2000). The rank order of affinities for the CysLT1 receptor expressed in COS-7 cells is LTD₄ >> LTE₄ = LTC₄, as reported by (Lynch et al., 1999), while binding studies for CysLT2 receptor showed an equivalent affinity for LTC₄ and LTD₄, and a lower binding affinity for LTE₄.
(Heise et al., 2000). Binding of radiolabeled cysLTs to the first cloned human CysLT receptor was shown to be inhibitable by antagonists such as zafirlukast, montelukast and pranlukast as well as by the dual antagonist BAY u9773, and the receptor was thus classified as being the human CysLT1 receptor. Binding to the second CysLT receptor (CysLT2) exhibited similar characteristics as the functionally defined CysLT2 receptor, in that it was resistant to CysLT1 receptor antagonists.

1.3.1 CysLT1 receptor

The full-length cDNA for human CysLT1 receptor has a 1014-bp sequence and encodes a protein of 337 amino acid residues with 4 potential N-glycosylation sites. Analysis of the amino acid sequence indicated homology to the seven-transmembrane-spanning G-protein coupled receptors (GPCRs). In addition, hydrophobicity plot analysis showed the existence of seven hydrophobic regions, each containing approximately 20 to 30 amino acids, which are likely to represent the membrane-spanning domains found among the GPCRs (Figure 3).
Figure 3. Seven-transmembrane spanning structure of CysLT1 receptor (predicted by TMPred; http://www.ch.embnet.org/software/TMPRED_form.html)

Northern blot analysis showed weak transcript expression, predominantly in peripheral blood leukocytes, followed by spleen, lung, pancreas, small intestine, and a number of other tissues. Reverse-transcription-polymerase chain reaction (RT-PCR) has revealed the mRNA for the receptor in a number of cell lines, including THP-1 cells and the human lymphoblastic U937 cell line (Figueroa et al., 2001). By in situ hybridization, CysLT1 mRNA was found in normal lung peribronchial and peribronchiolar smooth muscle cells, as well as in some alveolar macrophages (Lynch et al., 1999).
Figueroa et al. (2001) identified, by immunohistochemistry, the presence of the CysLT1 receptor protein in normal human lung and normal human peripheral blood cells, in accord with the in situ expression of CysLT1 mRNA. Using peripheral blood cell markers, they found that the receptor is expressed in cells of particular relevance to asthma and atopy, namely eosinophils, monocytes/macrophages and B lymphocytes, and in CD34+ granulocytic precursor cells. The expression of the CysLT1 receptor on eosinophils and monocytes, both of which are cell types capable of synthesizing CysLTs, suggested that both autocrine and paracrine activation may occur after stimulation by the appropriate inflammatory signal.

Murine CysLT1 is encoded by two transcripts: a short splice variant encoding a protein of 339 amino acids, which aligns well with human CysLT1 receptor; and a long variant encoding a protein extending 13 amino acids at the N terminus (Maekawa et al., 2001). The homology between human and murine CysLT1 is approximately 87% at the protein level. The mCysLT1 gene spans about 24 kb with 4 exons and 3 introns. Alternative splicing yields a short transcript containing exons 1 and 4 that encodes the short CysLT1 variant with initiator ATG codon in exon 4 or a long transcript containing all 4 exons with initiator ATG in exon 3. Human CysLT1 receptor cDNA does not have an upstream translation start site and is unlikely to have a similar long transcript. The sequence for rat and pig CysLT1 are also known and they also show high similarity to human CysLT1 (Figure 4).
Figure 4. Alignment of CysLT1 receptor from different species using Dialign and Boxshade servers: human CysLT1 – ltl-hum (GI: 20138087), murine CysLT1 – ltl-mus (GI: 31542449), rat CysLT1 – ltl-rattus (GI: 16758452), pig CysLT1 – ltl-sus (GI: 20137602). The consensus sequence is written below the aligned receptors, with consensus matching residues in upper case, non-matching residues in lower case. The residues in bold are the consensus for the NLS, which is conserved among all the four species.
As deduced from its amino acid sequence, CysLT1 contains no consensus tyrosine kinase phosphorylation site, one consensus casein kinase (CK) 2 phosphorylation site at position 326-329 and multiple serine and threonine residues in the cytoplasmic loops and C-terminal tail, four of which are within consensus phosphorylation sites for protein kinase C (PKC) and three within a consensus sequence for PKA. Interestingly, the C-terminus tail contains a putative bipartite nuclear localization sequence (NLS) at positions 310-324.

1.3.2 CysLT2 receptor

CysLT2 is a GPCR with 38% amino acid identity to CysLT1. CysLT2 mRNA is found in lung macrophages, airway smooth muscle, cardiac Purkinje cells, adrenal medulla cells, placenta, spleen, brain and peripheral blood leukocytes (Heise et al., 2000). Evidence suggests that the CysLT2 receptor has biological significance in the cardiovascular system. High levels of CysLT2 mRNA were detected in the human atrium and ventricle and intermediate levels in the coronary artery (Kamohara et al., 2001). Furthermore, in situ hybridization revealed that CysLT2 mRNA was expressed in myocytes, fibroblasts, and vascular smooth muscle cells.
1.3.3 Protein structure and ligand binding of CysLT receptors

CysLT receptors have long been recognized as GPCRs prior to their cloning (Mong et al., 1984), based on the fact that binding of the ligands to the receptors is enhanced by divalent cations but inhibited by sodium ions and non-hydrolyzable GTP analogs.

There is no binding model currently available for leukotrienes. Based on the ligand structure, eicosanoid receptors have been classified into a subfamily of rhodopsin-β2 adrenergic receptor-like receptors. CysLT receptors also bear some homology to the purinoreceptor P2Y family, and UDP indeed appears to be an agonist at CysLT1 (Mellor et al., 2001).

1.3.4 Signaling through the CysLT1 receptor

Stimulation of GPCRs, which couple to second messenger generation via multiple heterotrimeric G proteins (consisting of Gα and Gβγ subunits), can result in 'direct' effects on a range of signaling proteins, including phospholipase C (PLC), adenylate cyclase and ion channels (Gilman, 1987).

Although there are many gene products encoding each subunit (20 α, 6 β, and 12 γ gene products are known), four main classes of G proteins can be distinguished: Gs, which activates adenylate cyclase; Gi, which inhibits adenylate cyclase; Gq,11, which activates phospholipase C; and G12 and G13, of unknown function. G proteins are inactive in the GDP-bound, heterotrimeric
state and are activated by receptor-catalyzed guanine nucleotide exchange resulting in GTP binding to the α subunit. GTP binding leads to dissociation of Gα-GTP from Gβγ subunits, and to activation of downstream effectors by both Gα-GTP and Gβγ subunits (Hamm, 1998).

Members of the GPCR family are grouped into different categories dependent on the particular G protein subtypes that they predominantly interact with. Thus, receptors that couple to Gs proteins stimulate adenylate cyclase in many cells, while Gq/11 coupled receptors can mobilize intracellular Ca2+ via activation of phospholipase C.

Since it has been known that CysLT1 receptor signals through elevation of intracellular calcium concentrations (Chan et al., 1994), each of the reported cloning studies used calcium mobilization studies to measure receptor function in vitro. In CysLT1 transfected cells, the potency order for the three cysLTs measured by Ca2+ concentration increase was LTD4 > LTC4 ≥ LTE4 (Lynch et al., 1999; Sarau et al., 1999), while in cells transfected with CysLT2 the rank order was LTC4 = LTD4 > LTE4 (Heise et al., 2000; Takasaki et al., 2000).

Intracellular signaling pathways of CysLT receptors have not been studied thoroughly. Pertussis toxin, which is known to uncouple Gβγ protein signaling from receptors (Wess, 1998) did not change LTD4-induced functional responses in either CysLT1 cRNA injected Xenopus laevis oocytes (Lynch et al., 1999) or CysLT1-transfected HEK-293 cells (Sarau et al., 1999),
indicating that the receptors in these cells were coupled to G_{q/11} family members.

Early studies also showed G_{q/11} coupling in some cell types expressing CysLT1 endogenously (basophilic leukemic cell line from rat - RBL-1 and sheep tracheal smooth muscle primary culture cells) (Mong et al., 1988a; Mong et al., 1988b).

In differentiated human U937 cells (Saussy et al., 1989), intestinal 407 cells (Sjolander et al., 1990), and human monocytic leukemia THP-1 cells (Hoshino et al., 1998), coupling to both the G_{q/11} and the G_{i/o} family was observed. Therefore, CysLT1 G-protein coupling is possibly cell-type dependent, and the availability of G proteins may account, in part, for the differences.

Studies performed in our laboratory on COS-7 cells transfected with CysLT1 cDNA showed increased inositol phosphate (IP) production in response to LTD_{4} stimulation. When cotransfected with the receptor, both G_{a9} and G_{a11} subunits of G proteins augmented IP production, thus pointing again to a main signaling pathway which is coupled to G_{a9/11} proteins (D.J Dupré and C. Le Gouill, unpublished data).

A signaling pathway through G_{a9/11} subunits activates phospholipase Cβ (PLCβ). PLCβ cleaves phosphoinositol biphosphate (PIP_{2}) into inositol triphosphate (IP_{3}) and diacylglycerol (DAG), both of which play second messenger roles in intracellular signaling cascades. IP_{3} interacts with its receptors on the endoplasmic reticulum (ER) and induces Ca^{2+} release from endoplasmic reticulum stores into the cytosol, which in turn couples to and
activates calmodulin. In addition, DAG and calcium together activate PKC, which in turn catalyses phosphorylation of a large number of proteins.

Receptor desensitization terminates or attenuates receptor signaling. This process is the consequence of a combination of different mechanisms, including uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Bouvier et al., 1988), the internalization of cell surface receptors to intracellular compartments (Anborgh et al., 2000) and the down-regulation of the total cellular complement of receptors due to reduced synthesis and/or lysosomal and plasma membrane degradation of receptors (Valiquette et al., 1990; Valiquette et al., 1995). The extent of receptor desensitization varies from complete termination of signaling to the attenuation of agonist potency and maximal responsiveness and is regulated by a number of factors that include receptor structure and cellular environment (Ferguson, 2001).

Three families of regulatory molecules have been found to participate in desensitization of GPCRs: second-messenger-regulated kinases (e.g. PKA and PKC), G-protein coupled receptor kinases (GRKs) and the arrestins. GRKs are single subunit Ser/Thr protein kinases and there are seven family members cloned to date (GRK1-7)(Ferguson, 2001). They phosphorylate GPCRs at both serine and threonine residues localized within either the third intracellular loop or carboxyl-terminal tail domains. GRK activity is regulated by a complex series of phosphorylation events, depending on the types of
kinase and receptor implicated. For instance serine phosphorylation of GRK2 by PKC has a potentiating effect on its activity and plasma membrane translocation (Chuang et al., 1995), while GRK5 activity is reduced upon PKC-mediated phosphorylation (Chuang et al., 1996).

Agonist-activated receptor phosphorylation by GRKs promotes the binding of the cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from G proteins (Benovic et al., 1987). In contrast, second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs, but also indiscriminately phosphorylate receptors that have not been exposed to agonist (Lohse et al., 1990). However second messenger-dependent protein kinase and GRK activities may not be independent from each other, since in the olfactory system, inhibition of either kinase family results in the complete abolition of olfactory receptor desensitization (Schleicher et al., 1993; Boekhoff et al., 1994).

The mechanism by which arrestins regulate the desensitization of GPCRs involves both the physical uncoupling of GPCRs from G proteins and the targeting of the receptors for endocytosis (Ferguson, 2001).

To date, four arrestin family members have been identified, and they can be divided into two groups based on sequence homology, function and tissue distribution: 1) visual arrestin (arrestin1) (Shinohara et al., 1987) and cone arrestin (Craft et al., 1994) and 2) β-arrestins (βarrestin1/arrestin2 and β-arrestin2/arrestin3) (Lohse et al., 1990; Attalamadal et al., 1992). Visual and cone arrestins have a very restricted expression pattern, being localized in
retina and pineal gland, while β-arrestins are ubiquitously expressed outside of retina.

Arrestins preferentially bind to agonist-activated and GRK-phosphorylated GPCRs, but agonist-independent arrestin association may also be observed (Anborgh et al., 2000). This recruitment to the agonist-occupied receptors often terminates or attenuates G-protein-dependent signaling of GPCRs by physical uncoupling from the G-proteins.

Arrestins play an adapter role and mediate receptor internalization, by recruiting proteins implicated in the endocytic machinery: clathrin, adapter protein 2 (AP-2), N-ethylmaleimide-sensitive fusion protein (NSF). In the last years, evidence has been accumulating to indicate that arrestins play novel roles as adapters, which recruit additional signaling molecules to ligand activated receptors, such as JNK3, ASK1, Src, ERK, and Raf kinases (Miller and Lefkowitz, 2001).

In this respect, receptor signaling and desensitization are to be considered as two intimately linked aspects of receptor function, and mechanisms viewed as “desensitizing” with respect to one signaling pathway may be “activating” with respect to another.

Little is known yet about the intracellular signalling of the CysLT1 receptor and even less about the mechanisms regulating the expression of the receptor and its intracellular trafficking and desensitisation. The presence of the multiple potential PKC and PKA phosphorylation sites within the third intracellular loop and the C-terminal tail strongly suggest possible
homologous and heterologous desensitization and subsequent internalization of the CysLT1 receptor.

An intriguing detail is the presence of a putative bipartite NLS motif in the C-terminal tail of CysLT1. Given that an increasing number of GPCRs have been demonstrated to be expressed in the nuclear compartment or to translocate to the nucleus in both agonist dependent or independent manner (Lu et al., 1998; Marrache et al., 2002; Gobeil et al., 2003a; Gobeil et al., 2003b; Lee et al., 2004), the functionality of this sequence and its potential role in receptor signaling constitutes a very interesting research topic. In this respect, aspects about nuclear transport mechanisms and its importance will be detailed in the following section.
Part 2 Nucleocytoplasmic Transport

2.1 Principles of nucleocytoplasmic transport

The nuclear envelope divides eukaryotic cells into nuclear and cytoplasmic compartments, uncouples transcription from translation and thereby necessitates nucleocytoplasmic transport (Mattaj and Englmeier, 1998). tRNAs, mRNAs and rRNAs, for example, need to be exported from the nucleus to the cytoplasm, where they participate in translation. Conversely, virtually all nuclear functions are dependent on proteins that are imported from the cytoplasm. The nuclear compartment also represents the final destination for many import substrates, such as histones or polymerases. Numerous other proteins, however, pass through nuclei only transiently. Ribosomal proteins, for example, are first imported, assemble in the nucleoli with rRNAs, and finally are exported as ribosomal subunits to the cytoplasm (Venema and Tollervey, 1999).

Macromolecular transport between the nucleus and cytoplasm proceeds through nuclear pore complexes (NPCs) and is normally receptor mediated. Importin beta-type transport receptors account for most, but not all, nuclear transport pathways. They constitute a diverse protein super family and occur in two forms, import mediators (importins) and exportins. They circulate between nucleus and cytoplasm, recognize cargo molecules and transfer
them from the one side of the nuclear envelope (NE) to the other. The transfer through the NPC is energy-dependent, and requires GTP hydrolysis by a small GTPase called Ras-related nuclear protein (Ran) (Rexach and Blobel, 1995). Substrate loading and release is guided by a gradient of Ran-GTP across the NE, whereby a high nuclear Ran-GTP concentration favors cargo loading onto exportins and substrate displacement from importins, while cytoplasmic conditions with low Ran-GTP levels release substrates from exportins but allow importin-cargo complexes to form (Mattaj and Englmeier, 1998; Weis, 1998; Gorlich and Kutay, 1999) (Figure 5).

![Diagram of nuclear protein import cycle](image)

**Figure 5.** A model for the importin-dependent nuclear protein import cycle, and its coordination by Ran (adapted from Gorlich and Kutay, 1999)
2.2 Nuclear envelope and nuclear pore complexes

The nuclear envelope is a double-membrane barrier that is continuous with the endoplasmic reticulum (ER). The import of proteins into the nucleus occurs through the nuclear pore complexes which allow diffusion of small molecules (up to 40 kDa) and can accommodate the active transport as large as several million Dalton in weight or up to 39nm in diameter (Peters, 1983; Pante and Kann, 2002).

The vertebrate NPC has a mass of ~120 MDa (30 times bigger than a ribosome) and is one of the biggest macromolecular assemblies in the eukaryotic cell. Although it is likely to consist of almost 1000 polypeptides, it is made up of only 30-50 different proteins. The nuclear pore consists of two integral membrane proteins and a large cast of nucleoporins recruited from the cytoplasm. Together these are assembled at points of fusion between the inner and outer nuclear membranes. The nuclear pore structure consists of a scaffold of eight spokes, a central transporter, eight cytoplasmic filaments and eight nuclear filaments intersecting at a distal ring, termed the nuclear basket (Vasu and Forbes, 2001) (Figure 6).
Figure 6. Structure of the nuclear envelope and nuclear pore (adapted from Hinshaw et al., 1992)

2.3 Nuclear transport signals (NLS)

Shuttling of proteins between intracellular compartments is mediated by specific signaling motifs, which are both essential and sufficient. There are two different nuclear transport signals: the leucine-rich nuclear export signals (NES), and the nuclear localization signals (NLS). The latter are short peptide sequences that are required for the nuclear localization. They can have various locations in the protein and are not cleaved after nuclear import. NLS need to be distinguished from signal peptides (like the "targeting" signals), which are cleaved once the protein has reached the target compartment or subcellular structure, and are usually located at the N-terminus of the protein.
Many nuclear proteins contain a nuclear localization signal (NLS) in their primary amino acid sequence that is recognized by the cellular transport machinery.

Two different types of basic NLS were at first described, which follow a consensus motif in a large number of proteins.

The first type of NLS (classical NLS) was discovered in the simian virus 40 (SV40) large tumor antigen, and consists of a heptapeptide which contains five basic amino-acid residues (\textsuperscript{126}PKKKRKV\textsuperscript{132}) (Kalderon \textit{et al.}, 1984a; Kalderon \textit{et al.}, 1984b; Lanford and Butel, 1984). Substitution of a critical residue, namely K\textsuperscript{128} (underlined) from the SV40 NLS leads to loss of functionality (Kalderon \textit{et al.}, 1984a).

The first bipartite type of NLS was discovered and described in \textit{Xenopus laevis} nucleoplasmin (\textsuperscript{155}KRPAATKKAGQAKKKK\textsuperscript{170}) and consists of two basic clusters (bipartite NLS). The consensus of this type of NLS is: two basic residues (arginine and/or lysine), separated by a 10-12 variable residue spacer from the second cluster, in which 3 out of 5 residues must be basic (Dingwall and Laskey, 1991; Robbins \textit{et al.}, 1991).

The presence of an upstream cluster in the bipartite NLS appears to relax the stringent sequence requirements of monopartite signals. Indeed, the Thr\textsuperscript{128} mutant of the SV40 T antigen that is defective in nuclear import can be rescued by placing two basic amino acid residues ten residues upstream of the defective NLS (Makkerh \textit{et al.}, 1996), presumably converting an ineffective single basic cluster into an active bipartite signal.
Both types of NLS are recognized by an importin α/importin β heterodimer. Importin α itself contains in its N-terminal region a bipartite NLS (IBB-domain, Importin β binding domain) with a highly basic content, which is specifically recognized by Importin β. Importin β may be actually considered as the import mediator.

Gathering data from the classical and bipartite NLS have lead to the conclusion that NLS are rich in basic residues. This hypothesis was confirmed in the last years, by the discovery of more and more functional NLS, which show only few similarities with the classical NLS. For example, the NLS from yeast transcription repressor Matα2 (Hall et al., 1990) or the protooncogene c-myc NLS, both have critical flanking proline and aspartate residues. Both NLS are also specifically recognized by the importin α/β heterodimer. Further examples are the complex signals of U snRNPs (Fischer et al., 1993) or the ribosomal proteins NLS which consist of basic residues but do not follow either of the classical consensus patterns. The NLS of the ribosomal protein L23a consists of 43 amino acid residues and is recognized by at least four different import receptors. Such a signal is certainly longer than necessary if only a nuclear destination of the protein was to be “indicated”. It therefore has been suggested previously that importins not only direct their cargoes to the nuclear compartment, but might also serve a second purpose, namely shielding very basic cargoes against undesired interactions during transit to the nucleus (Jakel and Gorlich, 1998; Jakel et al., 1999).
A very recent study by Hachet and colleagues (Hachet et al., 2004) on *Xenopus* egg extracts, suggests that importin α, via interaction with NLS-containing proteins, functions in NE assembly, and that the correct balance of importin α and NLS protein is critical. Previous studies have already shown that the GTPase Ran plays a crucial role in NE assembly *in vitro* and *in vivo* (Hetzer et al., 2000). Further research is needed to decipher the complex roles of the nuclear transport receptors and of the NLS-bearing proteins.

2.4 Nuclear transport receptors

Three classes of transport receptors have been identified to date. The best-studied class is the family of importin β-like proteins (the importins and exportins, also called karyopherins). The second class comprises the small homodimeric nuclear transport factor 2 (NTF2)/p10, which imports the small GTPase Ran into the nucleus. The third class is the Tap/Mex67 family, which is involved in mRNA transport (Weis, 2002).

All known transport receptors shuttle between the cytoplasm and the nucleus and are characterized by their ability to specifically interact with a subset of nucleoporins (i.e. components of the nuclear pore) that contain characteristic phenylalanine/glycine (FG)-rich-repeat motifs. This concept of shuttling of import receptors applies to NLS-dependent import. In this model the heterodimeric import receptor initially binds its import substrate in the cytoplasm; it then
mediates the energy-independent docking reaction of the import substrate at the nuclear envelope and the consecutive transport into the nucleus, where the cargo is released from the transport receptor, and the import receptor finally returns to the cytoplasm without the cargo (Gorlich and Mattaj, 1996; Gorlich, 1997).

The NLS receptor consists of a ~60kDa subunit and a ~97kDa subunit. The small subunit that is primarily responsible for the NLS recognition has been named importin $\alpha$ (Gorlich et al., 1994) or karyopherin $\alpha$ (Moroianu et al., 1995). Importin $\alpha$ is a protein with hydrophilic N- and C-terminal regions and a large central domain that consists of tandemly repeated modules known as armadillo (arm) motifs (Gorlich et al., 1994; Weis et al., 1995). The arm-repeat domain is linked by a variable spacer to a small N-terminal region that is rich in conserved basic residues and is required for interaction with importin $\beta$ (Gorlich and Mattaj, 1996; Moroianu et al., 1996; Weis et al., 1996).

The larger subunit of the NLS receptor binds directly to nucleoporins and has been termed importin $\beta$ (Gorlich and Laskey, 1995) or karyopherin $\beta$ (Radu et al., 1995). The $\alpha$ subunit binds to the $\beta$ subunit via the short amino-terminal domain (~70 residues) named importin $\beta$-binding domain, IBB (Weis et al., 1996). In the absence of importin $\beta$ or of a NLS-bearing cargo protein, the IBB domain plays the role of an intrasterical self-regulatory sequence, by coupling to the NLS-binding sites (Catimel et al., 2001).

An additional soluble factor is needed to transfer the NLS-protein-receptor complex through the NPC, the GTP-ase Ran (Moore and Blobel, 1994b; Moore...
and Blobel, 1994a). Ran cycles between GTP- and GDP-bound states, which are found in a concentration gradient between nucleoplasm and cytoplasm. Ran in its GTP-bound state can form complexes with importin β and is able to break the heterotrimeric complex of NLS, protein and receptor, a reaction that might be used to terminate the translocation reaction at the nucleoplasmic side of the NPC (Rexach and Blobel, 1995).

2.5 Importin α proteins

In contrast to importin β for which only one gene has been identified in the organisms analyzed thus far, several isoforms of importin α in humans have been described. These include importin α1/hSRP1α (Weis et al., 1995), importin α3 (Kohler et al., 1997; Seki et al., 1997), importin α4/hSRPγ (Nachury et al., 1998), importin α5/hSRP1 (Cortes et al., 1994), importin α6 (Kohler et al., 1997), and importin α7 (Kohler et al., 1999).

2.5.1 Importin α1

Importin α1, also known as hSRP1α because of its homology to the yeast protein SRP1 (suppressor of RNA polymerase), was identified by Weis et al in 1995, and it was the first member of the importin α family to be cloned. It is able to bind to both monopartite and bipartite NLS bearing proteins and
mediate their nuclear import. Human multi-tissue Northern blot analysis revealed a wide expression for importin α1. High levels of mRNA were detected in kidney, liver, lung, placenta, brain and slightly lower levels in pancreas, skeletal muscle and heart muscle (Weis et al., 1995).

2.5.2 Importin α4

Importin α4 (hSRP1γ) was cloned and characterized a few years after importin α1, and shows around 48% identity on the amino acid level to importin α1. It is also able to bind specifically both types of NLS and to mediate their docking to the nuclear envelope. When compared to importin α1, nuclear import of NLS-bearing substrates was consistently lower when same amounts of active proteins were used. In contrast to importin α1, importin α4 shows a more tissue-specific expression pattern, being very abundant in skeletal muscle (representing probably more than 1% of the total protein in this tissue) and testis. Although mRNA was not detected in brain, high protein levels of importin α4 were detected in this organ, suggesting posttranscriptional regulation of importin α4 in brain (Nachury et al., 1998).
2.5.3 Importin α5

Cortes et al., in 1994 first identified importin α5 (hSRP1) as a homologue of the yeast SRP1 that was able to interact with RAG-1 and seemed to be bound to the nuclear envelope, thus helping nuclear localization of RAG-1. (O’Neill and Palese, 1995) demonstrated a year later its ability to interact with influenza virus nucleoprotein, and to mediate its nuclear import. It shows 46.7% amino acid identity to importin α4 and is ubiquitously expressed.

2.5.4 Importin α3 and importin α6

Cloning of both importins α3 and α6 was reported by Kohler et al., in 1997. Analysis of amino acid structure of the two proteins showed an identity about 50% with other members of the importin α family (importin α1 and 5). Their study also concluded that the overall identity between members of importin α family is about 33%.

Importin α3 mRNA was found to be highly expressed in testis, ovary, small intestine, and pancreas, but was barely detectable in kidney, thymus, colon, and peripheral blood leukocytes. In contrast, transcripts of importin α6 were detected only in testis. Analysis of protein expression levels in various tissues confirmed results obtained by mRNA analysis for the transcripts of the two importins.
2.5.5 Importin α7

Kohler et al. (1999) also reported the cloning of importin α7. Northern and Western blot analysis revealed its presence in a variety of tissues like heart, brain, lung, kidney, liver, ovary, testis and also demonstrated by in vitro nuclear import experiments, its capacity to mediate nuclear translocation for various NLS-bearing peptides.

2.6 Regulation of nuclear entry

Analysis of the rates and maximal extent of nuclear transport (Jans and Jans, 1994) has shown that nuclear transport is not exclusively determined by the NLS; rather, other sequences, and in particular phosphorylation sites, can act to modulate NLS function. Whereas, some proteins, such as histones, appear to be constitutively targeted to the nucleus, others are only targeted to the nucleus under specific conditions, often being present mostly in the cytoplasm. In particular, transcription factors regulating nuclear gene expression are subject to specific mechanisms regulating nuclear protein import. The advantages to the cell, of a conditionally cytoplasmic localization of transcription factors, include the potential to control its activity by regulating its nuclear uptake, and its direct accessibility to cytoplasmic signal-transducing systems. Thus, nuclear protein
import can be a key control point in the regulation of gene expression and signal transduction.

Examples of signal transduction pathways from extracellular hormonal signal to the nucleus ultimately leading to changes in regulation of gene expression include that of the glucocorticoid receptor, where, upon glucocorticoid hormone binding, the cytoplasmic receptor translocates to the nucleus to modulate gene transcription by direct binding to specific DNA sequences (Picard et al., 1988). Sites for the physiologically important casein kinase 2 (CK2) and the cyclin-dependent kinase cdc2, present close to the SV40 large T antigen NLS, regulate the kinetics of nuclear transport of the T-antigen, probably by modulating specific protein-protein binding affinities and recognition events (Rihs and Peters, 1989). The phosphorylation at the CK2 site increases the rate of NLS-dependent nuclear import, so that maximal accumulation within the nucleus occurs within minutes, which compares to the hours taken when phosphorylation by CK2 is prevented through mutation or deletion of the CK2 site. In contrast to the enhancing effect of the CK2 site, phosphorylation by cdc2 inhibits nuclear transport, drastically reducing the maximal level of nuclear accumulation (Jans et al., 1995). This complex regulatory system for SV40 T-antigen nuclear localization, involving two different kinases, demonstrates the existence of specific mechanisms regulating nuclear entry. The NLS is clearly not the sole determinant of nuclear localization; rather, the
kinetics of NLS-dependent nuclear import is regulated by phosphorylation in the vicinity of the NLS (Jans, 1995).

A number of proteins possessing apparently functional NLS are predominantly cytoplasmic due to the inaccessibility or masking of their NLS. This may be through interaction with another protein (e.g. a factor binding to the NLS region itself) or conformational effects whereby the NLS is masked by other parts of the molecule. NLS activity can also be directly affected by phosphorylation close to, or within an NLS, which masks or inactivates it through charge or conformational effects. Since phosphorylation appears to be a key event in triggering the nuclear translocation of a number of proteins, such as NF-AT and the glucocorticoid receptor, this may be a widespread mechanism (Jans, 1995).

2.7 G-protein coupled receptors and the nuclear compartment

Angiotensin II type 1 (AT₁) receptor, which contains a NLS in its cytoplasmic C-terminal tail, has been shown to translocate to the nucleus in brain neurons, after stimulation. However, this translocation seems to be cell specific given that the same receptor does not translocate to the nucleus in astroglial cells, under the same experimental conditions. The role of the nuclear translocation remains unknown (Lu et al., 1996).

Intranuclear δ-opioid binding sites have been reported (Belcheva et al., 1993) and muscarinic acetylcholine receptors can be found on nuclear membranes
of epithelial, endothelial and CHO cells (Lind and Cavanagh, 1995). Functional receptors for prostaglandin E2 (EP1, EP3 and EP4) have been localized to the nuclear envelope and recently the platelet activating factor (PAF) receptor has also been shown to be expressed at the nuclear level (Marrache et al., 2002). Agonist-independent nuclear localization was demonstrated recently for the apelin, angiotensin AT1 and bradykinin B2 receptors (Lee et al., 2004). Although, the potential role for these receptors is speculative, they have been shown to be functional, in that they can induce kinase activation and gene transcription. It has been well established that the necessary signaling molecules are present in the nuclear environment, such as G-proteins (Rubins et al., 1990; Takei et al., 1994), phospholipase C (Kim et al., 1996; Cocco et al., 1999a; Cocco et al., 1999b) and PKC (Rosenberger et al., 1995).

Since the substrate and the enzymatic components needed for leukotriene synthesis are situated at the nuclear envelope (Woods et al., 1993), the presence or translocation of the CysLT1 receptor at the nuclear level seems both possible and logical, and could facilitate autocrine and intracrine signaling (Bandeira-Melo et al., 2002), signal amplification and/or “second messenger” function.

In this respect, the objectives of our study were to characterize the putative NLS of the CysLT1 receptor by the means of its ability to interact with importins, and to study the expression and intracellular trafficking of CysLT1 receptor upon agonist stimulation.
MATERIALS AND METHODS

Reagents

Oligonucleotides were synthesized by Sigma-Genosys (Oakville, ON), PWO polymerase was from Roche Molecular Biochemicals (Mississauga, ON), restriction endonucleases and modifier enzymes were from Promega (Madison, WI), Roche (Mississauga, ON), Boehringer (Mannheim, Germany), New England Bio Labs (Pickering, ON) and Fermentas (Burlington, ON, Canada) – see Table 2; bovine serum albumin was from Sigma-Aldrich (Oakville, ON), FuGENE6 transfection reagent from Roche (Mississauga, ON), LTD₄ was obtained from Cayman Chemical (Ann Arbor, MI), anti-His₆-G antibody from Invitrogen (Burlington, ON), anti c-Myc from ATCC (Manassas, VA), rabbit polyclonal anti-human C-terminal CysLT1R antibody was developed and characterized in collaboration with Cayman Chemical (Ann Arbor, MI); rabbit IgG isotype control was obtained from Southern Biotechnology Associates (Birmingham, AL) Goat anti-mouse-HRP-conjugated antibody from Amersham Biosciences, rhodamine-conjugated goat anti-rabbit IgG from Bio Can Scientific (Mississauga, ON), and ECL Western Blotting detection reagent was from Amersham Biosciences.
Generation of recombinant His$_{6}$-tag truncated importin $\alpha$ proteins

Full-length human importin $\alpha$ 1 (PKW230), 4 (PKW263) and 5 (PKW268) cDNAs were kind gifts from Prof. Karsten Weis (University of California, Berkeley). The truncated importin $\alpha$ 1 (Imp $1\alpha$ΔNT, residues 65-530), was excised from the original cDNA (PKW 230) using PstI and was subcloned into His$_{6}$-tag expression vector pRSET C (Invitrogen) using the same restriction site. The right orientation was checked by digestion with XhoI.

The truncated importin $\alpha$ 4 (Imp $4\alpha$ΔNT, residues 67-522), was obtained by excising residues 66-521 with Dral from PKW263, and was subcloned in pGEM 3Z (Promega) previously opened with HincII, thus regenerating the STOP codon eliminated by Dral. Further, the truncated importin was amplified from pGEM3Z-imp4$\alpha$ΔNT using the following primers: myc-imp4fwd (5'-ggcagatctggaacagaaactgattagcgaagaagacctgaatctggaagctattatg-3') and SP6. The PCR-amplification product was cut with BglII/HindIII and subcloned into pRSET B vector previously digested with BamHI/HindIII, giving rise to a construct bearing an N-terminal c-myc tag.

Importin $\alpha$ 5 with N-terminal deletion (imp5$\alpha$ΔNT, residues 71-539) was amplified using the following primers: delNT IMP5A (5'-ggcgcggctcaattaataacatggag-3') and IMP5A RVS (5'-gggaattctctaagctggaacacatc-3'). The amplification product was then subcloned in pRSET B using Eco1CRI and EcoRI. A construct also bearing an N-terminal c-myc tag was then generated by inserting, using BamHI restriction site, a fragment consisting of two annealed primers (Myc-BamHI-
FWD: 5'-gatcagctggtcagcacagcaagagacgctgag-3' and Myc-BamHI-RVS: 5'-gatcagctggtcagcacagcaagagacgctgag-3').
Tagging of importin 4 and 5 α with the c-myc epitope was done for future subcloning purposes, like transfer of the truncated importin cDNAs in mammalian expression vectors and use in transfection experiments.

**Extraction and purification of His$_6$-proteins**

The His$_6$-tag fusion proteins were isolated from the E.coli strain BL21codon$^+$ (Stratagene) transformed with purified cDNA of Imp1αΔNT, Imp4αΔNT, and Imp5αΔNT. Bacteria were initially grown in 2xYT medium at 37°C for 3h (OD$_{600}$) in the presence of 100μg of ampicillin, and subsequently induced with 1mM IPTG (isopropyl-1-thio-beta-D-galactopyranoside, Sigma, Oakville, ON) for 3h at 22°C. Since the importins seem to be toxic for bacteria, induction at 22°C yielded a higher level of protein than induction at 37°C. The cells were collected by centrifugation at 4000rpm for 15min at 4°C.

Cells from 40ml bacterial cultures were lysed in 10ml lysis buffer (LB) in the presence of 0.1mg/ml of the protease inhibitor AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride, Sigma), 8μg lysozyme (Sigma) and 7μl β-mercaptoethanol for 30min on ice. The bacterial lysate was cleared by a 30min centrifugation in a Beckmann-Coulter centrifuge, at 13,000rpm. The clear lysate was incubated with 200μl Ni$_2^+$-charged Sepharose beads for at least 1h at 4°C,
and then washed three times with 50ml of wash buffer (WB). Purified proteins were then eluted with 1ml elution buffer (EB) and concentrated using Millipore microfilters (10kDA cut-off). For long term storage, protein suspensions were supplemented with 50% glycerol and stored at -80°C.

**Generation and expression of GST-fusion proteins**

The C-terminal tail of the CysLT1 receptor (amino acids 287-337), was excised with MfeI-XhoI from the full-length CysLT1 cDNA (pCMV-intron-myc-CysLT1, a construct created in our laboratory by C. Le Gouill, using pCMV-intron vector, a kind gift from Prof. Brian Talbot, University of Sherbrooke) and was subcloned into pGEX 4t3 vector (Amersham Biosciences) using EcoRI+XhoI to yield the pGEX 4t3 LT1-CT plasmid.

To obtain the C-terminal tail of the CysLT1 receptor with the alanine substitution in position lysine\(^{323}\), we used the following primers: LT1-K323A-FWD 5'-gtgacttatgtaccagccagccagccttttgc-3', and LT1-K323A-RVS 5'-ggcaagagggcctggctggtgcataagtcac-3'. A first PCR reaction was performed on pCMV-intron-myc-CysLT1, using T7 and LT1-K323A-RVS primers, and a second one, on the same cDNA, using SP6 and LT1-K323A-FWD. The two yields of the PCR reactions were isolated on agarose gels and used as templates for a subsequent PCR reaction using T7 and SP6 primers, to generate the entire mutant receptor. We also generated a variant of the
pUC19 vector (New England Biolabs) which we called pUCZ and which contained a modified multiple cloning site. The mutant C-terminal tail was excised MfeI-Xhol from the purified PCR product and subcloned using the same restriction sites in pUC Z. To generate the pGEX 4t3 vector containing the cDNA for the K323A mutant C-tail (LT1-CT-K/A), we used the same strategy, as for the WT.CysLT1-tail.

The entire K323A receptor was also reconstructed in pUC Z. The N-terminal third of the receptor was subcloned HindIII-MfeI by excising it from pCMV-intron-myc-CysLT1. The MfeI-MfeI fragment, corresponding to the middle part of the receptor was then inserted in the pUC Z construct, after dephosphorylation of the MfeI opened vector using alkaline phosphatase (Roche). The right orientation of the middle fragment was verified using HindIII-Mscl. The reconstructed receptor was then subcloned in pCMV-intron-myc vector, using HindIII-EcoRI (see Figure 7 for schematic representation of the cloning strategy).

This mutant was used as a template for generating a second mutant of the receptor and the C-tail, in which R$^{310}$K$^{311}$ were substituted with alanine (oligos: LT1 RK/AA FW 5'- cgtctacatttgccgccattttgtcc-3' and LT1 RK/AA RVs 5'-ggacaaagatggggcgaatgtagacag-3'). The strategy was similar to the CysLT1-K323A mutant, and the triple mutant GST-fusion C-terminal tail (LT1-CT-3xmut), was obtained by subcloning the MfeI-Xhol fragment in pGEX 4t3 vector.
Figure 7. Cloning strategy for the CysLT1 receptor C-tail and its mutants. A – Schematic representation of the two consecutive PCR reactions performed to generate the CysLT1 cDNA containing a point mutation in the C-terminal tail. B – Location of the restriction sites on the PCR-amplified fragment containing CysLT1 receptor cDNA, which were used for cloning in pUC Z vector. C – The final structure of the three constructs containing either the entire mutant CysLT1 cDNA (pUC Z and pCMV) or the mutant C-terminal tail cDNA in the GST-fusion expression vector (pGEX 4tl3).
A peptide coding for the putative bipartite NLS of CysLT1 (bNLS with the sequence: FRKHSLSVTYYPRKKLASL – basic residues are in bold) was obtained by subcloning of two annealed primers (LT1-bNLS-fwd 5′-gatcctttcgcaaaacatagccctccagcgtaccttgtggccgcaaaaagcgctctct-3′ and LT1-bNLS-rvs 5′-aattcagagggcctttttgaggggcacatatagctgacagctctgtgac-3′) into pGEX 4t3 vector using BamHI+EcoRI.

The Xenopus laevis nucleoplasmin bipartite NLS (NP-NLS, with the sequence: VKRPAATKKAGGAKKKK – basic residues are in bold) was subcloned in pGEX 4t3 using the same strategy and the following primers: NP-NLS-fwd 5′-gatcgcgtgaaacgccccggcctcgaacaaaaagccggcgcctcgtcgtc-3′ and NP-NLS-rvs 5′-aattcagcctttttttggttggcgcggctttttttggtttgtgcggcgcgttacg-3′.

A short peptide, similar to the second basic cluster of the putative NLS of the CysLT1 receptor (LT1-NLS, sequence: VPRKKASLPE – basic residues are in bold) was obtained by subclonig of two annealed primers (LT1-NLS-fwd: 5′-aattcgtgcggcgcggacaagcacgctgccggagaataatctgcagc-3′ and LT1-NLS-rvs: 5′-tgagcggcagcttccgggacagcgtcggcttggcgcggcgcgcagc-3′) into pGEX 4t1 vector using EcoRI +Xhol. Another peptide was generated using the same strategy (LT1-NLS-K/A-fwd: 5′-aattcgtgccggcggcggcggaaagcgagtgcgtcggagaataatctgcagc-3′ and LT1-NLS-K/A-rvs: 5′-tgagcggcagcttccgggagcgtcggcttggcgcgcggcgcagc-3′), in which the residue corresponding to lysine\(^{323}\) was replaced by alanine (LT1-NLS-K323A).

The Simian Virus 40 large T antigen NLS (SV40-NLS, sequence SPKKKRKVEAS) was expressed in pGEX 4t1, using the same strategy as for
LT1-NLS and using the following annealed primers: SV40-NLS-fwd: 5'-aattcagcccgaaaaaaacgaaagtgaagcagctaatctgcagc-3' and SV40-NLS-rvs: 5'-tcgagctgcagattgctgctccactttgcgtttttttcggg-3'.

Extraction and purification of GST-fusion proteins

The GST-fusion proteins were extracted and purified from E. coli strain BL21 codon+ transformed with purified cDNA of the constructs above. Bacteria were grown in 2xYT medium at 37°C for 3h in the presence of 100 μg ampicillin and subsequently induced with 1mM IPTG for another 3h at 37°C. The bacterial pellets were collected by centrifugation at 4,000 RPM for 15 min at 4°C. The pellets were resuspended in 3 ml of GST-lysis Buffer, in the presence of 0.1 mg/ml AEBSF and 8μg of lysozyme. For complete lysis of the cells, the resuspended pellets were alternatively frozen and thawed in liquid nitrogen. 7 ml of PBS were then added and the lysis was left to proceed on ice for 30min. Bacterial lysates were cleared by centrifugation at 13,000rpm for 30min at 4°C. The cleared lysates were then incubated with 200μl Glutathione-Sepharose 4B (Amersham Biosciences), for at least 1h at 4°C. The beads were recovered by centrifugation at 1,000rpm for 2min at 4°C, and subsequently washed three times with 50ml PBS. Finally, the beads were resuspended in 1ml PBS containing 0.1% BSA and 0.1mg/ml AEBSF. For their storage (2 weeks at 4°C), 0.1mg/ml AEBSF and 10μg BSA were added to the slurry.
The GST-fusion protein bound to the beads was quantified by SDS-PAGE and Coomassie-blue staining, using a BSA scale. For this, 50μl slurry for each of the GST-fusion peptides were washed twice with 1ml PBS, and then resuspended in 100μl 1xSDS-PAGE buffer. A successive dilution scale ranging from 0.25μg/10μl to 4μg/10μl was prepared using a 4μg/μl stock BSA solution (Roche) and 1xSDS-PAGE. 10μl from each of the GST-fusion proteins samples and from the BSA standards were loaded and resolved by SDS-PAGE. Gels were stained using Coomassie Blue for 1h at room temperature, and then destained using a 10% acetic acid and 30% methanol aqueous solution. After drying in a Bio-Rad Gel Dryer, gels were used to quantify the amount of purified GST-proteins by comparing the intensity of GST-fusion proteins bands with those of the BSA standards.

**Pull-Down assay using the GST-fusion proteins and His₆-proteins**

The glutathione-sepharose-4B-beads with the bound proteins were cleared by washing with PBS, to eliminate BSA. A quantity of beads corresponding to 10μg of bound GST-fusion protein were put in 500μl final reaction volume with each of the three different purified importins, using a pull down buffer (PD buffer). The mixtures were incubated with agitation overnight at 4ºC. For the GST-fusion proteins which were more concentrated by the means of μg protein/μl beads, we
compensated the quantity of beads introduced in the Pull-Down reaction, with plain Glutathione-Sepharose-4B.

The next day, the supernatants were collected by centrifugation at 800rpm at 4°C for 2min; the bead-pellets were then washed four times with 1ml cold PD buffer. The beads were mixed with 30μl 1xSDS-PAGE sample buffer each, boiled for 2-5min and centrifuged for 1min at 13,000rpm.

The cleared samples were loaded and separated on 10% SDS-PAGE gel, and the proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), according to the standard protocols.

Blocking of non-specific sites was done by incubating the membranes for 1h at 4°C in 5% skim milk solution in PBS.

The membranes were probed using 1:5000 Anti-His-G mouse monoclonal antibody (Invitrogen) in 5% skim milk in PBS (overnight at 4°C), washed three times for 20min in PBS and then blotted with 1:4000 goat anti-mouse antibody conjugated to HRP in 5% skim milk in PBS (1h at 4°C).

Membranes were washed three times for 20 minutes with PBS and then visualized using ECL Western Blotting System (Amersham Biosciences).

**Phosphorylation of GST-fusion proteins**

Beads containing 3μg of GST-fusion proteins were washed once with PBS and were incubated 30min at 30°C, in 1ml phosphorylation buffer in the presence of
20U of bovine PKA catalytic subunit (Sigma) for each sample (250U PKA were previously reconstituted in 500μl DTT 39mM) and 2mM ATP (Roche). The reaction was terminated by removing the phosphorylation buffer and washing the beads with PD buffer, and the beads were subsequently used for Pull-Down assays.

Control phosphorylation assays were performed as described, using γ-32P-ATP (Perkin Elmer Life Sciences, Boston MA). Beads were washed using PD buffer, were mixed with 1xSDS-PAGE buffer and loaded and separated on a 10% SDS-PAGE gel. The gel was dried in a Bio-Rad Gel Dryer and was exposed to autoradiographic film (Perkin Elmer) for 2h at -80°C.

Cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle's Medium (Invitrogen) with high glucose, supplemented with 10% fetal bovine serum (Sigma) and 100 μ/ml garamicin. For a six well plate, \(10^6\) cells were plated in each well, and were transfected the next day, using 2.5μl FuGENE6 transfection agent and 2.5μg plasmid DNA per well. For a 100mm Petri plate, 0.8-1x10^6 cells were plated, and transfected using 16μl FuGENE6 and up to 16μg DNA. The next day after transfection, the cells were replated (at a density of 0.5-0.75x10^5 cells per ml) in 6-well plates containing 25 mm sterilized coverslips. Coverslips were pretreated with 70% ethanol and distilled water then sterilised by flame, and placed in the
wells. They were left to dry and then incubated for 20 min at 37°C with sterile 0.2% gelatine solution. After incubation the gelatine solution was aspirated and the coverslips were left to dry before use.

Fluorescence and confocal microscopy

COS-7 cells were transfected and then plated on coverslips as described. For the transfections we used pCMV-intron-myc-CysLT1 and arrestin3-GFP (a kind gift from Prof. Benovic, Thomas Jefferson University, Philadelphia, PA). 40h after transfection supernatants were eliminated and cells were stimulated with 10^{-7}M LTD₄ in DMEM containing 0.1%BSA. Unstimulated cells were pretreated only with DMEM containing 0.1% BSA. Cells were washed three times with HBSS (Sigma) pH 7.4, and then fixed for 15min at room temperature with paraformaldehyde 2% in PBS. The cells were permeabilized with 0.1% saponin in PBS for 15min, non-specific sites were blocked with 5% skim milk in PBS, for 15min, and then with 0.1M glycine in PBS for the same time (all three steps were performed at room temperature). Cells were then incubated overnight at 4°C with a 1:1000 dilution of rabbit polyclonal anti-CysLT1 antibody (C-terminus) or the rabbit IgG isotype control. The antibodies were diluted in a solution of 2% BSA in PBS with 0.1% saponin. The next day, the cells were washed three times with BSA 2% in PBS, and then incubated for 1h at room temperature with a goat anti-rabbit rhodamine-conjugated secondary antibody, using the same dilution
buffer, and the same ratio (1:1000). Coverslips were then washed three times with BSA 2% in PBS, and three times with PBS alone, then mounted on glass slides, using Vectashield H-1000 fluorescence mounting medium (Vector laboratories, Burlington, ON). The cells were then analyzed using an Axioscop 2 fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Photomicrographs were captured using 40x or 63x oil immersion objectives and a cooled SPOT color digital camera (Diagnostic instruments Inc, St. Sterling Heights, MI). The images were processed using SPOT software.

Confocal microscopy analysis was performed on a Molecular Dynamics (Sunnyvale, CA) Multi-Probe 2001 confocal argon laser scanning system equipped with a Nikon Diap epifluorescence inverted microscope. Scanned images were transferred on a Silicon Graphics Indy 4000 work station equipped with Molecular Dynamics Imagespace analysis software.
Appendix

5X Native purification Buffer (NPB)
250mM Na₂HPO₄
2.5M NaCl

1X NPB
200ml 5X NPB
800ml H₂O
pH 8.0

Elution Buffer (EB)
50mM Na₂HPO₄
500mM NaCl
250mM Imidazole
pH 8.0

Lysis Buffer (LB)
EB diluted to 10mM Imidazole with NPB 1X
0.1% Triton X-100
pH 8.0

Wash Buffer (WB)
EB diluted to 10mM Imidazole with NPB 1X
1% Triton X-100
pH 8.0
GST-lysis Buffer
2.2 g Glucose
2.5ml Tris-HCl 1M pH 8.0
2.0ml EDTA 1M, pH8.0
100 ml H₂O

PD buffer
150 mM NaCl
10mM Tris HCl pH 7.4
1mM EDTA
1mM DTT (added before use)
10% glycerol
1% NP-40
0.1mg/ml AEBSF (added before use)

Phosphorylation buffer
50 mM Tris-HCl pH 7.5
12 mM MgCl₂
2 mM ATP (added before use)

PBS 1X
80g NaCl
2g KCl
26.8 Na₂HPO₄
2.4g KH₂PO₄
H₂O to 1000ml
pH 7.4
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<tr>
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<tr>
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Table 2. Restriction enzymes
RESULTS

Identification of the putative NLS of the CysLT1 receptor

Detailed analysis of the CysLT1 receptor amino acid sequence performed using the ScanProsite online application (http://ca.expasy.org/tools/scanprosite/, Hulo et al., 2004) and PSORT II online application (http://psort.ims.u-tokyo.ac.jp/form2.html, Nakai and Horton, 1999) indicated the presence of a putative NLS motif in the C-terminal tail between residues 310-326 (Figure 8A). The NLS is surrounded by numerous potential phosphorylation sites for kinases, such as PKA, PKC and CK2 (Figure 8B), which might be of significance in view of the fact that phosphorylation close to, or within the NLS, is known to regulate NLS function.

Expression of GST-fusion proteins

To investigate the capacity of the putative NLS motif present in the C-terminal tail of the CysLT1 receptor to specifically interact with different members of importin α family, we expressed and purified several peptides carrying different amino acid sequences.
Figure 8. Structure and expression of GST-fusion proteins. A - Putative bipartite NLS of the CysLT1 receptor, and B - potential phosphorylation sites within the intracellular loops and the C-terminal tail of the CysLT1 receptor. C - Structure of the GST-fusion proteins carrying the NLS: CT - C-tail of CysLT1; CT K/A - K^{323}A mutant of the C-tail; CT-3xMut - triple mutant of the C-tail, bNLS - bipartite NLS; bNLS K/A - K^{323}A mutant of the bNLS; NLS - second basic cluster of the bipartite NLS, NLS K/A - K^{323}A mutant of the NLS (GST is expressed as a N-terminal fusion peptide to the fragments represented above; black bands represent the relative positions of the basic residues within the constructs, and white bands the point mutations). D+E - structure of the pUC Z vector and the sequence of the modified multiple cloning site.
The C-terminal tail of the CysLT1 receptor (LT1-CT) and its mutants (LT1-CT-K/A and LT1-CT-3Xmut) were subcloned in pGEX 4t3 vectors to yield GST-fusion peptides (Figure 8C) and were expressed in *E.coli* under IPTG induction.

Difficulties in obtaining the mutants for the C-terminal tail of the CysLt1 receptor led us to modify the multiple cloning site (MCS) of the pUC19, an *E. coli* cloning vector. One feature of pUC19 vector is that its MCS is in frame with the *lacZα* gene, allowing screening for insertions using α-complementation. In this respect we designed two DNA primers, which were annealed and then inserted in the MCS which was almost entirely removed previously by digestion with restriction enzymes EcoR I and Sal I (Figure 8D). The new multiple cloning site contains the MfeI unique restriction site that was used to excise the C-terminal tail of CysLT1 receptor from the PCR-amplification product, to subclone it in pUCZ and then transfer it in pGEX 4t3 (Figure 8D and 7E).

Another GST-fusion peptide coding just for the putative bipartite NLS (bNLS with the sequence 309FRKHSLLSVTVPRKKASL 327) and a short peptide spanning just the second basic cluster of the putative NLS (NLS, with the sequence 320VPRKKASLPE 329) were also expressed and purified as described (Figure 8C). Mutants for the lysine 323 to alanine (VPRAKASLPE; FRKHSLLSVTVPRAKASL) were also constructed, using the same strategies.
We also expressed and purified two functional and well characterized NLS: the classical monopartite SV40 large T-antigen NLS (Kalderon et al., 1984a), and the bipartite NLS from *Xenopus laevis* nucleoplasm (Robbins et al., 1991).

We used these two peptides as positive controls for the *in vitro* interaction of the putative NLS of CysLT1 receptor with importins α 1, 4 and 5. The GST-fusion proteins were purified on Glutathione-Sepharose beads using the described protocol, and SDS-PAGE and Coomasie-blue staining using a BSA scale served us to assess the purity and the amount of bound proteins.

**Expression of recombinant truncated importins**

Previous studies have demonstrated that *in vitro* interaction between importin α and a NLS-bearing substrate can be impaired if full-length importin α is used (Fanara P et al., 2000) (Figure 9A).

We therefore expressed, using pRSET vectors, N-terminal truncated importin 1α, 4α and 5α that were bearing an N-terminal poly-histidine-tag (Figure 9B). After expression in *E.coli*, under IPTG induction, and purification on Ni²⁺-charged beads, the three N-terminal truncated importins were eluted from the beads using an elution buffer containing a high concentration of imidazole (250mM), and then concentrated using microcentrifuge filter columns.
Figure 9. Structure and expression of importin α1, 4 and 5. A – General structure of importin α proteins, with the auto-inhibitory N-terminal importin β-binding domain (IBB domain) and the 8 armadillo domains. B – Schematic view of the His6-tagged importins expressed in pRSET vectors. C – Western blots with the purified importins, using an anti-His-G antibody; the N-truncated importins have an approximate size of 60kDa. The lower bands (*) are truncated forms of His6-tagged importins.
Western blot analysis of the purification products, using an anti-His_{6}-G antibody revealed the three N-truncated importins as proteins with an approximate size of 60kDa (Figure 9C).

The expression level for importin \( \alpha 1 \) was always higher than for importin \( \alpha 4 \); importin \( \alpha 5 \) had the lowest expression level. Additional fragments with an approximate size of \( 30kDa \), which might be incomplete translation or degradation products, were co-purified with all the three importins.

**The C-tail of CysLT1 receptor interacts with importins \( \alpha 1, \alpha 4 \) and \( \alpha 5 \)**

To test whether the C-terminal tail of the CysLT1 receptor is able to interact with importin \( \alpha \) proteins, we carried out Pull Down assays, using the purified GST-fusion proteins and the three importins described above.

Both importins \( \alpha 1 \) and \( \alpha 4 \) interacted with the C-terminal tail of CysLT1 in a comparable manner to the SV40 NLS. The interaction of the small NLS with importins 1 and 4 had a much lower intensity, and was not consistently reproduced. Importin \( \alpha 5 \) demonstrated a different pattern of interaction; it did not interact with the SV40 NLS, but it was able to couple to the C-tail of the receptor. The K/A mutant of the small NLS, was not able to interact with any of the three importins \( \alpha \) (Figure 10).
Figure 10. Interaction between the C-terminal tail of the CysLT1 receptor and importin α1, α4 and α5. A Pull-Down assay was performed as described in 'Materials and methods', using N-terminally truncated importin α1, α4 and α5. The purified proteins were incubated with equivalent amounts of GST (first lane), the GST fused to the C-tail of the CysLT1 receptor (lane 2), the SV40 NLS (lane 3), the second basic cluster of the putative NSL of the CysLT1 receptor (lane 4), or its K/A mutant (lane 5). Lane 6 was loaded with a quantity of importin α equivalent to 1/5 of the input in each reaction. Western-bLOTS were performed with anti-His6-G antibody. (n=3)
Since the C-tail showed a very strong capacity for interacting with the importins, we disrupted the second basic cluster of the bipartite NLS, by replacing lysine\textsuperscript{323} with alanine (CT K/A). The K/A mutant of the C-tail showed only a slight decrease in affinity for the importins (Figure 11).

Given that the small NLS did not show a strong capacity of binding to the different importins, we introduced in our experiments a GST-fusion peptide coding for the bipartite NLS of the CysLT1 receptor. We considered that the nucleoplasmia bipartite NLS would make a good positive control for our experiments.

The bipartite NLS (bNLS) of the CysLT1 receptor showed a very low affinity for the three importins, being almost comparable to the background binding of simple GST.

Since the K\textsuperscript{323}A mutation in the C-tail did not disrupt binding for the importins, we next created a triple mutant of the C-terminal tail, in which R\textsuperscript{310}, K\textsuperscript{311}, and K\textsuperscript{323} were replaced with alanine (CT 3xMut). This last mutant lacks the first basic cluster, and the second one is disrupted.

As it can be seen in Figure 12, the disruption of the NLS in the triple mutant of the receptor C-tail was not able to inhibit interaction with the three importins. The slight decrease in binding that can be seen for importins 4 and 5, illustrated in this experiment, was not reproductible.
Figure 11. Replacement of lysine 323 with alanine does not inhibit binding of importin α1, α4 and α5 by the C-tail. A Pull-Down assay was performed, using importins α1, α4 and α5, which were incubated with equal amounts of NP-NLS (lane 1), LT1-CT (lane 2), LT1-CT K/A (lane 3), bNLS (lane 4), bNLS K/A (lane 5), SV40 (lane 6), NLS (lane 7), NLS K/A (lane 8) and plain GST (lane 9). Western-bLOTS were performed with anti-His<sub>6</sub>-G antibody. (n=3)
Figure 12. The triple mutant of the CysLT1 C-tail preserves binding capacity to the importins. A Pull-Down assay was performed, using importins α1, α4 and α5, which were incubated with equal amounts of NLS (lane 1), bNLS (lane 2), GST (lane 3), SV40 (lane 4), NP-NLS (lane 5), Lt1-CT (lane 6), Lt1-CT K/A (lane 7), and LT1-CT 3xMut (lane 8). Western-blots were performed with anti-His6-G antibody. (n=3)
Given that phosphorylation of sites close to the NLS is known to modulate binding affinity for importin α we wanted to determine whether phosphorylation of residues situated within the C-terminal tail of the receptor could affect binding to the three importins α. In this respect we used the catalytic subunit of PKA to phosphorylate *in vitro* the C-terminal tail of CysLT1 and then performed Pull-Down assays.

Preliminary results suggest that in the case of importin α1 and α5, phosphorylation within the CysLT1 C-terminal tail does not modulate binding, when compared to the case in which the C-tail was incubated only with the phosphorylation buffer, but without PKA and ATP. On the other hand, phosphorylation of the C-tail, increases binding affinity for importin α4 (Figure 13A).

*In vitro* phosphorylation of the C-tail by PKA was confirmed in parallel experiments, using γ⁻³²P-ATP (Figure 13B).
Figure 13. Modulation of in vitro importin α binding through phosphorylation within the C-tail. A – Pull-Down assays were performed using previously phosphorylated or non-phosphorylated GST-coupled C-tail of the receptor and the three importins. After incubation for 30 minutes at 30°C in phosphorylation buffer containing catalytic subunit of PKA and ATP (+), or just in phosphorylation buffer (-), the GST-beads were washed once with Pull-Down buffer, and after that the Pull-Down assays were done as described in materials and Methods. Numbers represent difference (%) in binding between unphosphorylated and phosphorylated C-tail for importin α1, 4 and 5 determined by densitometry. B – Phosphorylation of the C-terminal tail of the CysLT1 receptor by PKA; the C-tail of the receptor (LT1-CT) and the GST peptide (GST) were phosphorylated in the same experimental conditions as for the Pull-Downs, but γ-32P-ATP was used instead; samples were then washed and separated on 10% SDS-PAGE gel, and the gel was subsequently exposed to film. (n=2)
CysLT1 receptor is expressed on the cellular membrane and also in the perinuclear space in transiently transfected cells

Since our *in vitro* experiments suggested a possible interaction between the CysLT1 receptor and members of the importin α family of nuclear transport receptors, we analyzed the intracellular distribution and trafficking of the receptor in COS-7 cells transiently expressing the CysLT1 receptor. Previous studies by FACScan performed in our laboratory on transiently transfected COS-7 cells, revealed the fact that 22-25% of the transfected cells were actually expressing the receptor on the plasma membrane (D.J. Dupré and D. Gingras, unpublished data).

Fluorescence microscopy studies on COS-7 cells transiently transfected with the cDNA of the CysLT1 receptor revealed the membrane distribution of the receptor, which is characteristic for GPCRs. Interestingly, a large amount of receptor was detected in intracellular compartments, and especially in what seems to be the perinuclear space, creating a fine contour of the nucleus (Figure 14).
Figure 14. Cellular distribution of CysLT1 receptor in transiently transfected COS-7 cells. COS-7 cells were seeded and grown on gelatin pretreated glass-coverslips in 6-well plates and were transfected with 2 μg DNA using FuGENE6 transfection agent. 40h post-transfection, cells were fixed and permeabilized, and immunocytochemistry was performed using rabbit polyclonal anti-CysLT1 (C-terminus), and goat anti-rabbit rhodamine-conjugated antibodies. Coverslips were mounted on glass slides and were subsequently analyzed using fluorescence microscopy. (n=5)
CysLT1 receptor internalization upon agonist stimulation

Inositol-phosphate production assays carried out in our laboratory using different concentrations of LTD₄ have demonstrated that a maximal response, by the means of IP production, is achieved when COS-7 cells expressing the CysLT1 receptor were stimulated for 45 minutes with a concentration of 10⁻⁶M of LTD₄. We stimulated the transfected COS-7 cells with LTD₄, at concentrations of 10⁻⁶ and 10⁻⁷M, and have concluded that both agonist concentrations were able to induce receptor internalization. Thus, for further experiments we used an agonist concentration of 10⁻⁷ and stimulated the cells for different periods of time, varying between 10 and 40 minutes.

As seen in Figure 15A, unstimulated cells expressed the CysLT1 receptor on the cellular membrane. Stimulation with 10⁻⁷M of LTD₄ induced receptor internalization within 10 minutes (Figure 15B). At 30 minutes, internalization of the receptor was still evident as seen in Figure 15C.

We next wanted to determine if, like for other GPCRs, arrestins might be implicated in the internalization process. For this, we cotransfected COS-7 cells with the cDNAs of CysLT1 and arrestin3-GFP, and stimulated them with LTD₄ for 30 minutes.

Confocal microscopy analysis revealed, in unstimulated cells, the membrane distribution for the CysLT1 receptor, and a uniformly distributed arrestin3 inside the cytoplasm. Upon stimulation, agonist induced internalization of CysLT1 promoted also aggregation of arrestin3 and colocalization with the receptor in the
suggesting endocytic vesicle formation and arrestin-dependent internalization (Figure 16).

**Figure 15.** Agonist-induced internalization of CysLT1 receptor in transiently transfected COS-7 cells. COS-7 cells were grown and transfected as described in Materials and Methods. 40 hours post-transfection, cells were stimulated with $10^{-7}$M LTD$_4$, for 10 or 30 minutes. Immunofluorescent labeling was performed with rabbit polyclonal anti-CysLT1 and goat anti-rabbit rhodamine-conjugated antibodies. The slides were then analyzed using a fluorescence microscope. A - unstimulated COS-7 cells expressing CysLT1 receptor; B and C - COS-7 cells stimulated with LTD$_4$ ($10^{-7}$) for 10 and 30 minutes, respectively. (n=5)
Figure 16. LTD₄-induced internalization of CysLT1 receptor and arrestin3 recruitment. Cells were grown and cotransfected as described in Materials and Methods, with cDNA of CysLT1 and arrestin3-GFP. 40h after transfection, cells were stimulated for 40 minutes with LTD₄ 10⁻⁷, fixed and stained with anti-CysLT1 antibody and rhodamine-conjugated secondary antibody, mounted on glass slides and analyzed using a confocal microscope. A-C - Distribution of CysLT1 receptor and arrestin3-GFP in unstimulated cells. D-F - CysLT1 receptor internalization and arrestin3 recruitment upon LTD₄ stimulation. (n=4)
DISCUSSION

Given that amino acid sequence analysis of the CysLT1 receptor revealed the presence of a putative bipartite NLS in its cytoplasmic tail, in this study we aimed to characterize its functionality by studying its ability to interact with different members of importin α family of proteins.

The *in vitro* approach used for the characterization of interaction capacity of NLS with importin α is a commonly used technique and provides details about the residues that are involved in the interaction.

We expressed different peptides carrying the NLS and the entire cytoplasmic tail of CysLT1, and also N-terminally truncated importin α1, α4 and α5. The use of N-truncated importin α is motivated by the fact that when the complete protein is used in the absence of importin β, the internal NLS found in the N-terminal IBB-domain plays an auto-inhibitory function, and competes with the bait NLS (Kobe, 1999).

Our Pull-Down results demonstrate that the cytoplasmic tail of the CysLT1 receptor interacts with importin α1, 4 and 5, in a comparable manner to the classical NLS of the SV40 large T antigen and the bipartite NLS of *Xenopus laevis* nucleoplasmin.

This observation is in accordance with the structural requirements for recognition of NLS by importin α proteins (Dingwall and Laskey, 1991; Robbins *et al.*, 1991; Fontes *et al.*, 2000).
However, the fact that the 'small' NLS was not able to interact with the importins may be explained by the reduced number of basic residues (3) in its sequence, which does not seem to be sufficient for it to play the role of a classical type of NLS. The 'small' NLS was predicted as a putative monopartite NLS by the PSORT online application. PSORT uses the following two rules to detect it: 4 residue pattern composed of basic amino acids (K or R), or composed of three basic amino acids (K or R) and H or P; the 7 residue pattern starting with P and followed within 3 residues by a basic segment containing 3 K or R residues out of 4 residues. On the other hand, the consensus for a monopartite SV40-type NLS, K(K/R)x(K/R) where x is any aminoacid, (Conti et al., 1998), is not matched by the second basic cluster of the bipartite NLS (321PRKK324) in the CysLT1.

Another interesting observation was the relatively low binding affinity of the GST-fusion peptide expressing the bipartite NLS. It is also known that the surrounding amino-acid sequence plays an important role in the NLS recognition by importin α. This is true not only for the linker residues but also for 'outsider' residues, especially the ones situated N-terminally to the NLS (Fontes et al., 2003a; Fontes et al., 2003b).

Indeed, point mutations targeting the NLS in the CysLT1 C-terminal tail were not able to disrupt importin binding, which suggests involvement of residues surrounding the putative NLS. This is consistent with reports for the SV40 NLS, which, when expressed as a fusion peptide containing a 15-residue N-
extension of its original molecular context, shows a stronger binding to importin \( \alpha \) than just the NLS-alone (Hubner et al., 1997).

Moreover, the existence of a variety of different NLS types supports the idea of an extended binding sequence recognized by importin \( \alpha \). Annilo et al. have demonstrated that efficient nuclear localization of human ribosomal protein S7 depends not only on a minimal NLS (a tetrapeptide) but it requires the presence of an additional 17-residue upstream sequence (Annilo et al., 1998). Another upstream cluster of three basic residues that exists in the C-terminal tail of CysLT1, between positions 303-305 (RKR), might also play a role in importin coupling.

Additional \textit{in vitro} studies are necessary to establish the molecular determinants of coupling to importins. One approach could be to use truncated forms of the cytosolic tail, which will lack the second basic cluster of the bipartite NLS or the entire NLS. Once the region responsible for interaction is identified, point mutations to replace other residues will finally clarify the molecular determinants for importin \( \alpha \) binding to the CysLT1.

Another aspect touched by our study was to verify if phosphorylation of sites within the C-tail of the receptor might modulate the interaction with importins \( \alpha \). To date, none of the potential phosphorylation sites situated within the CysLT1 C-tail has been shown to be phosphorylated. We demonstrate, here, that the C-terminal tail of the receptor is phosphorylated \textit{in vitro} by PKA and our results suggest that \textit{in vitro} PKA phosphorylation within the CysLT1
cytosolic tail may play a role in importin α binding via the enhancement of binding for importin α4.

Gorlich and Kutay affirm that direct regulation of the interaction between an NLS-cargo and importin α requires that the phosphorylation site is within, or adjacent to, the NLS (Gorlich and Kutay, 1999).

Indeed the NLS of CysLT1 receptor is flanked by, or contains, within its sequence three potential phosphorylation sites for PKA (RKLSTFKHSLSSVTVPRKKASLPEKGEI CV337; the putative phosphorylation sites are in bold and underlined; basic clusters of NLS are in bold).

It is generally accepted that both second messenger-dependent protein kinases (e.g. PKA, PKC) and GRKs phosphorylate serine and threonine residues within the intracellular loops and cytosolic tail domains of GPCRs (Ferguson, 2001). Our study is the first to demonstrate in vitro PKA phosphorylation of the C-terminal tail of the CysLT1 receptor, suggesting possible functional implication of these sites in receptor signaling.

We have not yet identified which sites are phosphorylated in vitro and further studies, using mutants of the C-tail for the various potential phosphorylation sites, are necessary to elucidate both the functionality of each of the sites and their specific implication in importin α binding.

PKA phosphorylation might be a consequence of LTD₄-induced activation of the kinase via an increase in cellular levels of cAMP, as demonstrated (Sjolander and Gronroos, 1994; Gronroos et al., 1998).
On the other hand, if nuclear import of the receptor has to be considered, the role of phosphorylation in this phenomenon can not be excluded, since it is known that in some cases, phosphorylation within, or near, the NLS does not affect binding to the nuclear transport receptor, but plays enhancing or inhibitory roles in nuclear import (Hubner et al., 1997; Fontes et al., 2003b). Confirmation by in vivo studies will be also necessary. One possibility would be immunoprecipitation of CysLT1 receptor from nuclear extracts. Unfortunately, the low expression level of CysLT1 receptor renders this approach very difficult. Another solution might be to co-express the C-terminal tail of the receptor with the importins, or the use of chimeric receptors, which, on the backbone of a highly expressed receptor, will bear the cytosolic tail of CysLT1. The use of chimeric receptors is a commonly used approach in assignment of specific roles to the individual loops and C-terminus in the signaling and intracellular trafficking of receptors (Castro-Fernandez and Conn, 2002; Yu et al., 2002; Gupte et al., 2004).

Immunogold labeling of the receptor and electron microscopy on intact cells or isolated nuclei could also constitute an alternative. Indeed, this approach was used successfully to confirm localization of GPCRs (EP3, EP4, PAF, LPA1 receptors) on the nuclear membrane or inside the nucleus (Bhattacharya et al., 1999; Marrache et al., 2002; Gobeil et al., 2003a).
A second part of our study involved fluorescence and confocal microscopy to provide insights in the cellular and subcellular distribution of CysLT1 receptor, as well as intracellular trafficking upon agonist stimulation.

Bandeira-Melo *et al.* have demonstrated intracrine CysLT-receptor mediated signaling in eosinophils (Bandeira-Melo *et al.*, 2002). Permeabilized eosinophils responded by the means of IL-4 release to LTC$_4$ and LTD$_4$ stimulation, in contrast to intact eosinophils in which cysLTs were not able to induce IL-4 release. These results suggest involvement of an intracellular receptor in cysLT signaling.

Preliminary studies in our laboratory, have suggested the presence of CysLT1 receptor in intracellular compartments, in some populations of peripheral blood leukocyte populations (B lymphocytes, monocytes and monocyte-derived macrophages) (M. Thivierge and S. Turcotte, unpublished data).

Our results show that CysLT1 receptor is not only expressed on the plasma membrane, but also in intracellular compartments in COS-7 cells transiently transfected with the cDNA of CysLT1. The localization in what seems to be the perinuclear space is a very interesting finding, but further research, to determine the exact localization of the receptor is needed.

Agonist activation of a GPCR not only results in the G-protein-dependent activation of effector systems, but also sets in place a series of molecular interactions that allow for feedback regulation of G protein coupling, receptor
endocytosis and signaling through G protein-independent signal transduction pathways (Ferguson, 2001).

Here we demonstrate that LTD₄ induces CysLT₁ receptor internalization. In addition, our results strongly suggest implication of arrestins in this process, by the means of arrestin recruitment.

In the vast family of G-protein coupled receptors, a diversity of internalization pathways has been described. Many GPCRs induce translocation of arrestins and subsequently colocalize with them in intracellular compartments (vasopressin V₂ receptor) (Oakley et al., 1999), whereas other GPCRs (β₂-adrenergic receptor) induce the translocation of arrestins but do not colocalize intracellularly after internalization (Zhang et al., 1999). Other receptors, like the AT₁ receptor, internalize without mobilizing arrestins but can use the arrestin system if arrestins are overexpressed (Zhang et al., 1996).

The arrestin-dependent internalization pathway is by far the best-characterized route of internalization for GPCRs, implicating clathrin-coated pits. In this system, arrestins play the role of adaptor molecules that target activated GPCRs to endocytic coated pits (Ceresa and Schmid, 2000).

The results of our experiments demonstrate a change in intracellular arrestin distribution pattern, upon LTD₄ stimulation, by the means of agglomeration in what seem to be cytoplasmic vesicles. This is also supported by the fact that in cells that do not express CysLT₁, stimulation with LTD₄ does not induce the same effect on arrestins (data not shown).
Further characterization of arrestin implication in CysLT1 internalization is needed. Two commonly used tools are dominant-negative constructs of the GTPase dynamin and arrestins. Overexpression of dominant negative forms of dynamin blocks clathrin- (Damke et al., 1994) and caveolae-mediated endocytosis (Dessy et al., 2000), and dominant-negative arrestins are able to block recruitment of GPCRs to endocytic vesicles (Orsini and Benovic, 1998). These approaches together with construction of mutant CysLT1 receptor (e.g. C-terminal deletion, or substitution mutants, like for the PAF receptor (Chen et al., 2002) should elucidate internalization pathways adopted by the CysLT1 receptor and bring further details on this important aspect of receptor function.

In what concerns a possible nuclear translocation or perinuclear/nuclear localization of CysLT1 receptor, there are several aspects to have in view. First, there is substantial evidence that supports the presence of cell surface GPCRs (e.g. angiotensin II, somatostatin, PAF, prostaglandin E₂) in perinuclear or nuclear domains of various cells, either in a constitutive manner or following stimulation with extracellular agonists (Lu et al., 1998; Marrache et al., 2002; Gobeil et al., 2003b). In most cases, however, neither the mechanism of GPCR intracellular distribution nor the functional consequence of receptor internalization and nuclear translocation has been explicitly established, with the exception, perhaps, of the AT₁ receptor.
As suggested for AT₁ receptors, the presence of putative NLS within the C-terminal segments of GPCRs is believed, although equivocally, to pertain to their nuclear sequestration via an importin-dependent mechanism (Lu et al., 1998). However, it has to be mentioned that nuclear translocation for AT₁ receptor has been demonstrated to be cell-type dependent, which might also be the case for CysLT₁ receptor.

On the other hand, receptors like LPA₁, for instance, do not bear an NLS sequence, but were demonstrated to be expressed in perinuclear/nuclear compartments, suggesting that other nuclear import mechanisms may prevail or that biogenesis and/or endocytosis mechanisms might be implicated (Gobeil et al., 2003a).

However, intracellular expression and functionality of GPCRs, which were at first defined as plasma membrane receptors, becomes more and more evident, thus adding complexity to the physiological roles that can be played by this class of proteins. Functionality was documented for PGE₂ nuclear receptors in that they affect transcription of genes such as inducible nitric-oxide synthase (iNOS) and intranuclear calcium transients (Bhattacharya et al., 1999). iNOS and cyclooxygenase-2 (COX-2) gene expression was shown to be evoked by agonist stimulation of PAF receptors present on isolated nuclei from porcine cerebral microvascular endothelial cells (Marrache et al., 2002). Nuclear LPA₁ receptor was also shown to modulate iNOS gene expression and to induce increases in Ca²⁺ transients in purified liver nuclei (Gobeil et al., 2003a).
The presence of a putative NLS in the C-terminal tail of CysLT1 and its capacity to bind importin α, point to a possible nuclear translocation of the receptor. Another possibility that may be taken into consideration is the secondary role as chaperones that can be played by importin α proteins (Jakel et al., 2002). In this respect, association of importin α with the cytosolic tail of the receptor might be of importance in receptor conformation or signaling activity. From this point of view, it would be interesting to compare functionality between wild-type and a mutant CysLT1 receptor that lacks binding capacity to importin α.

Very recent studies provide evidence for another role played by importin α, namely its implication in nuclear envelope assembly during mitosis in conjunction with NLS-containing partner proteins (Hachet et al., 2004). Although it is a new and yet poorly described role for an importin α-NLS-cargo coupling, a possible implication of CysLT1 NLS in mitosis of certain cell types could be also investigated.

Considering all the above data, it is likely that the CysLT1 receptor could interact with importin α under physiological conditions. However, additional studies are needed to elucidate the molecular determinants for importin α binding, to identify phosphorylated residues that modulate this interaction and to detail the intimate processes of cellular expression and trafficking of the CysLT1 receptor, and eventually to link the importinα binding to subsequent functions accomplished by the receptor.
CONCLUSIONS

In the present work, we studied the ability of the CysLT1 receptor cytosolic tail to interact with importin α proteins, and we examined sub-cellular expression and trafficking of the receptor.

We have shown that importin α proteins bind the C-terminal tail of the CysLT1 receptor, and that the molecular determinants of this interaction are not restricted only to the putative NLS motif found within the tail, but probably extends to upstream basic clusters.

We also demonstrated for the first time, by in vitro phosphorylation, the possible functionality of potential PKA phosphorylation sites within the CysLT1 C-tail, and the increase of binding for importin α4 through phosphorylation.

We further demonstrated that CysLT1 is expressed both on the plasma membrane and in intracellular compartments in transiently transfected COS-7 cells.

Finally, we showed that CysLT1 receptor internalizes upon LTD4 stimulation, and that stimulation determines arrestin recruitment, thus supporting a role for these adaptor molecules in CysLT1 receptor intracellular trafficking.
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