

The cytokine interleukin-5 (IL-5) effects cotransport of its receptor subunits to the nucleus in vitro

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Abstract Interleukin (IL)-5 is central in regulating eosinophilia in allergic disease and parasitic infections. We have recently shown that human (h) IL-5 both possesses a functional nuclear localization signal capable of targeting a heterologous protein to the nucleus and localises to the nucleus of intact receptor-expressing cells. In this study, the extracellular domains of the hIL-5 α - and β -receptor subunits were expressed in baculovirus, fluorescently labelled and assayed for nuclear targeting in vitro in the absence and presence of IL-5. The β -subunit, which lacks IL-5 binding activity, only accumulated in the nucleus in the presence of both the hIL-5 binding α -subunit and hIL-5. The IL-5-binding α -subunit showed similar results. IL-5 thus effected nuclear transport of its α - and β -receptor subunits apparently through a 'piggy back' mechanism, raising the possibility that IL-5's nuclear signalling role may be to cotarget its receptor subunits to the nucleus. This is the first demonstration of nuclear protein piggy back transport in vitro.

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Key words: Interleukin-5; Nuclear protein import; Confocal laser scanning microscopy; Piggy back transport; Cytokine receptor

1. Introduction

The cytokine interleukin (IL)-5 appears to be a quite specific differentiation factor for the eosinophil cell lineage [1–6], studies with an IL-5 deficient mouse suggesting that the only obligatory role of IL-5 may be in controlling eosinophilia in response to allergic diseases and certain parasitic infections [3,4]. The receptor for IL-5 comprises an α -subunit which recognises IL-5 specifically, and a β -subunit which lacks ligand binding activity but which is essential for signal transduction [7,8]. The high affinity IL-5 receptor comprises both α - and β -subunits. The IL-5 β receptor subunit is shared with the related IL-3 and granulocyte-macrophage-colony stimulating factor (GM-CSF) receptors [7], with IL-3, IL-5 and GM-CSF exhibiting overlapping activities on certain cell types in vitro as a direct consequence [7,8]. Two major signalling pathways mediating IL-5's effects through the receptor β -subunit are the JAK2 (Janus kinase 2) tyrosine phosphorylation cascade leading to activation of STAT 1,3 and 5 (signal transducers and activators of transcription 1, 3 and 5) [9–13], and the Ras-Raf-1-MEK (mitogen-activated protein kinase – MAPK – or Erk kinase) protein kinase pathway [11,14]. The existence of these two pathways does not explain IL-5's

very specific role in eosinophil differentiation and development, since IL-3 and GM-CSF, which 'do not have such distinct roles, also activate both [9,10,15]. Differences in receptor expression (see Ref. [16]) similarly do not explain IL-5's obligatory role in controlling the eosinophil lineage.

One aspect of specificity in terms of IL-5-signalling mechanisms additional to those emanating from the shared β -receptor subunit resides in the fact that both human (h) and mouse (m) IL-5, in contrast to IL-3 and GM-CSF, possess sequences homologous to nuclear localization signals (NLSs) (see Ref. [17]). NLSs are the sequences sufficient and necessary to effect nuclear targeting of the proteins carrying them to the nucleus (see Refs. [18,19]). We have recently shown that both hIL-5 and mIL-5 can localise in the nucleus in intact living receptor-expressing cells subsequent to endocytosis, as well as target to the nucleus in in vivo and in vitro nuclear transport assays [20]. We also showed that the hIL-5 NLS is capable of targeting a heterologous protein to the nucleus both in vivo and in vitro, implying that it is the targeting signal responsible for IL-5 accumulation in the nucleus in intact cells [20]. Dimeric baculovirus-expressed hIL-5 is about 30 kDa, which is below the molecular mass cut-off (45 kDa) for an NLS-requirement to enter the nucleus [18,19], the possession of an NLS by IL-5 thus possibly being necessary to target a larger complex of proteins to the nucleus in a 'piggy back' type of transport. That non-NLS-containing proteins can be targeted to the nucleus through binding to an NLS-containing protein has been demonstrated for a number of nuclear proteins including high mobility group proteins and DNA polymerase subunits [18,19,21–24]. Importantly, the transport of one NLS-containing molecule can thus effect nuclear localization of a complex of specifically interacting signalling molecules [18,19].

In this study we investigate nuclear transport of the hIL-5 receptor subunits in vitro in the absence and presence of IL-5 and/or the other receptor subunit using labelled and unlabelled baculovirus-expressed hIL-5 and soluble forms of the receptor subunits. The results show that IL-5 is capable of cotransporting its receptor subunits to the nucleus in semi-intact cells, thus representing the first demonstration of nuclear protein piggy back transport in vitro. The results imply that there may be a nuclear signalling role for IL-5 and its cotargeted receptor subunits additional to pathways linked to the membrane receptor system.

2. Materials and methods

2.1. Chemicals and reagents

The amino labelling reagent 6-(fluorescein-5-(and-6)-carboxamido)-

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hexanoic acid, succinimidyl ester (SFX) was from Molecular Probes. Other reagents were from the sources previously described [20,25].

2.2. Cell culture

The FDC-P1 hR α cell line is a murine factor-dependent cell line expressing the hIL-5 receptor α -subunit which is responsive to IL-5 owing to the presence of the mIL-5 receptor β -subunit [20]. FDC-P1 hR α cells were cultured in RPMI Medium supplemented with 10% FCS and the corresponding factor. Cells of the HTC rat hepatoma tissue culture line were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS as described previously [20,25].

2.3. Baculovirus expression and purification of IL-5 and receptor subunits

hIL-5 was expressed using the baculovirus system in Sf9 insect cells, purified by gel filtration and passage through Mono Q as described previously [20,26]. cDNA fragments encoding the external domains of the hIL-5 α - and β -receptor subunits (residues 1–321 and 1–419 of the mature forms, respectively) were derived by PCR and subcloned into the transfer vector pBacPAK8 (Clontech). These recombinant proteins were also expressed using the baculovirus system, and purified by gel filtration followed by ion exchange chromatography on Mono Q. The purity of the proteins was routinely verified by SDS polyacrylamide gel electrophoresis.

2.4. Fluorescent labelling

IL-5 was labelled using the covalent amine-labelling reagent SFX as described previously [20], whereby labelled hIL-5 retained greater than 50% biological activity. The external domains of the hIL-5 α - and β -receptor subunits were similarly labelled with SFX, with subsequent passage through a NAP-5 column (Pharmacia) to separate the labelled protein from free dye [20]. Protein samples were concentrated using 'Ultrafree-MC' filter units (Millipore) [20].

2.5. Nuclear transport assay

In vitro nuclear import kinetics at the single cell level, using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy (CLSM), was as described previously in detail [25,27] and in particular for hIL-5 [20]. Where combinations of receptor subunits and IL-5 were used, preincubation (15 min at room temperature) of the proteins to achieve optimal IL-5 binding (the α -receptor subunit at 3.2 mg/ml and IL-5 and β -receptor subunit at 2 mg/ml) was carried out prior to addition to the transport assay (a subsequent dilution of 2 in 5). Image analysis of CLSM files and curve fitting to the equation $F_n/c(t) = F_n/c_{\max} \times (1 - e^{-kt})$ were performed as described [20,25,27]. $F_n/c_{\max}$ is the maximal level of nuclear accumulation whilst the first-order rate constant (k) is the rate at which $F_n/c_{\max}$ is reached.

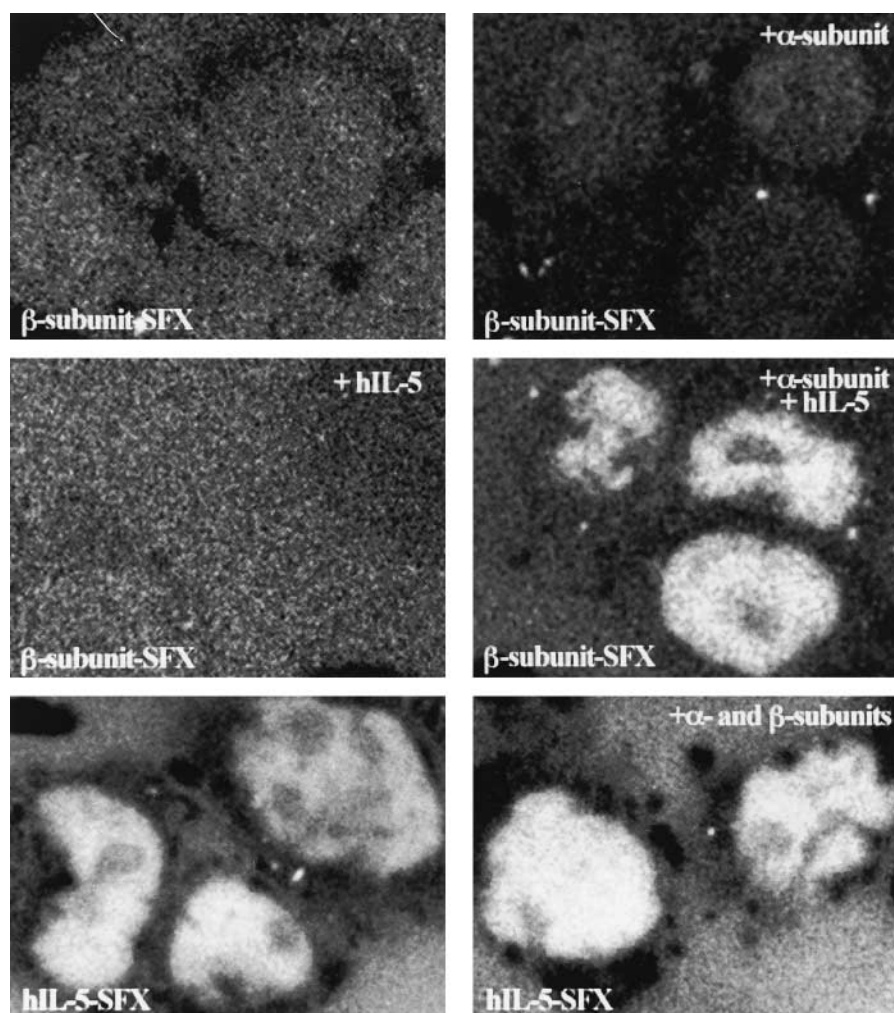


Fig. 1. Visualization of nuclear uptake of the hIL-5 β -receptor subunit in the absence and presence of hIL-5 and the hIL-5 α -receptor subunit in vitro. Results are shown for nuclear import of hIL-5- β -receptor-SFX preincubated (15 min, room temperature) in the absence (top left panel) or presence (top right panel) of unlabelled hIL-5 α -receptor subunit, unlabelled hIL-5 (middle left panel), or both (middle right panel), as indicated, in mechanically perforated HTC cells after 12–16 min at room temperature. Results are compared to those for hIL-5-SFX (bottom panels) in the absence and presence of unlabelled hIL-5 α - and β -receptor subunits, as indicated, after 6–8 min at room temperature.

3. Results

3.1. IL-5 nuclear import in the presence of IL-5 receptor subunits

To carry out subcellular localization studies, hIL-5 and soluble extracellular domain forms of the hIL-5 α - and β -receptor subunits (see Section 2) were expressed in baculovirus. Consistent with previous studies on the soluble form of the hIL-5 α -receptor subunit [28], a 1:1 complex between the extracellular domain of the hIL-5 α -receptor subunit and hIL-5 was observed on non-denaturing polyacrylamide electrophoretic gels (not shown); a complex of the β -receptor subunit extracellular domain with hIL-5 was not observed.

hIL-5 was fluoresceinated with SFX as previously to yield a fluorescent derivative which retained biological activity [20], and nuclear import kinetics assessed *in vitro*, results being identical to those observed previously (Figs. 1 and 2, and see Table 1 for pooled data [20]). To test transport in the presence of the hIL-5 receptor subunits, hIL-5-SFX was allowed to bind to unlabelled soluble α -receptor subunit in the presence of hIL-5 β -receptor subunit extracellular domain, and nuclear import assayed (Figs. 1 and 2; Table 1). No marked difference in the maximal nuclear accumulation of hIL-5-SFX was noted in the presence of receptor subunits compared to in their absence (Fig. 1, bottom panels; Fig. 2, top panel). The import rate, however, was about 50% slower (Table 1), implying that the receptor subunits may have bound to IL-5 and been cotransported to the nucleus through a piggy back mechanism [18,19,21–24], with the slower nuclear import kinetics attributable to the larger size of the protein complex compared to IL-5 alone.

3.2. Nuclear co-import of IL-5 receptor subunits in the presence of IL-5

The hIL-5 receptor subunits were labelled with SFX, and their nuclear import examined in the absence or presence of combinations of unlabelled hIL-5 and receptor subunits. Results for nuclear import of hIL-5 β -receptor-SFX are shown in Figs. 1 and 2. hIL-5 β -receptor-SFX alone did not accumulate in the nuclei of mechanically perforated HTC cells to any significant degree (F_n/c_{max} of about 1, indicating equal concentrations in nucleus and cytoplasm, consistent with its molecular mass of ca. 40 kDa, below the cut-off value of 45 kDa for passive nuclear entry [18,19]), and did not show any marked nuclear uptake in the presence of either hIL-5 or hIL-5 α -receptor alone (Figs. 1 and 2). The latter was not unexpected, since the hIL-5 β -receptor is unable to bind IL-5 in the absence of the α -receptor subunit. However, in the presence of both hIL-5 and the hIL-5 α -receptor, quite striking nuclear accumulation of hIL-5 β -receptor-SFX was observed (Fig. 1, middle right panel), maximally to levels about 3-fold those in the cytoplasm (see Fig. 2, bottom panel). The rate of accumulation was about 2.5-fold slower than that of hIL-5-SFX in the absence of receptor subunits (see Table 1), being both consistent with the uptake of a larger protein complex, and comparable to that of hIL-5-SFX in the presence of the α - and β -receptor subunits. The clear implication was that the hIL-5 β -receptor could be targeted to the nucleus by hIL-5 through a piggy back mechanism requiring the hIL-5 binding α -receptor subunit in order to bring the β -subunit into the complex.

Similar experiments were performed with labelled hIL-5 α -

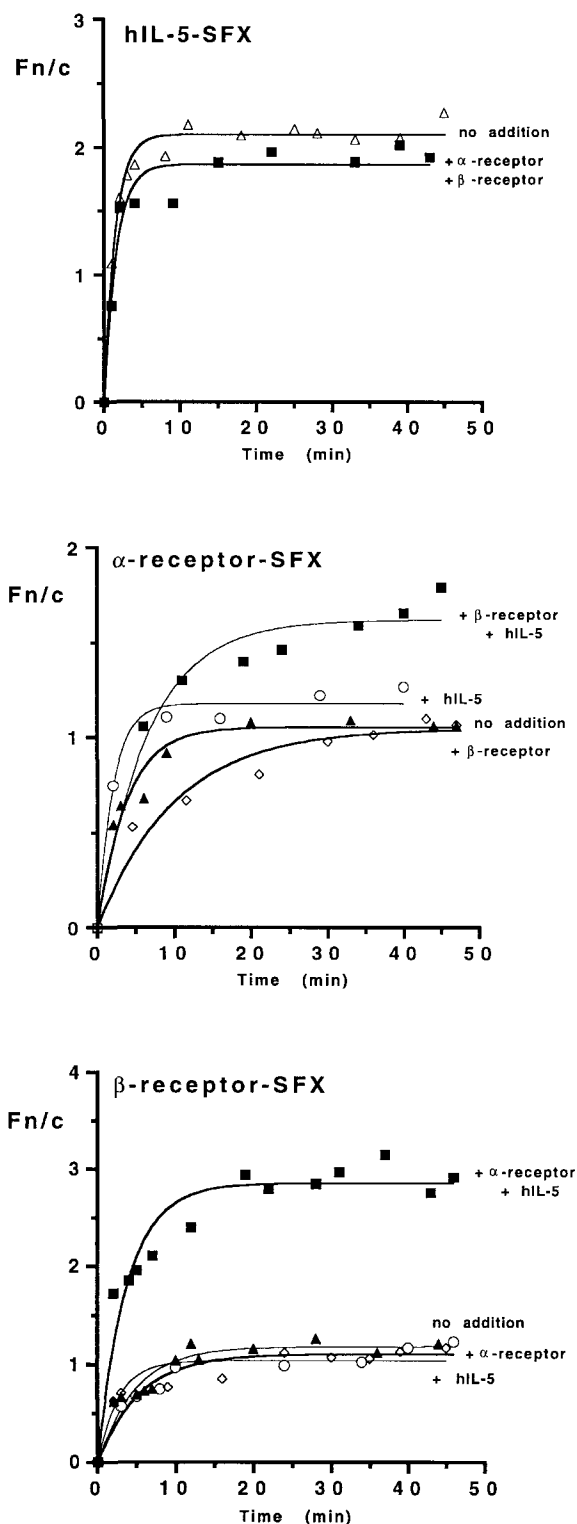


Fig. 2. Nuclear import kinetics of hIL-5 and the hIL-5 receptor subunits in the absence and presence of hIL-5 and receptor subunits *in vitro*. Quantitative results for the treatments described in Fig. 1 (see also Section 2) are shown for hIL-5-SFX (top panel), hIL-5 α -receptor-SFX (centre panel), and hIL-5 β -receptor-SFX (bottom panel), as indicated. Measurements were performed using image analysis as described in Section 2 [20] and represent the average of two separate experiments, where each point represents the average of 5–7 separate measurements for each of F_n and F_c , respectively, with autofluorescence subtracted. Collated data are presented in Table 1.

Table 1
In vitro nuclear import kinetics of hIL-5 and hIL-5 receptor subunits

Protein	Nuclear import parameter ^a		
	$F_n/c_{\max}$	k (10^{-3})	n
hIL-5-SFX	2.16 ± 0.19	924 ± 302	6
hIL-5-SFX (+α- and β-receptor)	1.86 ± 0.06	620 ± 108	2
hIL-5 α-receptor-SFX	1.06 ± 0.04	262 ± 38	1 ^b
hIL-5 α-receptor-SFX (+β-receptor)	1.05 ± 0.05	100 ± 20	1 ^b
hIL-5 α-receptor-SFX (+hIL-5)	1.18 ± 0.04	491 ± 83	1 ^b
hIL-5 α-receptor-SFX (+β-receptor+hIL-5)	1.73 ± 0.05	186 ± 28	1 ^b
hIL-5 β-receptor-SFX	1.14 ± 0.18	260 ± 70	2
hIL-5 β-receptor-SFX (+α-receptor)	1.17 ± 0.08	460 ± 120	2
hIL-5 β-receptor-SFX (+hIL-5)	1.10 ± 0.05	190 ± 30	2
hIL-5 β-receptor-SFX (+α-receptor+hIL-5)	2.81 ± 0.19	370 ± 80	2
20 kDa dextran	0.94 ± 0.03	ND ^c	4
70 kDa dextran	0.16 ± 0.03	ND ^c	3

^a Raw data (see Fig. 2) were fitted for the function $F_n/c(t) = F_n/c_{\max} \times (1 - e^{-kt})$ [20,25,27], where $F_n/c_{\max}$ is the maximal level of accumulation at steady state in the nucleus, and t is time in min. An $F_n/c_{\max}$ of 1.0 (e.g., 20 kDa dextran) indicates nuclear entry and equilibration between nucleus and cytoplasm, with values below 1 (e.g., 70 kDa dextran) indicating exclusion from the nucleus. The values shown are the mean $\pm$ SEM, with n indicated.

^b SE from curve fit [25,27].

^c ND, not determined.

receptor (see Fig. 2 and Table 1). hIL-5 α-receptor-SFX did not accumulate significantly in the nucleus either in the presence or the absence of the β-receptor subunit or hIL-5 alone (Fig. 2, middle panel; Table 1). The combination of hIL-5 and β-receptor subunit had a marked effect, leading to maximal levels of nuclear accumulation of about 1.7-fold; the fact that accumulation under these conditions was not higher was attributable to the fact that hIL-5 α-receptor-SFX was in a slight molar excess relative to IL-5 (see Section 2), and thus not all the α-receptor could bind stoichiometrically to IL-5 and be piggy-backed into the nucleus. The fact that hIL-5 in the absence of the β-receptor subunit did not effect hIL-5 α-receptor-SFX nuclear import implied that the high affinity receptor (i.e., α- and β-subunits) was required for the latter, due to the fact either that the lower affinity of the α-receptor-subunit alone for ligand was insufficient to enable piggy back transport of the α-receptor-subunit, or that the trimeric complex as such was required for cotransport. Overall, the results strongly supported the idea that the hIL-5 receptor subunits can be cotargeted to the nucleus by IL-5.

4. Discussion

A variety of studies have put the traditional concept of hormone/cytokine action being exclusively confined to events at the level of the membrane into question, with clear evidence for significant nuclear signalling roles for ligands such as prolactin, platelet derived growth factor (PDGF), fibroblast growth factor (FGF), growth hormone (GH) and insulin (see Ref. [17]). Like molecules of the PDGF and FGF classes [17,29,30], IL-5 possesses a functional NLS and is able to target to the nucleus in intact receptor-expressing cells [20].

Nuclear targeting of IL-5 may be the basis of IL-5's highly specific signal transduction role with respect to regulation of the eosinophil lineage and recruitment of eosinophils to the sites of inflammation, additional to the signalling functions it shares with IL-3 and GM-CSF through the common β-receptor subunit.

Although IL-5 is of sufficiently small size not formally to require an NLS in order to enter the nucleus, the fact that its NLS is capable of targeting the heterologous protein β-galactosidase (476 kDa) to the nucleus both in vivo and in vitro [20] implies that the NLS may be functionally important in the context of a protein complex larger in size than IL-5 alone. Our results for α- and β-receptor subunit cotransport to the nucleus in vitro by hIL-5 here strongly imply that the IL-5-NLS may be required to enable the nuclear entry of the α- and β-receptor subunits in a ligand-receptor complex. Membrane forms of other polypeptide hormone receptors such as those for IL-1 and nerve growth factor can be found in the nucleus subsequent to endocytosis at the plasma membrane ([31,32]; see Ref. [17]), and it seems likely that the membrane-associated IL-5 receptor subunits could follow a similar pathway.

Based on the results here, a possible role of IL-5 in the nucleus would appear to be to target its receptor subunits to the nucleus, where they could modulate gene expression through modifying the activity of nuclear TFs; eg. through binding of the IL-5 β-receptor subunit to SH2/SH3 domain-carrying proteins. Nuclear receptor subunits could thus modulate nuclear tyrosine phosphorylation, and thereby act in analogous fashion to prolactin which, subsequent to receptor-mediated endocytosis [33], appears to have a specific signal transduction role in the nucleus through activating nuclear

PK-C [34]. Alternatively, IL-5 together with its receptor subunits may be directly involved in binding to chromatin, possibly in analogous fashion to GH which, together with the soluble GH binding protein form of the GH receptor, may bind specific DNA sequence elements directly [35,36].

In summary, this study represents the first formal, direct demonstration of nuclear protein piggy back transport in vitro. Our observations raise the possibility that the primary role of IL-5 nuclear targeting is to cotransport its receptor subunits, and perhaps other complexed signalling molecules, to the nucleus. Future work in this laboratory will seek to determine the precise signalling role of IL-5, together with its specific receptor subunits, in the nucleus.

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