A functional bipartite nuclear localisation signal in the cytokine interleukin-5

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Abstract Interleukin (IL)-5 is central in regulating eosinophilia in allergic disease and parasitic infections. We have identified a bipartite nuclear localisation signal (NLS) within amino acids 95–111 of human IL-5 (hIL-5), also present in mouse IL-5 (mIL-5). hIL-5 and mIL-5 were labelled fluorescently, and nuclear uptake subsequent to membrane binding and internalisation by intact receptor expressing cells visualised and quantified using confocal laser scanning microscopy. hIL-5 and mIL-5 were shown to be transported to the nucleus in in vivo and in vitro nuclear protein import assays. The hIL-5 NLS was able to target a heterologous protein to the nucleus both in vivo and in vitro. Mutations within the proximal arm of the NLS abrogated nuclear targeting activity, confirming its bipartite nature. The results imply a nuclear signalling role for IL-5 additional to pathways linked to the membrane receptor system.

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

1. Introduction

The cytokine interleukin (IL)-5 appears to be a quite specific differentiation factor for the eosinophil cell lineage [1–6], recent studies with an IL-5 deficient mouse [3,4] suggesting that the only obligatory role of IL-5 may in fact be in controlling eosinophilia. Eosinophils and IL-5 are of particular importance in the pathogenesis of allergic diseases [5] and certain parasitic infections [6]. The receptor for IL-5 consists of 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]. Interestingly, the IL-5 β receptor subunit is shared with the related IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) receptors [7], and IL-3, IL-5 and GM-CSF exhibit overlapping activities on certain cell types in vitro as a direct consequence [7,8]. Two major signalling pathways appear to mediate IL-5's effects essentially through the receptor β subunit. These 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) microtubule-associated protein kinase pathway [11,14].

The elucidation of these two signalling pathways does not explain IL-5's very specific role in the differentiation and development of eosinophils, since IL-3 and GM-CSF, which do not have such distinct roles, also activate the JAK2/STAT (e.g. [10,15]), and Ras-Raf-1-MAP kinase signalling cascades (see [9]). Differences in receptor expression (see [16]) also do not appear to explain the apparently obligatory role of IL-5 in controlling the eosinophil lineage. This specificity in terms of IL-5-signalling mechanisms additional to those emanating from the β-receptor subunit shared with IL-3 and GM-CSF must therefore reside in particular properties of the IL-5 molecule itself or its α-receptor subunit, whose cytoplasmic domain has been shown in several studies to be essential for signal transduction (e.g. [12,15,17]).

We have previously noted that both human (h) and mouse (m) IL-5 possess sequences homologous to nuclear localisation signals (NLSs), and in particular bipartite NLSs (see [18]; see Table 1), which have a very distinctive structure of two arms of basic residues separated by a spacer of 10–12 amino acids [19]. This sequence is absent from both IL-3 and GM-CSF. NLSs are the short polypeptide sequences sufficient and necessary to effect nuclear targeting of the proteins carrying them to the nucleus (see [20,21]), and have been identified in several polypeptide hormone ligands specific for membrane integral receptors and shown to have an important signalling role (see [18]).

This study focuses on the nuclear targeting capacity of baculovirus-expressed IL-5 in intact cells expressing the IL-5 receptor, as well as in in vivo and in vitro nuclear transport assays, and the ability of its NLS to target a heterologous protein to the nucleus in vivo and in vitro. The results show that IL-5 is capable of localising in the nucleus in semi-intact as well as whole cells and open up the possibility of a signalling role for IL-5 in the nucleus, additional to that at the membrane.

2. Materials and methods

2.1. Chemicals and reagents

The amino labelling reagent 6-(fluorescein-5-(and-6)-carboxamido)-hexanoic acid, succinimidyl ester (SFX) and the sulfhydryl labelling reagent 5-iodacetamido-fluorescein (IAF) were from Molecular Probes. Other reagents were from the sources previously described [22–24].

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Abbreviations: IL-5, interleukin-5; IL-3, interleukin-3; GM-CSF, granulocyte macrophage colony stimulating factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; NLS, nuclear localisation sequence; SFX, 6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester; IAF, 5-iodacetamido-fluorescein; CLSM, confocal laser scanning microscopy

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2.2. Cell culture

The FDC-P1 hRα 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. It was derived from FDC-P1 cells [25] by electroporation with the hIL-5 receptor α subunit cDNA [26] subcloned into the expression vector pEFBOS [27], and selected as being hIL-5-dependent for growth. The FDC-P1 mRα cell line is identical to FDC-P1 hRα, except that the mIL-5 receptor α subunit cDNA was used inserted into the expression vector pcDNAIneo (Invitrogen). FDC-P1 hRα and FDC-P1 mRα cells were cultured in RPMI Medium supplemented with 10% FCS and the corresponding factor. Cells of the HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7288C) line were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS as described previously [23,24].

2.3. Baculovirus expression and purification of IL-5

hIL-5 and mIL-5 were expressed using the baculovirus system in Sf9 insect cells as described previously [22]. Recombinant proteins were purified by gel filtration using Sephadex G-25 and then by passage through Mono Q at pH 7.4, followed by binding at pH 9.2, and subsequent elution using a 0–350 mM NaCl gradient.

2.4. β-Galactosidase fusion proteins

Plasmids expressing the wild type hIL-5-NLS β-galactosidase fusion proteins were derived by oligonucleotide insertion into the *Sma*I restriction endonuclease site of the plasmid vector pPR2 [28]. The resultant fusion protein contains hIL-5 amino acids 93–112 (see Table 1) fused N-terminal to the *Escherichia coli* β-galactosidase enzyme sequence (amino acids 9–1023). The plasmid expressing the hIL-5-NLSmut β-galactosidase fusion protein (see Table 1) was derived by oligonucleotide site-directed mutagenesis of the hIL-5-NLS β-galactosidase fusion protein expressing construct using the Clontech Transformer Kit, as described previously [24].

1 mM isopropyl-β-thiogalactoside was used to induce expression of β-galactosidase fusion proteins in *E. coli*. They were purified by affinity chromatography as described previously [24,28].

2.5. Fluorescent labelling

IL-5 was labelled using the covalent amine-labelling reagent SFX, whereby 100 μg of freshly prepared SFX (5 mg/ml in DMSO) was added to 200 μg of protein (in phosphate-buffered saline (PBS)) and incubated at room temperature for 1.5 h (in the dark). PBS was then added to bring the sample volume to 500 μl, and the total volume loaded on to a NAP-5 column (Pharmacia) to separate the labelled protein from free dye. The sample was eluted with 1 ml of PBS, and protein samples concentrated using 'Ultrafree-MC' filter units (Millipore). Labelled hIL-5 retained greater than 50% biological activity (1.23×10^6 ED_{50} U/ml) compared to unlabelled material as assessed in thymidine incorporation assays [29], whilst labelled mIL-5 also retained biological activity (1.1×10^5 ED_{50} U/ml).

β-Galactosidase fusion proteins were labelled with IAF as described previously [24,28].

2.6. Nuclear transport assays

In vivo and in vitro nuclear import kinetics at the single cell level, using microinjected or mechanically perforated HTC cells respectively, in conjunction with confocal laser scanning microscopy (CLSM), was as described previously in detail [23,24,30]. In the case of in vivo assays, HTC cells were fused with polyethylene glycol (PEG) about 1 h prior to microinjection to produce polykaryons and injected using a Narshige IM-200 pneumatic microinjector and Leitz micromanipulator [24]. The in vitro assay was carried out in the presence of 45 mg/ml cytosolic extract (untreated reticulocyte lysate; Promega Cat. No. L415A) and an ATP regenerating system [24,30]. Image analysis of CLSM files, and curve fitting to the equation $Fn/c\ (t) = Fn/c_{max}*(1-e^{-kt})$ was performed as described [23,24,28,30]. Fn/c_{max} is the maximal level of nuclear accumulation whilst the first order rate constant (k) is the rate at which Fn/c_{max} is reached.

3. Results

3.1. Nuclear localisation of IL-5 in receptor expressing cells

To carry out subcellular localisation studies, baculovirus expressed hIL-5 and mIL-5 were fluoresceinated with SFX to yield fluorescent derivatives. The derivatives retained biological activity in proliferation assays with mouse FDC P1 cells expressing the cDNAs for the hIL-5 (FDC P1 hRα cells) or mIL-5 (FDC P1 mRα cells) α-receptor subunits respectively in addition to the endogenous IL-5 β-receptor subunit. Both derivatives were found to be able to bind specifically to and be internalised by the appropriate FDC P1 cells as visualised by CLSM. A 10-fold excess of unlabelled IL-5 could compete for binding (see Fig. 1A, compare left and right panels for total and non-specific binding respectively), demonstrating that the process was receptor-mediated. Fig. 1B comprises the quantitative data for membrane and nuclear fluorescence due to specific (values for non-specific binding subtracted) binding and uptake of hIL-5-SFX by FDC P1 hRα cells. Membrane binding was rapid (maximal at about 10 min), with membrane-bound ligand diminishing with time thereafter at 37°C due to internalisation (Fig. 1B). A second 'peak' of membrane binding activity appeared to be present 22 h after ligand addition, presumably the result of receptor recycling and appearance of new receptors on the membrane surface. Specific nuclear fluorescence was detectable as soon as 45 min after ligand addition at 37°C, reaching maximal levels at around 5–6 h (see Fig. 1B, and data not shown), at which time nuclear concentrations were higher than those at the membrane. As expected, specific nuclear (as opposed to membrane) fluorescence was not measurable even up to 4h incubation at 4°C (Fig. 1B, and not shown), implying that nuclear localisation occurs subsequent to receptor internalisation, which is inhibited at 4°C [31,32].

3.2. Nuclear accumulation of hIL-5 and mIL-5 in vivo and in vitro

The results from the whole cell experiments indicated that subsequent to binding and internalisation, IL-5 is able to make its way to the nucleus. In order to confirm that IL-5 possesses nuclear targeting activity, the nuclear import kinetics of hIL-5-SFX and mIL-5-SFX were measured in estab-

Table 1
Bipartite NLSs in human and mouse IL-5 compared to those in SWI5, N1N2 and nucleoplasmin, and hIL-5 sequences in the hIL-5-NLS-β-galactosidase fusion proteins used in this study

Protein	Bipartite NLS[+]	
	I--10 aa--I	
SWI5	**KK**YENVVIKRSP**RKR**GR**PRK**^{655}	[23]
N1N2	**RKKR**KTEEESPLKD**KAKKSK**^{554}	[19]
nucleoplasmin	**KR**PAATKKAGQA**KKKK**LD**K**^{174}	[19]
CONSENSUS	**KK**-10-12 aa-**KKK** **RR** **RRR**	
	I---12 aa---I	
mouse IL-5	LI**KK**YIDRQKEKCGEE**RRR**TRQ^{113}	
human IL-5	LI**KK**YIDGQKKKCGEE**RRR**VNQ^{114}	

B) hIL-5 sequences in hIL-5-NLS-β-galactosidase fusion proteins

IL-5-NLS-β-gal	LI**KK**YIDGQKKKCGEE**RRR**V
IL-5-NLSmut-β-gal	LI**tt**YIDGQKKKCGEE**RRR**V

[+]The single letter amino acid code is used, and the proximal and distal arms of basic residues of the bipartite NLS are in bold. References for confirmed NLSs are indicated (see also [19,21]). aa, amino acids.

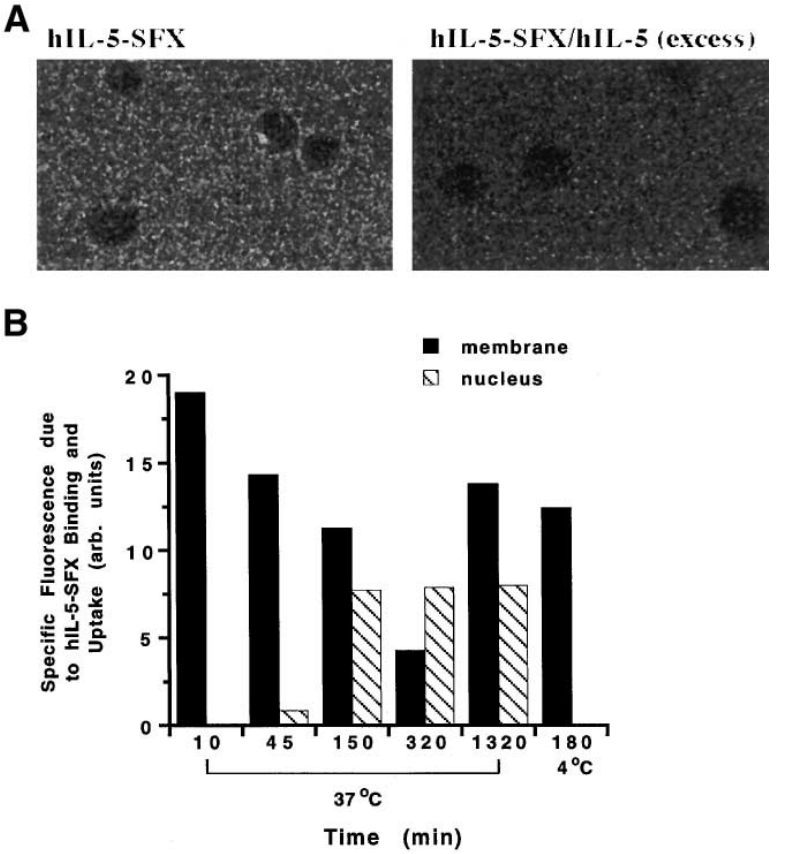

Fig. 1. Binding and uptake of human interleukin-5 by FDC-P1 hRα cells. A: Visualisation of the membrane binding of hIL-5 to FDC-P1 hRα cells. Cells were washed 4 times in factor-free RPMI and then incubated for 60 min at 37°C with 975 nM hIL-5-SFX in the absence and presence of a 10-fold excess of unlabelled hIL-5, prior to mounting and visualisation of fluorescence using CLSM. B: Specific membrane and nuclear fluorescence due to membrane binding and internalisation of fluorescently labelled hIL-5 by FDC-P1 hRα cells. Cells were treated as in A, prior to mounting and examination using CLSM. Image analysis was performed to quantitate membrane and nuclear fluorescence in the absence (fluorescence due to total binding) and presence (fluorescence due to non-specific binding) of unlabelled hIL-5, with the latter subtracted from the former to give a measure of the fluorescence ('specific fluorescence') due to specific binding. Incubations were all carried out at 37°C, except that of 180 min at 4°C (right-most column). Results represent at least 28 measurements for each of the fluorescent measurements of total and non-specific binding, with the S.E.M. less than 3.2% the value of the mean.

lished in vivo (microinjected HTC rat hepatoma cells) [23,24,28] and in vitro (mechanically perforated HTC cells [24,30]) nuclear transport assay systems using CLSM. Both ligands accumulated rapidly in the nucleus both in vivo (Fig. 2A, top panels and Fig. 2B, panel A) and in vitro (Fig. 3) to levels over 2-fold those in the cytoplasm, steady state being achieved within 1–3 min.

3.3. *The hIL-5 NLS can target a heterologous protein to the nucleus*

We had previously identified putative bipartite NLSs in the sequences of both hIL-5 (amino acids 95–111) and mIL-5 (amino acids 94–110) [18] (see Table 1). To test whether these sequences might play a role in the nuclear targeting of IL-5 observed in intact cells, as well as in in vivo and in vitro nuclear transport systems, β-galactosidase fusion protein expressing plasmid constructs were derived containing either the wild type hIL-5-NLS, or a mutant version in which lysines 95 and 96 were substituted by threonine residues (see Table 1), thus neutralising the proximal arm of the bipartite NLS; similar mutations abolish the nuclear targeting activity of other bipartite NLSs such as those listed in Table 1. Nuclear import of the hIL-5-NLS fusion protein derivatives was examined both in vivo (Fig. 2) and in vitro (Fig. 3), the hIL-5 NLS being capable of targeting the heterologous *E. coli* protein β-galactosidase (476 kDa) to the nucleus in both assay systems. The maximal levels of hIL-5-NLS-β-Gal nuclear accumulation in vitro were comparable to those of hIL-5-SFX (Fig. 3B), although the rate of accumulation was slower, consistent with the larger size of the fusion protein compared to IL-5. These results were similar to those for a β-galactosidase fusion protein containing the SWI5 bipartite NLS [23]. Although not as rapid as that of hIL-5-SFX, nuclear accumulation of hIL-5-NLS-β-Gal in microinjected cells could be readily visualised (Fig. 2A, bottom left panel) and quantified (Fn/c of 1.3 about 90 min after injection; Fig. 2B). The hIL-5-NLSmut-β-Gal protein was completely excluded from the nucleus both in vivo and in vitro (Fn/c_{max} of about 0.5, indicative of exclusion from the nucleus; Fig. 2FIg. 3) even up to 150 min in vivo (not shown), confirming the bipartite nature of the NLS. Although its significance is unclear at this stage, an intriguing observation was the fact that at later time points in vitro, hIL-5-NLSmut-β-Gal appeared to preferentially localise around the periphery of the nucleolus (Fig. 3A). Similar although not so pronounced localisation around the nucleolar periphery was evident for hIL-5 and mIL-5 (Fig. 3A).

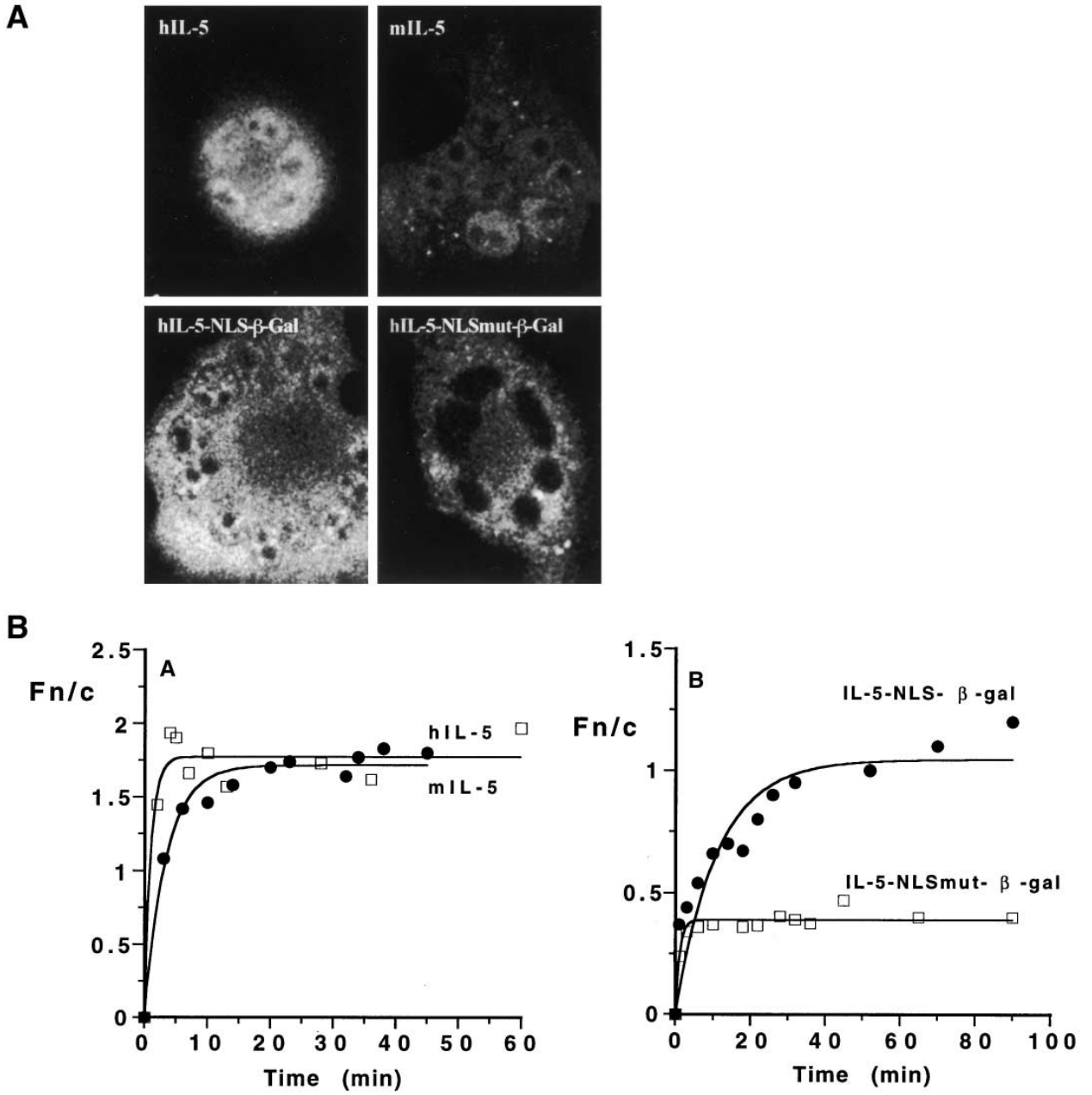

Fig. 2. Nuclear uptake of hIL-5, mIL-5 and hIL-5-β-galactosidase fusion protein derivatives in vivo. A: Visualisation of nuclear uptake in microinjected HTC cells. HTC cells were fused with PEG 1 h prior to microinjection, performed as described in Section 2. CLSM images are shown for cells 6–9 min (hIL-5 and mIL-5; upper panels) or 35–40 min (β-galactosidase fusion proteins; lower panels) after microinjection at 37°C. B: Nuclear transport kinetics of hIL-5 and mIL-5 (A) and hIL-5-β-galactosidase fusion protein derivatives (B) in microinjected HTC cells as measured by quantitative CLSM. Measurements, performed as described in Section 2 [23,24,28], represent the average of at least two separate experiments, where each point represents the average of 6–10 separate measurements for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted.

4. Discussion

A number of recent studies have encouraged reassessment of the traditional concept of hormonal action being confined to signal transduction events at the level of the membrane, indicating that polypeptide hormones such as prolactin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), growth hormone (GH) and insulin have significant nuclear signalling roles (see [18]). Molecules of the PDGF and acidic FGF (aFGF) classes have in fact been demonstrated to possess functional NLSs [33,34], which in the case of PDGF A and aFGF appear to be necessary for full mitogenic activity ([35,36]; see [18]). Our results here show that IL-5, in similar fashion to these growth factors, possesses a functional NLS capable of targeting a large (476 kDa) heterologous protein to the nucleus both in vivo and in vitro. The bipartite NLS we have defined for hIL-5 is presumably the sequence element responsible for targeting IL-5 to the nucleus in in vivo and in vitro nuclear transport assays, and in intact receptor expressing cells.

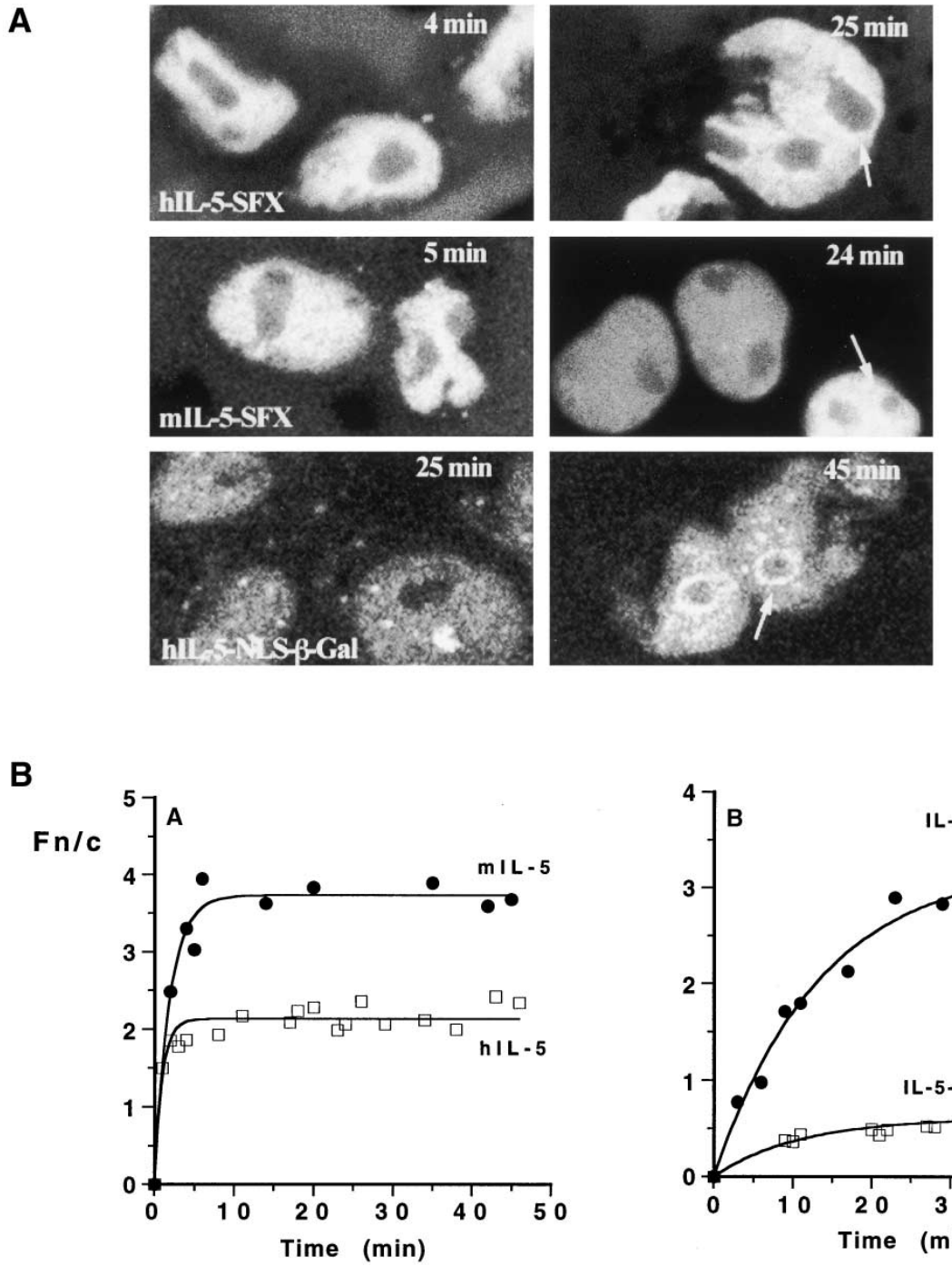

Fig. 3. Nuclear uptake of hIL-5, mIL-5 and hIL-5-β-galactosidase fusion protein derivatives in vitro. A: Visualisation of nuclear uptake in mechanically perforated HTC cells. Results are shown at the time points indicated in the presence of exogenously added cytosol and an ATP-regenerating system at room temperature. Nucleolar rim staining (indicated by the arrows) at later time points is shown in the right panels. B: Nuclear import kinetics of hIL-5 and mIL-5 (A) and hIL-5-β-galactosidase fusion protein derivatives (B) in mechanically perforated HTC cells as measured by quantitative CLSM. Measurements, performed as described in Section 2 [24,30], represent the average of at least 2 separate experiments, where each point represents the average of 5–8 separate measurements for each of Fn and Fc respectively, with autofluorescence subtracted.

This study shows that, in analogous fashion to ligands such as FGF, GH, interferon-γ [37], IL-1α [38], etc. (see [18]), IL-5 is capable of localising in the nucleus subsequent to binding to its specific receptor at the membrane and endocytosis. The amount of internalised ligand that ultimately makes its way to the nucleus (perhaps 20–25% of the total ligand originally bound) is likely to be significant in terms of signalling. The role of IL-5 in the nucleus may take the form either of influencing transcription directly by binding to chromatin in analogous fashion to basic FGF or GH ([39,40]; see also [18]), or through cotargeting bound receptor subunits and possibly other signalling molecules (e.g. JAKs) to the nucleus where

they may modulate the activity of nuclear transcription factors (see [18,41]). Nuclear targeting of IL-5 may conceivably 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 [3–6], additional to the signalling functions it shares with IL-3 and GM-CSF through the common β-receptor subunit.

In summary, our observations raise the intriguing possibility that the IL-5 NLS may be the basis of its unique signalling properties with respect to eosinophil differentiation etc., distinct from those of the related IL-3 and GM-CSF molecules. Future work in this laboratory will enlarge upon the observations reported in this study in trying to determine the unique signalling role of IL-5, with or without its specific receptor subunits, in the nucleus.

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