Growth inhibition signalled through the interleukin-4/interleukin-13 receptor complex is associated with tyrosine phosphorylation of insulin receptor substrate-1

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Induction of growth inhibition in human colorectal carcinoma cell lines by interleukin (IL)-4 and IL-13 was associated with the neophosphorylation of a 170 kDa cellular protein, identified as insulin receptor substrate-1 (IRS-1) by immunoprecipitation. Tyrosine phosphorylation of IRS-1 was also induced by insulin and insulin-like growth factor I. Sublines of colorectal carcinoma cells unresponsive to growth modulation by IL-4, IL-13 or insulin-like growth factor I-induced growth did not phosphorylate IRS-1. A functional, multimeric IL-4 receptor complex was present on all carcinoma cell lines with a subunit composition of 65 kDa, 75 kDa and the previously characterized 130 kDa band as demonstrated by affinity cross-linking with ^{125}I-labelled IL-4. The 65 kDa subunit is novel whereas the 75 kDa band represents the common IL-2 receptor γ-chain. The novel 65 kDa receptor was present as a double band and bound primarily ^{125}I-labelled IL-13. The present study demonstrates the involvement of a novel chain other than the γ-chain in the receptor complexes of IL-4 and IL-13 and post-receptor tyrosine phosphorylation of IRS-1. The association of IRS-1 with growth inhibitory signals in carcinoma cells suggests a novel mechanism of tumour growth control.

INTRODUCTION

Interleukin (IL)-4, a T_H2 lymphocyte-derived cytokine of 19 kDa, has pleiotropic immunomodulatory functions, inducing protein neoexpression on B-cells and endothelial cells and functioning as a co-stimulator of B-cell proliferation [1–4]. IL-4 also suppresses growth of non-haematopoietic tumour cells. IL-13, a recently discovered cytokine of 15 kDa, has similar structure, cellular origin and effects on various haematopoietic cell types to IL-4 [5,6]. Growth regulatory effects have not been described for IL-13. Insulin-like growth factor I, an 8 kDa peptide, in addition to its effects on glucose metabolism, is believed to promote both haematopoietic and non-haematopoietic cell growth and differentiation. We have previously shown that both insulin-like growth factor I and IL-4 regulate growth of a group of three responsive carcinoma cell lines but have no detectable effects on three other carcinoma cell lines [7,8]. The signal transduction of three responsive and unresponsive cell lines was investigated in the present study.

The major cytoplasmic target for the insulin and insulin-like growth factor I receptors in human and murine tissue cells is the 165–185 kDa phosphoprotein known as insulin receptor substrate-1 (IRS-1) [9–11]. IRS-1 is tyrosine phosphorylated in response to insulin and insulin-like growth factor I. Mice lacking the IRS-1 gene were found to be retarded in embryonal and postnatal growth and phosphorylated an alternative substrate, IRS-2 [10]. IL-4 is able to induce a rapid and striking tyrosine phosphorylation of IRS-2 (4PS) in various murine haematopoietic cell types [12–14]. Furthermore, IL-4 stimulation of a myeloid cell line transfected with the cDNA encoding IRS-1 resulted in the tyrosine phosphorylation of IRS-1 in murine systems [15]. In human systems, the activation of IRS-1 by the extracellular growth signal IL-4 and related factors is poorly understood, as are the growth suppressive effects of IL-4.

The IL-4 receptor is a 130 kDa transmembrane protein of the cytokine receptor family [16]. The primary binding site is specific for IL-4 binding, consistent with its unique, large intracellular domain being essential for most IL-4-specific functions. This domain lacks tyrosine kinase activity but was shown to contain sequence motifs capable of phosphoprotein binding [14,17]. The overlap between IL-4 inducible functions and those of IL-13 and to a lesser extent IL-2 suggests the presence of shared signal-transduction pathways for these cytokines. Partial sharing of the binding site at the cell membrane has been shown for IL-4 and IL-13 [6,18], as well as for IL-4 and IL-2 [19] but not for IL-13 and IL-2. It has been shown that the common receptor γ-chain of the IL-2 receptor, as well as of IL-7, IL-9 and IL-15 receptors [19–22], is a subunit that associates with the IL-4 receptor and is essential for signal mediation of certain lymphocyte functions [23,24]. Involvement of the common receptor γ-chain in the IL-4 and IL-13 receptors of non-haematopoietic cells is as yet unclear.

The goal of the present study was to define the signalling of the growth regulation of colonic carcinoma cell lines by IL-4 and IL-13. A key observation is the demonstration of a novel IL-4/IL-13 cell surface receptor subunit. Transduction signals for the inhibition of colon carcinoma cell growth are consistently associated with a rapidly induced neophosphorylation of tyrosine on IRS-1. The absence of IRS-1 neophosphorylation in cells unable to respond to growth modulation by IL-4, IL-13 or insulin-like growth factor I supports an important role for tyrosine-phosphorylated IRS-1 in the regulation of colon carcinoma cell growth. The panel of carcinoma cell lines used, defined with respect to cytokine responsiveness, provides an ideal

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; IRS-1, insulin receptor substrate-1.

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tool for studying the signal transduction pathways that regulate epithelial cell growth.

MATERIALS AND METHODS

Reagents

Recombinant human IL-4 (derived from the IL-4-transfected CHO cell line) with a molecular mass of 19 kDa and the mouse monoclonal antibodies S697 and S103 raised against human 130 kDa IL-4 receptor were provided by Schering Plough (Dardilly, France). Recombinant human IL-13 (CHO-derived) with a molecular mass of 15 kDa was provided by Dr. A. Minty (Sanofi Recherche, Montpellier, France). Rat monoclonal antibody TUGh4 raised against human IL-2 receptor γ-chain was provided by Dr. K. Sugamura (Sedai, Japan). The absence of the γ-chain from the IL-13 receptor complex was confirmed in experiments with the rabbit anti-human γ-chain polyclonal antibody provided by Dr. W. J. Leonard (National Heart, Lung and Blood Institute, Bethesda, MD, U.S.A.). Polyclonal rabbit anti-rat IRS-1, cross-reacting with human IRS-1, was purchased from UBI, Lake Placid, NY, U.S.A. Recombinant human insulin-like growth factor I and insulin-like growth factor II were purchased from AMS Biotechnology (Lugano, Switzerland). Cell lines HT29, WiDr and SW1116 were purchased from ATCC (Rockville, MD, U.S.A.), Co115 was obtained from Dr. B. Sordat (ISREC, Epalinges, Switzerland), LS513 from Dr. L. Suardet (ISREC) and Lisp-1 from Dr. D. Lopez (Ludwig Institute, São Paulo, Brazil). Human cell line HMC-1 was provided by Dr. J. Butterfield (Mayo Clinic and Mayo Foundation, Rochester, MN, U.S.A.). B16 mouse fibroblasts, transfected with human 130 kDa IL-4 receptor cDNA, were received from Dr. J. Banchereau (Schering Plough, Dardilly, France). Sodium orthovanadate was purchased from Sigma (St. Louis, MO, U.S.A.) and disuccinimidyl suberate from Pierce (Rockford, IL, U.S.A.).

Cell culture and ^{3}H incorporation

Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (GIBCO, Basel, Switzerland), supplemented with 2 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 5 % (v/v) fetal calf serum (Seromed, Berlin, Germany). Standard culture procedures (Myco Tect, GIBCO) consistently showed all cell lines to be free of mycoplasma contamination. The effect of proliferation regulated by IL-4 and IL-13 was assessed by using culture conditions previously described in detail [25]. Briefly, cytokines were added daily over a period of 6 days. For measurement of cell proliferation in 96-well plates, the cells were pulsed with thymidine ([^{3}H]TdR) (0.5 μCi per well) during the last 4 h of culture at 37 °C and 5 % CO_2 in air, harvested on filter papers and dried. The incorporated radioactivity was determined with an automatic filter-counting system (Inotech, Dottikon, Switzerland). All samples were measured in duplicate. The clear correlation of thymidine incorporation with colon carcinoma cell proliferation for IL-4 and insulin-like growth factor I has previously been demonstrated [8].

Cell staining by flow cytometric analysis

Cells were grown to confluency in 6-well dishes and then incubated in DMEM without supplements for 2 h. IL-4 (1 nM) was added directly to the medium containing 1 % BSA for indicated periods (10 or 60 min) before the cells were washed once in 20 mM Tris-buffered 150 mM saline solution, pH 7.4 (TBS), and detached briefly (less than 5 min) in a 0.05 % trypsin/0.02 % EDTA solution, buffered with PBS (20 mM phosphate-buffered 150 mM saline solution, pH 7.4) (Amimed, Birsfelden, Switzerland). Tumour cells (5×10^5) were incubated with monoclonal antibodies S697 or TUGh4 for 45 min at 4 °C, and washed twice in PBS, pH 7.5/1 % (w/v) BSA at 4 °C, containing 0.1 % NaN_3. Goat anti-mouse IgG–phycoerythrin R, diluted 1:30 (Sigma), was then applied for 30 min, followed by two washes in PBS/1 % BSA and resuspension in PBS/0.1 % NaN_3 immediately before measurement. Stained cells were excited by 488 nm argon laser light and analysed with a FACScan (Beckton Dickinson, Mountainview, CA, U.S.A.) using the LYSIS II software.

Analysis of phosphotyrosine-containing proteins by immunoblotting

Cells were grown to confluency in 24-well dishes and then incubated in DMEM without supplements for 2 h. IL-4 (0.1–1 nM), IL-13 (0.1–1 nM), insulin-like growth factor I (7.5 nM) or other cytokines as indicated were added directly to the medium containing 0.1 % BSA for 5 min, or indicated periods, and then the cells were washed once in TBS containing 100 nM sodium orthovanadate. Lysis in 0.1 ml of 2 % (v/v) Nonidet P40 lysis solution (buffered with 20 mM Tris, pH 7.4) supplemented with 0.1 % EDTA and 2 μg/ml each of aprotinin, leupeptin, pepstatin, PMSF and fresh orthovanadate was performed for 30 min at 4 °C, followed by centrifugation at 4 °C for 15 min at 12000 ***g***. The supernatant was subjected to SDS/PAGE (3–10 % gel gradient) and transferred to nitrocellulose. Rainbow-coloured high-molecular-mass standard from Amersham was used. Immunoblotting detection was performed at room temperature. After blocking in TBS/0.2 % Tween-20 buffer containing 0.25 % (w/v) gelatin for 30 min followed by TBS/Tween-20 buffer containing 2 % (w/v) milk powder for 30 min, the immunoblot was incubated with an affinity-purified anti-phosphotyrosine-specific monoclonal antibody (clone 66 from Sigma), diluted 1:2000 in TBS/Tween-20 buffer containing 1 % human serum albumin for 30 min. Membranes were washed for at least 1 h in TBS/Tween-20 buffer containing 2 % milk powder. For detection, peroxidase-labelled anti-mouse Fab fragments (Amersham), diluted 1:2000 in TBS/Tween-20 containing 2 % milk powder and 0.1 % (v/v) human serum, were incubated for 45 min and measurements were made using a fluorescence-based system (enhanced chemiluminescence, Amersham), using hypersensitive X-ray films (Amersham) with exposure for about 2 min.

Immunoprecipitation

For immunoprecipitation of membrane-bound proteins, cells [$(0.2–2) \times 10^6$ per 0.1 ml) were lysed in 20 mM Tris buffer, pH 7.5, containing 1 % (v/v) Nonidet P40. Lysis solution was supplemented with 0.1 % EDTA and 2 μg/ml each of aprotinin, leupeptin, pepstatin A and PMSF. After incubation for 30 min at 4 °C followed by centrifugation at 4 °C for 15 min at 12000 ***g***, the pellet was lysed a second time in TBS supplemented with 1 % Nonidet P40 and protease inhibitors. Pooled cell lysates were cleared with denatured pansorbin (from Calbiochem) that had been labelled with control IgG for 30 min. After incubation with specific antibody-labelled pansorbin for 2–12 h the precipitates were washed several times with lysis buffer, eluted with reducing PAGE loading buffer at 96 °C and subjected to SDS/PAGE (3–10 % gel gradient).

For non-specific interaction of antibody with ^{125}I-labelled ligand–receptor complexes, an irrelevant ligand was ^{125}I-labelled, cross-linked to the cell surface and applied to immunoprecipitation as negative control.

Iodination of IL-4 and IL-13

Iodination of IL-4 (2.5 μg) or IL-13 (2.5 μg) was performed by using 5 μg of Iodogen (Pierce, Rockford, MD, U.S.A.) in the presence of 1 mCi of $Na^{125}I$ (Amersham); free ^{125}I was removed by chromatography on a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS containing 1 % (w/v) BSA. ^{125}I-labelled IL-4 and IL-13 had specific activities of 60–70 and 90–100 μCi/μg respectively, and showed no loss of biological activity after labelling. The presented method thus provides a mild ^{125}I-labelling of the single tyrosine residue of IL-13; no amino acid modification, as performed by others (Phe^{43}-IL-13-GYGY; [26]), was needed.

Affinity cross-linking

Adherent carcinoma cells were detached by Ca/Mg-free PBS supplemented with 0.5 % EDTA or by short incubation (less than 5 min) in 0.05 % trypsin/0.02 % EDTA. Cells were washed three times in PBS containing 1 % BSA (with centrifugation at 2000 ***g*** for 5 min at 4 °C between washings), and for each sample 4×10^6 cells were resuspended in 0.2 ml of DMEM containing 1 % BSA and incubated with 0.5 nM ^{125}I-labelled IL-4 or 3 nM ^{125}I-labelled IL-13 for 1 h on ice. For competition a 100-fold excess of unlabelled ligand was added 20 min beforehand. The cell suspension was layered carefully on to 3 ml of 10 % (w/v) sucrose/PBS, kept at 4 °C and centrifuged in polycarbonate tubes at 20000 ***g*** for 2 min at 4 °C. The pellet was dissolved in 250 μl of PBS and cross-linking was performed for 15 min at room temperature by adding 5 μl of disuccinimidyl suberate (10 mg/ml in dimethylformamide, from Pierce, Rockford, MD, U.S.A.). The reaction was stopped with 750 μl of TBS, for 5 min at room temperature, followed by centrifugation at 10 000 ***g*** for 2 min at 4 °C and resuspension in 50 μl of lysis buffer supplemented with 2 % (v/v) Nonidet P40, 0.1 % EDTA, 1 μg/ml each of aprotinin, leupeptin, pepstatin and PMSF for 40 min on ice. After centrifugation at 4 °C for 15 min at 10000 ***g*** the resulting supernatant was analysed by SDS/PAGE (3–10 % gel gradient) under reducing conditions, followed by autoradiography (for 2–4 weeks) of dried gels at −70 °C, with X-ray Hyperfilm (Amersham).

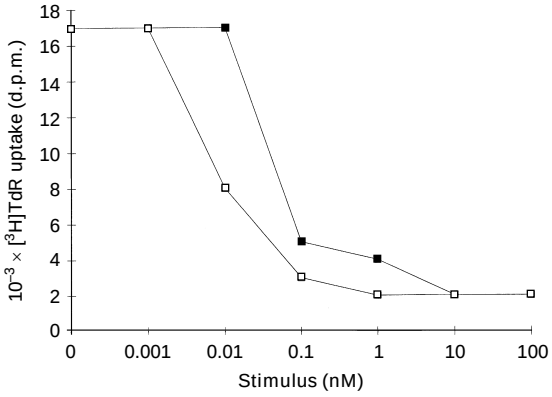

Figure 1 Potent carcinoma cell growth inhibition by IL-4 and IL-13

Colorectal carcinoma cell line LS513 was cultured in the presence of different concentrations of IL-4 (□) or IL-13 (■). Thymidine incorporation into cells is indicated as d.p.m.

RESULTS

IL-4 and IL-13 inhibit proliferation of colorectal carcinoma cells

We have previously described the inhibitory effects of IL-4 on the growth of human colorectal carcinoma cell lines [8]. Addition of increasing concentrations of IL-4 or IL-13 inhibited cell growth by 60 % or more in LS513 cells (Figure 1), as well as in HT29 and WiDr cells (results not shown). Cells were generally more sensitive to IL-4 than to IL-13. It was previously demonstrated that the same group of cell lines was able to respond to insulin-like growth factor I with growth stimulation [25]. Growth responses to IL-4 [8], IL-13 (results not shown) or insulin-like growth factor I were not detected for the three colorectal carcinoma cell lines Co115, SW1116 and Lisp-1.

Tyrosine phosphorylation correlates with IL-4-induced growth regulation

IL-4 (0.1 nM) induced strong tyrosine phosphorylation of a 170 kDa substrate. The immunoblot in Figure 2 using a mono-

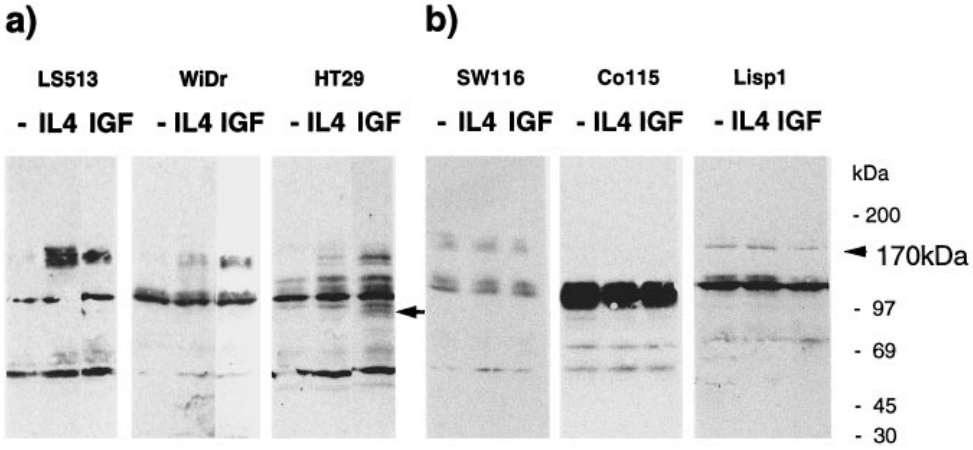

Figure 2 IL-4-induced tyrosine-phosphorylation of the 170 kDa substrate

Cell lines (**a**) LS513, WiDr and HT29 (responsive to IL-4 and insulin-like growth factor I) and (**b**) SW1116, Co115 and Lisp1 (non-responsive) were grown to confluency in 24-well plates, starved for 2 h in serum-free medium and stimulated for 5 min with 1 nM IL-4, 7.5 nM insulin-like growth factor I (IGF), or left unstimulated (—). Cell lysates were separated by SDS/PAGE (3–10 % gel gradient) and run under reducing conditions. Immunoblots were stained for tyrosine-phosphorylated proteins by using monoclonal PY-antibody clone 66 followed by peroxidase-labelled anti-mouse Fab fragments and the enhanced chemiluminescence detection system (2 min exposure to X-ray film). Molecular mass is indicated on the right of (**b**); the arrow on the right of (**a**) marks the 95 kDa IGF-receptor. Neophosphorylation of 170 kDa substrate is only seen in responsive cell lines.

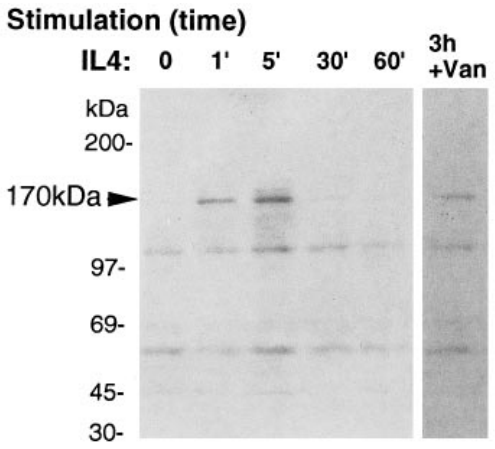

Figure 3 Kinetics of signalling pathway of IL-4

The cell line LS513 was stimulated with 1 nM IL-4 for the periods indicated. Dephosphorylation occurring after 30 min was blocked by 1 μg/ml orthovanadate (+Van). Cell cultivation and staining for tyrosine phosphorylated proteins on immunoblot were as indicated in Figure 2. Molecular mass is indicated on the left.

clonal anti-phosphotyrosine antibody shows that this phosphorylation event was strongly induced in the three growth-responsive cell lines (Figure 2a) but was not induced in non-responsive cell lines (Figure 2b). The phosphorylation was completely absent in Co115 cells, and weakly but constitutively detectable in SW116 and Lisp-1 cells without an observable induction pattern. Given the possible identity of the large 170 kDa phosphorylated band as the well-characterized IRS-1, stimulation with 7.5 nM insulin-like growth factor I was investigated. An identical protein at 170 kDa was phosphorylated. Weak induction of tyrosine phosphorylation was also seen on the 95 kDa receptor of insulin-like growth factor I (indicated by an arrow in Figure 2 for HT29 cells). The phosphorylation at 170 kDa, first evident 1 min after IL-4 stimulation, disappeared after 30 min. This dephosphorylation was driven by a protein phosphatase because it was blocked by the tyrosine phosphatase inhibitor, orthovanadate (Figure 3).

Identification of the 170 kDa substrate as IRS-1

Tyrosine phosphorylation of the 170 kDa protein substrate was shown for insulin, insulin-like growth factor II and IL-13, but not for IL-2 (Figure 4a). Immunoprecipitation of the 170 kDa phosphoprotein with a polyclonal antibody specific for IRS-1 identified the phosphoprotein as IRS-1 for both IL-4 and IL-13, and confirmed the identity of this protein as IRS-1 for insulin-like growth factor I (Figure 4b).

Down-regulation of IL-4 receptor expression after IL-4 binding

Flow cytometric analyses of the human IL-4 receptor, with the use of monoclonal antibodies (Figure 5) [17], demonstrated a homogeneous population of immunopositive carcinoma cells for both of the IL-4-responsive lines, LS513 (Figure 5a, column 1) and HT29 (column 2), and the non-responsive line Lisp-1 (column 3). IL-4 receptor expression was consistently down-regulated after 60 min with the use of an antibody that does not compete with IL-4 in receptor binding. The linear mean fluorescence intensity diminished from 95–135 arbitrary channels in unstimulated cells to 55–90 in stimulated cells and from intensity 70 to 30 in the responsive and non-responsive cell lines respectively. This internalization or removal of the IL-4 receptor from the cell surface was therefore independent of cell line responsiveness to IL-4.

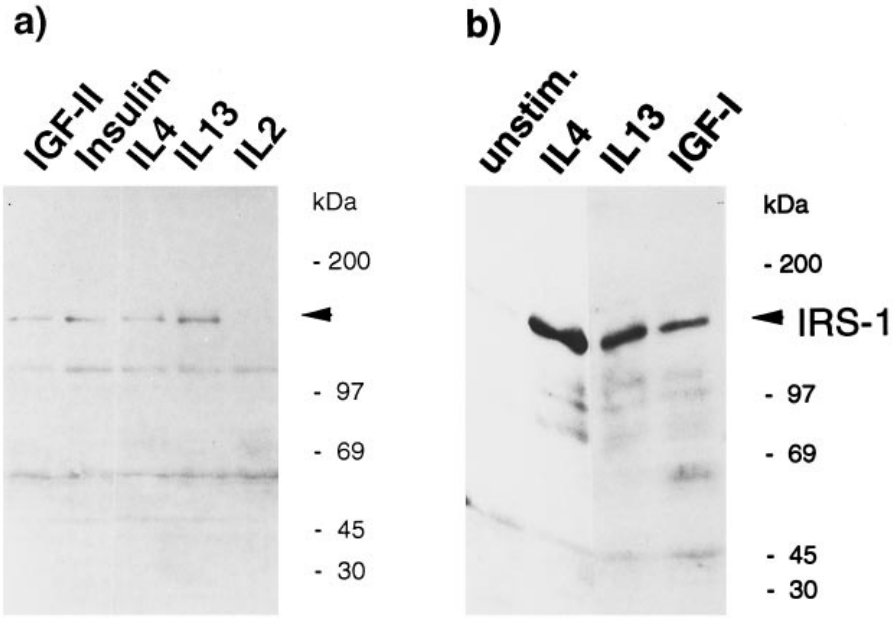

Figure 4 Major IRS-1 signal transduction pathway of IL-4 is shared with IL-13

Cell line LS513 was stimulated with 7.5 nM insulin-like growth factor II (IGF-II), 1 μM insulin, 1 nM IL-4, 1 nM IL-13 or 10 nM IL-2. (**a**) Cell cultivation and staining for tyrosine phosphorylated proteins on immunoblot were as indicated in Figure 2. (**b**) Immunoprecipitation using IRS-1 polyclonal antibody was followed by staining for tyrosine phosphorylated proteins. Molecular mass is indicated on the right. unstim., unstimulated cells.

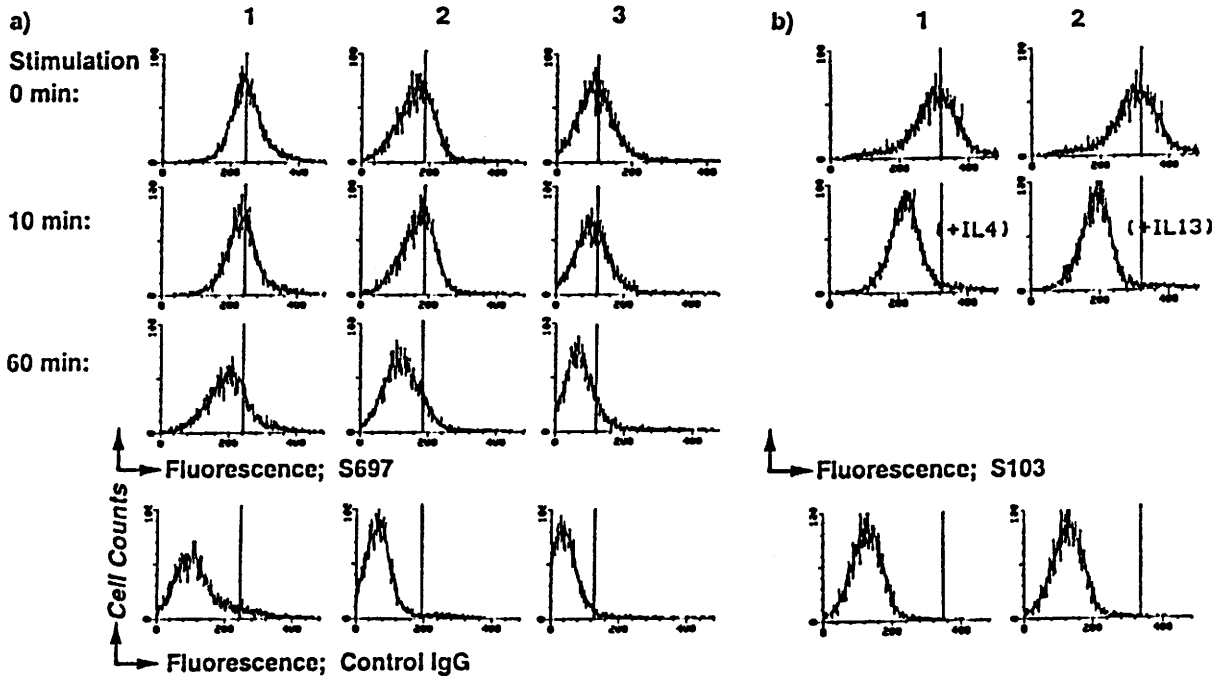

Figure 5 Flow cytometric analysis of 130 kDa IL-4 receptor in colon carcinoma cells

Flow cytometric immunofluorescence with monoclonal IL-4 receptor-specific antibodies, S697 or S103 is shown. S103 competes with IL-4 for receptor binding, whereas S697 does not. The density of the antigen on the cell surface is given on the abscissa, and the frequency of stained cells on the ordinate. (**a**) IL-4-responsive lines LS513, HT29 (columns 1 and 2 respectively) and the non-responsive Lisp-1 (column 3) with the use of S697. Cells were cultured to confluency and stimulated with 1 nM IL-4 for 0, 10 and 60 min. (**b**) HT29 cells were stimulated with 1 nM IL-4 (column 1) or IL-13 (column 2). Prebound unlabelled IL-13 and IL-4 prevented IL-4 receptor antibody from binding. This figure is a representative example of three consistent experiments.

Figure 5(b) shows that binding of an IL-4 receptor antibody, competitive with IL-4 in receptor binding, is also sterically inhibited by pre-ligation with IL-13.

Radioligand cross-linking of the subunits of the functional IL-4 receptor site

The overlap in receptor subunit composition was investigated in cell lines with documented growth responsiveness and non-responsiveness to IL-4. ^{125}I-labelled IL-4 bound predominantly to the cloned and characterized 130 kDa IL-4 receptor subunit in responsive and non-nresponsive cell lines, in addition to demonstrating minor binding to two bands of lower molecular mass (Figure 6, lane 2). The molecular mass of chemically cross-linked receptors was calculated from the relative migration on PAGE with subtraction of the 19 kDa bound ligand from the apparent mass of the radioactive bands. The identity of 130 kDa band was defined by using monoclonal antibodies specific to the IL-4 receptor and murine fibroblast-transfectants (see Figure 8c) of a cDNA encoding the human haematopoietic cell 130 kDa IL-4 receptor [27]. For Figure 6 the 130 kDa receptor was immunoprecipitated, along with accessory chains from colon carcinoma cell lysates, by using the non-competitive monoclonal antibody (lane 2). Non-binding IgG was employed to control for antigen-recognition specificity (lane 1).

Among the accessory IL-4 binding proteins, the 75 kDa band was shown to be the IL-2 receptor γ-chain, because it was immunoprecipitated with antibodies specific for the γ-chain. Only minor amounts of the cognate 130 kDa IL-4 receptor were co-immunoprecipitated (Figure 6, lane 3). The 65 kDa protein represents a novel binding protein clearly distinct from the 130 and 75 kDa chains.

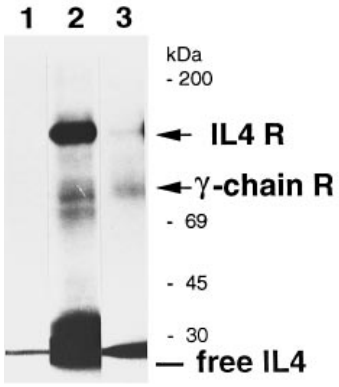

Figure 6 Multimeric IL-4 binding site identified as the cognate 130 kDa receptor and common γ-chain

Affinity cross-linking is shown of adherent carcinoma cell line HT29, which was cultured to confluency and labelled with 0.5 nM ^{125}I-labelled IL-4. Immunoprecipitates with non-binding control IgG (lane 1), IL-4 receptor antibodies (lane 2) and γ-chain antibodies (lane 3) were separated by SDS/PAGE (3–10% gel gradient), run under reducing conditions. Molecular mass is indicated at the right of the figure, and was calculated for receptors (R) by subtracting 19 kDa for bound IL-4 ligand.

Characterization of the receptor subunits for ^{125}I-labelled IL-13

^{125}I-labelled IL-13 bound specifically to a protein of molecular mass 65 kDa (Figure 7a, lane 3). When highly resolved, the IL-13 receptor appeared as a distinct double band. The SW1116 cell line, which was non-responsive to IL-13 and IL-4, also demonstrated the specific binding of ^{125}I-labelled IL-13 (Figure 7b, lane 2) to the same protein bands as the responsive lines. This novel

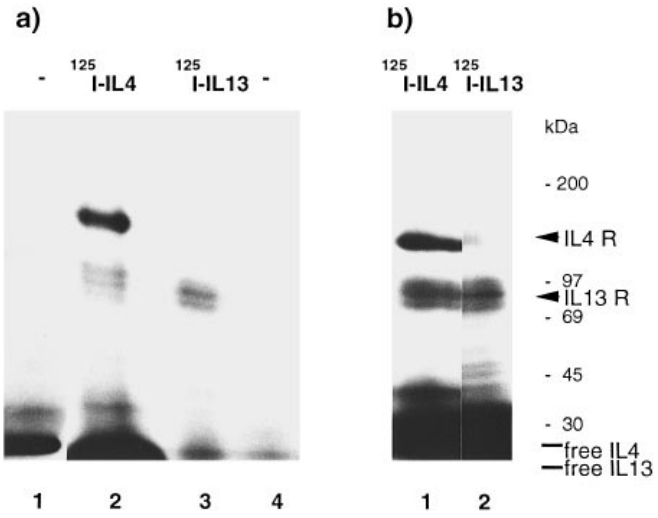

Figure 7 Multimeric IL-13 binding site primarily composed of a novel 65 kDa double band

Affinity cross-linking of the cell lines (**a**) WiDr (IL-4 responsive) and (**b**) SW1116 (IL-4 unresponsive) with 0.5 nM ^{125}I-labelled IL-4 or 3 nM ^{125}I-labelled IL-13. A 100-fold excess of unlabelled IL-4 was used to compete for ^{125}I-labelled IL-4 binding [lane 1 in (**a**)]. Excess IL-13 also successfully competed for ^{125}I-labelled IL-13 binding [lane 4 in (**a**)]. Cell lysates were separated by SDS/PAGE (3–10% gel gradient), run under reducing conditions and exposed to X-ray films. Molecular mass is indicated on the right of the figure, and was calculated for receptors (R) by subtracting 19 kDa for bound IL-4 or 15 kDa for bound IL-13.

putative IL-13 receptor was not immunoprecipitated by monoclonal antibodies to the cognate 130 kDa IL-4 receptor (results not shown). The 65 kDa double band was clearly distinct from the 75 kDa IL-4 receptor γ-chain, in that specific antisera to the γ-chain failed to immunoprecipitate this double band (results not shown). The binding specificity of the protein doublet at 65 kDa is therefore consistent with that of the putative IL-13 receptor.

IL-4 partly shares the multimeric receptor complex with IL-13

Sharing of the novel 65 kDa receptor was shown by cross-competition cross-linking studies between IL-4 and IL-13. The binding of ^{125}I-labelled IL-4 was blocked by unlabelled IL-13 (Figure 8a, lane 2). Conversely, binding of ^{125}I-labelled IL-13 was specifically blocked when cells were preincubated with an excess of unlabelled IL-4 (Figure 8a, lane 4). That the receptor sharing of IL-4 and IL-13 is not restricted to adherent cells is documented in a non-adherent human mast cell line (Figure 8b, lane 3). IL-13 receptor bound neither IL-2 (lane 4) nor IL-10 (lane 5).

The 130 kDa IL-4 receptor subunit is shared by IL-4 and IL-13, however, to a smaller extent than that seen for the 65 kDa receptor. Binding of ^{125}I-labelled IL-4 to the 130 kDa receptor was markedly diminished, but not blocked, after preincubation with excess unlabelled IL-13 (Figure 8a, lane 2). Binding of ^{125}I-labelled IL-13 to the 130 kDa receptor could not be detected by using transfectants (Figure 8c, lane 2) (smaller IL-4 receptor bands in lane 1 are truncated transfection forms [28]).

DISCUSSION

IL-4 and IL-13 were characterized as strong growth suppressants in responsive human colorectal carcinoma cells. IL-4-mediated growth inhibition has also been documented for non-neoplastic astrocytes, human umbilical vein endothelial cells, and cell lines from renal carcinoma, breast carcinoma and melanoma [4,29–32]. Consistent intracellular tyrosine phosphorylation events were associated with growth suppression. Because phosphorylation of a 170 kDa substrate identified as IRS-1 was restricted to the cytokine-responsive colorectal carcinoma lines and was absent from three non-responsive colorectal carcinoma cell lines, it is probably an important signal-transducing event in growth regulation of these cells.

Two phosphoproteins in the molecular mass range 165–180 kDa, IRS-1 and IRS-2 (4PS), have been cloned and characterized [9,33,34]. The tyrosine phosphorylation of both substrates

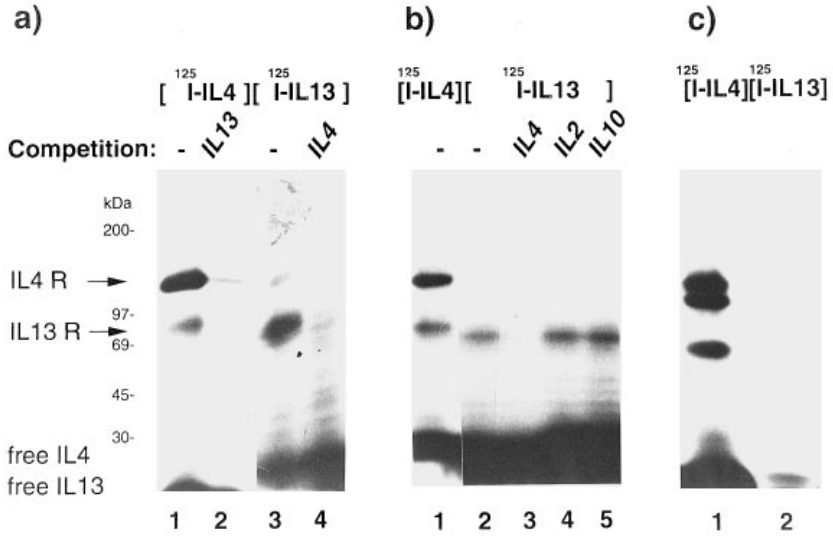

Figure 8 Reciprocal binding of IL-4 and IL-13 to the shared 65 and 130 kDa receptors

Affinity cross-linking of (**a**) the adherent colon cell line LS513, (**b**) non-adherent mast cell line HMC-1 and (**c**) transfectants of 130 kDa IL-4 receptor cDNA, employing 0.5 nM ^{125}I-labelled IL-4 or 3 nM ^{125}I-labelled IL-13. A 100-fold excess of unlabelled IL-13 was added to ^{125}I-labelled IL-4 (indicated on lane 2 in (**a**) to assess potential cross-competition. Excess unlabelled IL-4 [lane 4 in (**a**) and lane 3 in (**b**)], IL-2 or IL-10 [lanes 4 and 5 in (**b**)] was added with ^{125}I-labelled IL-13. Cell lysates were separated by SDS/PAGE (3–10% gel gradient), run under reducing conditions, and exposed to X-ray films. Molecular mass is indicated at the left of the figure, and was calculated for receptors (R) by subtracting 19 kDa for bound IL-4 and 15 kDa for bound IL-13.

is inducible by growth factors and interleukins, such as insulin-like growth factor I, insulin and IL-4. IRS-1 is a major phosphoprotein of non-haematopoietic cells, whereas IRS-2 phosphorylation is induced principally in murine haematopoietic cell types [9,12–14,35]. Consistent with the present study is the identification in non-haematopoietic human tumour cells of the neophosphorylation of IRS-1 after stimulation by IL-4, IL-13 and insulin-like growth factor I. The large number of potential phosphorylation sites on IRS-1 (21 sites) [36] could permit different patterns of phosphorylation for inhibitory or stimulatory signals. Phosphorylation induced after growth stimulation was detected for proteins other than IRS-1, at 95 kDa and 60 kDa in the present study.

IL-4-induced tyrosine phosphorylation events have been shown for Jak kinases-1 and 3 in human T-lymphocytes [20,37] and for certain transcription factors [38,39]. IL-4-induced dephosphorylation of an unidentified minor 80 kDa protein has also been documented in human haematopoietic cell lines [40]. IL-4 does not effect the activity of serine/threonine kinases such as p21ras, raf and mitogen-activated protein kinase [28]. Tyrosine phosphorylation of the cognate 130 kDa IL-4 receptor, as shown in previous cell stimulation experiments [41], was not detected in the present study by *in vitro* kinase phosphorylation assays on the carcinoma cells (results not shown). Neophosphorylation of proteins on tyrosine was, however, limited to the protein identified as IRS-1 in the present study.

A sequence motif present on the cytoplasmic portion of the 130 kDa IL-4 receptor was recently characterized as essential for signal transduction through IRS-1 in a study with site-directed mutagenesis [14]. Colonic carcinoma cells preincubated with IL-13 are unable to bind an antibody specific for the IL-4 receptor. Because IL-13 does not itself bind the IL-4 receptor on colon carcinoma cells, it is possible that IL-13 bound to the putative 65 kDa IL-13 receptor, which, we hypothesize, lies in close proximity to the IL-4 receptor, sterically prevents binding to the IL-4 receptor. Binding of IL-13 to its 65 kDa putative receptor, might, in a manner analogous to IL-2 binding to the IL-2 receptor α-chain, signal through the cognate IL-4 receptor, as does IL-2 through the IL-2 receptor β- and γ-chains.

The novel 65 kDa receptor on the adherent carcinoma cells was demonstrated as a clearly resolvable double band after ^{125}I-labelled IL-13 chemical cross-linking. Only a single dominant band at 65 kDa could be resolved in a non-adherent mast cell line. A similar binding protein shared by IL-4 and IL-13 has recently been described [18,26,42] but the presently documented association of this receptor complex with growth inhibitory signals in adherent epithelial cells represents a novel aspect of the biology of IL-13 and IL-4.

Participation of the common IL-2 receptor γ-chain, originally discovered in the IL-2 receptor complex of lymphocytes, was considered likely in the IL-4 receptor complex on colon carcinoma cells. A γ-chain antibody was able to immunoprecipitate ^{125}I-labelled IL-4 cross-linked receptor proteins. This IL-4 receptor complex, including the γ-chain, transduces growth-inhibitory signals in colonic carcinoma cells, in contrast with growth-stimulatory signals transduced through this complex in lymphocytes [23]. The common γ-chain was not detected in the ^{125}I-labelled IL-13 cross-linked receptor complex and its absence seems to correlate with the lower affinity of IL-13 binding to receptors on the colon carcinoma cells (results not shown). It is well documented that the γ-chain increases the affinity of IL-4 binding to the IL-4 receptor complex [19]. The potency of growth inhibition induced by IL-13 was lower than that of IL-4. It has recently been shown that the γ-chain is required for IL-4-induced IRS-1 phosphorylation [19]. IRS-1-mediated growth inhibition by IL-13 of colon carcinoma cells, however, seems to occur in a γ-chain independent fashion, because binding of IL-13 appears to be restricted to the novel IL-13 receptor. Supporting this model is the work of Welham et al. [43], which showed that the γ-chain docking kinase, Jak3, was not required for IRS-1 signal transduction, whereas the 130 kDa IL-4 receptor substrate, Jak1, was essential [44].

The demonstration of one or more novel IL-13/IL-4 receptor proteins at 65 kDa in addition to the 130 kDa IL-4 receptor on colonic carcinoma cells suggests that overlapping multimeric complexes signal the growth-inhibitory effects of IL-4 and IL-13 in these cells. This growth control is associated with rapid phosphorylation of IRS-1 and provides a unique system for dissecting pathways regulating cell proliferation and tumorigenesis.

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