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Stoichiometry of the complex of human interleukin-4 with its receptor

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Abstract

A large number of cytokines have been shown to possess a four-helix bundle structure with a unique up-up-down-down connectivity. The receptors for this family of cytokines have been shown to be homologous as well, each possessing two tandem repeats of a fibronectin type III-like domain. The crystal structure of human growth hormone bound to the soluble portion of its receptor has served as the only experimentally-determined example of the interaction between the four-helix bundle cytokines and their receptors: two identical receptor subunits bind to different epitopes on the same growth hormone ligand. We have conducted a series of experiments to determine if this structural paradigm is true for interleukin-4 and interleukin-4 receptor. Native polyacrylamide gel electrophoresis and gel filtration chromatography reveal that interleukin-4 forms a tight 1:1 complex with the soluble portion of interleukin-4 receptor and is thus unlike the growth hormone system.

Key words: Interleukin-4; Interleukin-4 receptor; Cytokine; Stoichiometry

1. Introduction

Human interleukin-4 (hIL-4) is a 129 residue, T lymphocyte-derived cytokine which induces a large number of biological effects, activities centered around the stimulation of proliferation and differentiation of a variety of cell types (for reviews see [1]). IL-4 belongs to a large family of structurally homologous cytokines, each composed of a four-helix bundle. The helices are connected with the up-up-down-down topology unique to the family, one which can be further divided into a 'short' group containing on average 120–130 residues (IL-2, IL-4, GM-CSF) [2] and a 'long' group containing on average 180 residues (GH, IL-6, G-CSF, Onco M). The structure of IL-4 has been determined by four independent efforts yielding two NMR solution structures [3,4] and two crystal structures [5,6]. These have been compared in detail [7] and with other helical cytokines [8].

Mature human interleukin-4 receptor (hIL-4R) is an 800 residue protein with an extracellular domain of about 207 residues, a transmembrane domain of 24 residues, and an intracellular domain of 569 residues [9]. The receptor binds IL-4 with high affinity (K_d ~100 pM) and transfection of the hIL-4R cDNA into a murine T cell line is sufficient for conferring biological responsiveness to IL-4 [10]. Although no structure of hIL-4R has yet been determined experimentally, the extracellular domain shares significant sequence homology with a large number of cytokine, hormone, and neurotrophic factor receptors [11,12]. Members of this receptor superfamily all contain a tandem repeat of ~100 residue modules in their extracellular domains proposed to fold into fibronectin type III-like domains [13]. It is these modules that recognize the four-helix bundle cytokines leading to signal transduction.

At present the growth hormone system is the only cytokine–receptor complex for which the high resolution structure has been determined, and it has served as the structural paradigm for interactions between the four-helix bundle cytokines and their receptors. The structure of the human growth hormone (hGH) bound to the extracellular domain of human growth hormone receptor (hGHR) was solved by X-ray crystallography at 2.8 Å resolution by de Vos et al. [14]. The receptor structure reveals that the two tandem 100-residue modules conserved in the receptor family fold into two fibronectin type III-like domains connected by a hinge region. A single hGH molecule interacts with two hGH receptors to form the ternary complex hGH:$(hGHR)_2$ [14]. This type of complex was used for preliminary modeling of the interactions of IL-4 with its receptor [8,15]. In order to determine if hIL-4 and hIL-4R truly interact in a fashion analogous to the growth hormone system and to guide our crystallization efforts, we have undertaken a series of experiments to establish the stoichiometry of the hIL-4:hIL-4R complex.

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Abbreviations: hIL-4, human interleukin-4; shIL-4R, soluble human interleukin-4 receptor; IL-, interleukin-; hGH, human growth hormone; hGHR, human growth hormone receptor; GM-CSF, granulocyte macrophage colony stimulating factor; LIF, leukemia inhibitory factor; Onco M, Oncostatin M; CNTF, ciliary neurotrophic factor; Tris, Tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis.

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2. Materials and methods

2.1. Expression and Purification of hIL-4

Recombinant hIL-4 used in the gel filtration experiments was expressed in a yeast α-factor expression system and purified to homogeneity as previously described [16]. This version of recombinant hIL-4 includes the tetrapeptide Glu-Ala-Glu-Ala at the N-terminus. Protein was quantified by amino acid composition analysis on a Beckmann System 6300 High Performance Analyzer.

2.2. Preparation of hIL-4 Affinity resin

An hIL-4 affinity column was prepared by coupling recombinant hIL-4 to Affi-Gel 10 from Bio-Rad (Richmond, CA). Five milliliters of Affi-Gel was washed with 10 ml ice cold coupling buffer (25 mM $NaHCO_3$/100 mM NaCl, pH 8.5) and then transferred into an ice cold solution containing 10 mg hIL-4/ml Affi-Gel in coupling buffer (i.e. 50 mg hIL-4 total). The reaction was incubated at 4°C for 24 h. The Affi-Gel was next blocked with 1 M Tris pH 8.0 (0.1 ml buffer/ml Affi-Gel) for 1 h at 4°C. The hIL-4 affinity gel was then loaded into a column and washed with 3 column volumes of coupling buffer before equilibrating for soluble human IL-4R (shIL-4R) purification. The IL-4 coupling efficiency achieved was 97%.

2.3. Expression of shIL-4R

A soluble version of human IL-4R (shIL-4R) truncated at the extracellular/transmembrane domain junction was expressed in a baculovirus/insect cell expression system. The coding sequence for the N-terminal 209 residues of hIL-4R was cloned into the *Nhe*I site of the baculovirus transfer vector pJVP10 [17]. This vector directs the expression of the inserted gene under control of the polyhedrin promoter. Recombinant virus was generated after co-transfection of SF9 cells with transfer vector and viral DNA. Plaque-purified recombinant virus expressing shIL-4R was used to generate high-titer virus stocks. For the production of shIL-4R protein, SF21 or SF9 cells in suspension were infected with virus at a multiplicity of 0.5 in serum-free medium (EXCELL 400, JRH Biosciences, Lenexa, KS) and conditioned media containing secreted protein was harvested 4 days post infection. Unpurified conditioned supernatants contained shIL-4R at concentrations between 6–20 mg/l.

2.4. Purification of shIL-4R

The 5 ml IL-4 affinity column was equilibrated with at least 5 column volumes of 50 mM Na phosphate buffer, pH 7.2. The pH of the baculovirus shIL-4R supernatant was adjusted to pH 7.2 with concentrated NaOH. Fifty column volumes of the pH-adjusted supernatant was passed over the column at a flow rate of 2.5 ml/min. The column was then washed with 8 column volumes of 50 mM Na phosphate/1.5 M NaCl, pH 7.2, to remove non-specifically bound protein and then with an equal volume of 50 mM Na phosphate, pH 7.2, to remove the salt. Bound shIL-4R was eluted with 100 mM Na phosphate pH 3.0. The eluted shIL-4R was concentrated and de-salted using an Sigma Sephadex G-25 (medium fractionation range) in 10 mM NH_4HCO_3 followed by lyophilization. All steps were performed at 4°C. Protein purity was verified by PAGE, Western blot, and N-terminal sequence analysis and was quantified by amino acid composition analysis.

2.5. Polyacrylamide gel electrophoresis

The gels were run on a Pharmacia Phast System (Piscataway, NJ) under native conditions. Precast 20% homogeneous polyacrylamide gels were employed. Due to the high isoelectric point of hIL-4 (pI = 9.0), the electrophoretic separation of the protein required acidic buffer systems, and the polarity of the electrical field across the gel had to be reversed. The acidic conditions were provided by special buffer strips prepared with a β-alanine acetic buffer pH 4.1 as recommended by Pharmacia. These buffer strips in combination with the original buffer in PhastGel gave a pH of 4.2 during electrophoresis. The reversed polarity electrode assembly used for the migration was purchased from Pharmacia.

All the samples were prepared in 50 mM Na/K phosphate buffer, pH 7.0, and diluted with an equal volume of Pyromin Y, a tracking dye. The various mixtures of hIL-4 and shIL-4R were allowed to equilibrate at room temperature for 15 min before being applied to the gel. The gels were Coomassie stained.

2.6. Gel filtration

Protein samples were run on a pre-packed Pharmacia Hi-Load 16/60 Superdex 200 gel filtration column with a diameter of 1.6 cm and a length of 60 cm. The column was equilibrated at room temperature in 50 mM Na phosphate buffer, pH 7.2; the flow rate was maintained at 1.0 ml/min by a Schimadzu HPLC pump. The eluant was monitored by a UV detector at a wavelength of 280 nm. Pharmacia small molecular weight protein standards were used to calibrate the column: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). A plot of K_e vs. log MW for two standard runs resulted in a straight line with a correlation coefficient of $R^2 = 0.99$. K_e was defined as elution volume/column volume. All samples were prepared in 50 mM Na phosphate buffer, pH 7.2. The two mixtures, 1 mol hIL-4:1 mol shIL-4R and 1 mol hIL-4:2 mol shIL-4R, were prepared at protein concentrations of ~1 mg/ml and were allowed to equilibrate in the phosphate buffer at room temperature for 15 min before being applied to the column. All samples had protein concentrations between 0.5–1.0 mg protein/ml and sample volumes between 1–2 ml.

3. Results

We established the stoichiometry of the complex of hIL-4 with shIL-4R by separating mixtures of hIL-4 and shIL-4R (in ratios 1:1, 1:1.5 and 1:2) using gel electrophoresis under native conditions at pH 4.2 (Fig. 1). At a 1:1 ratio of hIL-4 to shIL-4R, the mixture migrated as one single band whose position on the gel was different from the position obtained with either hIL-4 or shIL-4R alone. This band corresponds therefore to the complex of hIL-4 with shIL-4R. When the ratio of hIL-4 to shIL-4R is lower than 1:1 (1:1.5 in lane 4 and 1:2 in lane 5), an excess of free shIL-4R was present as well as the complex of hIL-4 with shIL-4R. The same experiment has been done at pH 8.8 (using buffer strips at pH 8.8 and the normal polarity electrode assembly). Under these conditions, hIL-4 does not enter into the gel be-

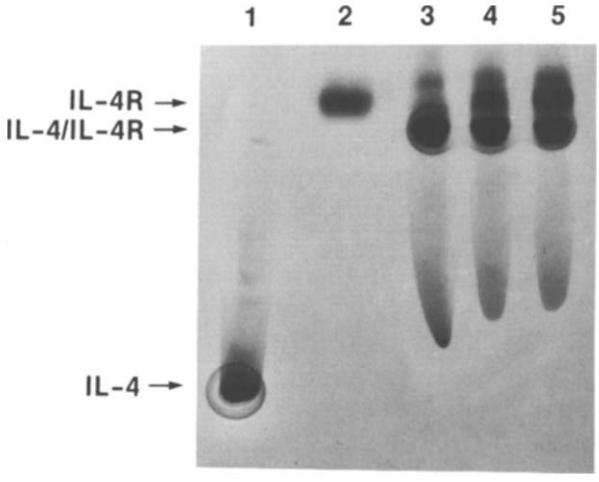

Fig. 1. Polyacrylamide gel electrophoresis analysis in native conditions at pH 4.2 obtained with mixture of hIL-4 and shIL-4R in varying ratios showing that hIL-4 forms a 1:1 complex with the extracellular domain of hIL-4R. The gel was run as described in section 2. Lane 1, hIL-4; lane 2, shIL-4R; lane 3, mixture of hIL-4 and shIL-4R in an 1:1 ratio; lane 4, mixture of hIL4 and shIL-4R in an 1:1.5 ratio; lane 5, mixture of hIL-4 and shIL-4R in an 1:2 ratio.

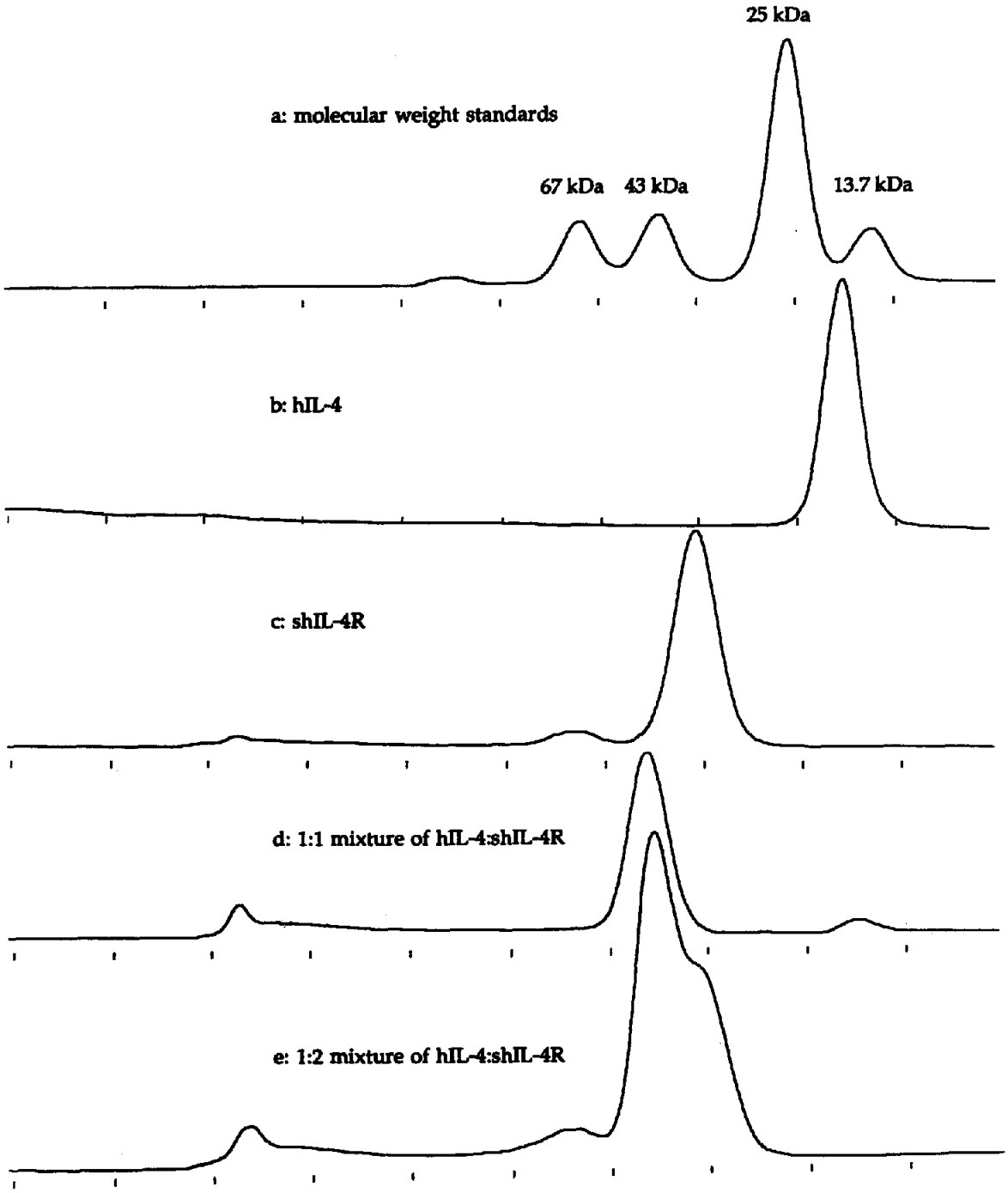

Fig. 2. Chromatographs of the gel filtration runs with A_{280} on the *y*-axis and time on the *x*-axis, tick marks are at 4 min intervals. a: protein molecular weight standards: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa); b: hIL-4; c: shIL-4R; d: 1:1 mixture of hIL-4 and shIL-4R; e: 1:2 mixture of hIL-4 and shIL-4R.

cause of its positive charge, but the results obtained with the various mixtures of hIL-4 and shIL-4R are the same as the results described previously at pH 4.2 (data not shown).

We utilized gel filtration chromatography as an alternative experimental means to verify the results obtained from the native gel analysis. First hIL-4 and shIL-4R were run independently on the calibrated Superdex 200 column to determine their individual apparent molecular weights. The molecular weight of recombinant hIL-4 calculated from its amino acid sequence is 15.4 kDa. The cytokine eluted from the column with an apparent molecular weight of 16.7 kDa (Table 1).

The shIL-4R sample resulted in a major peak and a minor peak corresponding to ~90% and 10% of the total protein (Fig. 2C). The peaks have apparent molecular

weights of 37.5 kDa and 71.1 kDa, respectively (Table 1). Although the smaller molecular weight obtained is greater than the molecular weight of shIL-4R calculated from its amino acid sequence (23.9 kDa), the protein is glycosylated at six N-linked glycosylation sites and runs with an apparent molecular weight of 35–36 kDa on a denaturing polyacrylamide gel (data not shown). This glycosylated shIL-4R apparent molecular weight is very close to the value of 37.5 kDa obtained for the major peak. The larger apparent molecular weight of the minor peak is in the molecular weight range one would expect for a dimer of shIL-4R. When run at 4°C, the major and minor peaks are reversed with ~90% of the total protein running as a dimer (data not shown).

When a 1:1 mixture of hIL-4 and shIL-4R was run over the column, a single peak eluted with an apparent molecular weight of 50.9 kDa, indicating all of the protein had combined into a single complex. A slight molar excess of hIL-4 was noted as a minor peak which eluted at 16.6 kDa. When a 1:2 mixture of hIL-4 and shIL-4R was run over the column the same peak observed in the 1:1 mixture eluted at 46.6 kDa and peaks corresponding to excess shIL-4R were also observed at the monomer (37.4 kDa) and dimer (74.4 kDa) positions (Fig. 2). No peaks were observed with an apparent molecular weight greater than shIL-4R dimer as would be expected for a 1:2 complex of hIL-4 to shIL-4R.

4. Discussion

These results clearly show that hIL-4 binds to the extracellular domain of hIL-4R in a 1:1 ratio forming a tight monomeric complex, hIL-4:shIL-4R. In this particular case, ligand-induced receptor homodimerization as described for growth hormone [18] does not occur. This is not surprising given recent results which indicate that the IL-4:IL-4R complex interacts with a second receptor molecule, IL-2Rγ [19,20].

IL-2Rγ appears to be a common component in the receptor complexes for IL-2, IL-4, IL-7 and possibly IL-9, IL-13, and IL-15 [19–24]. However, the signalling duties are not completely relegated to the common receptor subunit. In the case of IL-4, it appears that there are IL-2Rγ-independent signalling events and some IL-2Rγ-dependent events [20,25,26]. Therefore IL-4 should logically form a tight 1:1 complex with its specific receptor subunit, hIL-4R.

For cytokines sharing a common receptor chain, formation of a heterodimeric receptor complex will likely be the rule rather than receptor homodimerization. Two different receptor chains recognizing different epitopes on a cytokine is certainly easier to imagine than the amazing and unique case of identical receptor chains recognizing disparate cytokine epitopes as observed with hGH:$(hGHR)_2$ [14]. Our findings clearly emphasize that although the large family of cytokines and their receptors are related, the specific ways in which they interact are unique.

Table 1
Apparent molecular weights obtained from gel filtration chromatography

Run	Average K_e[a,b]	Average apparent mol. weight (kDa)
hIL-4	0.762 ± 0.026	16.7 (13.7–20.4)[c]
shIL-4R (major)	0.659 ± 0.023	37.5 (31.2–45.0) monomer
(minor)	0.577 ± 0.027	71.1 (57.5–87.9) dimer
1:1 mixture hIL-4:shIL-4R	0.620 ± 0.025	50.9 (41.9–61.7) 1:1 complex
1:2 mixture hIL-4:shIL-4R	0.659 ± 0.023	37.5 (31.2–45.0) monomer
	0.631 ± 0.024	46.6 (38.6–56.3) 1:1 complex
	0.571 ± 0.028	74.4 (59.9–92.3) dimer

[a] All reported values are the averages of two measurements.
[b] K_e = protein elution volume/column volume.
[c] Uncertainty in K_e value gives associated range of apparent molecular weight shown in parantheses.

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