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SOLUTION ASSEMBLY OF CYTOKINE RECEPTOR ECTODOMAIN COMPLEXES.

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INTRODUCTION: For the majority of single transmembrane-spanning cell surface receptors, signal transmission across the lipid bilayer barrier involves several discrete components of molecular recognition. The interaction between ligand and the extracellular segment of its cognate receptor (ectodomain) initiates either homomeric or heteromeric association of receptor subunits. Specific recognition among these subunits may then occur between ectodomain regions (1), within the membrane by interhelical contact (2) or inside the cell between cytoplasmic domains (3, 4). Any or all of these interactions may contribute to the stability of the signaling complex. It is the characteristics of ligand binding by the ectodomains of these receptors, however, that controls the heteromeric or homomeric nature and the stoichiometry of the complex.

Cytokines and their receptors belong to a growing family of macromolecular systems that exhibit these functional features and share many structural similarities as well (5). Interleukin-2 is a multifunctional cytokine (6) that represents, perhaps, the most complex example to date of ligand recognition among the hematopoietin receptor family. The high affinity interleukin-2 receptor (IL-2R, $K_d = 1 \times 10^{-11}$ M) is composed of three different cell surface subunits, each of which participates in ligand recognition (7). The 55 kDa α -subunit binds IL-2 with a K_d of 1×10^{-8} M. The larger 75 kDa β -subunit and the recently discovered 64

kDa γ -subunit combine to form the intermediate affinity site ($K_d = 1 \times 10^{-9}$ M). It is the cooperative binding of IL-2 by all three proteins on the surface of activated T- lymphocytes, however, that ultimately results in crosslinking of the β - and γ -subunits and signaling via association of their cytoplasmic domains (4). Although the high-affinity IL-2R functions as a heterotrimer, heterodimers of the receptor subunits are also physiologically important. The α/β heterodimer or “pseudo-high affinity” receptor captures IL-2 as a preformed cell surface complex (8) while the β/γ intermediate affinity site exists, in the absence of the α subunit, on the majority of natural killer cells (8).? shouldn't you reference Caligiuri et al.?

In an effort to facilitate structure-function analysis of IL-2 and its receptor, we have begun to employ a strategy of directed and stable solution assembly of receptor ectodomains (9, 10). The goal of these studies is to prepare stable complexes of cytokine receptor ectodomains of defined composition and stoichiometry and that mimic the ligand binding characteristics of the equivalent cell surface receptor sites. We have chosen coiled-coil molecular recognition to direct the formation of these complexes. As result of more than 20 years of study (11-13), the noncovalent coiled - coil interaction is now well understood. Recent reports stimulated by the rediscovery of this motif as the “leucine zipper” in eukaryotic transcription factors (14), have defined the critical parameters controlling both stability and stoichiometry of complex formation (15 -17). In fact, others have previously employed sequences based on transcription factor leucine zippers to direct complexes of antibody segments (18, 19). We chose idealized coiled-coil motifs due to their inherently greater stability, thus eliminating the need for additional covalent linking.

DESIGNS: The design of our hydrophobic heptad repeats employed Leu residues at both the first and fourth (a and d positions) of the heptad. Leucines at these positions favor the formation of trimeric complexes (16, 20). Therefore we employed seven repeats of the heptad **LEALEKK** fused to the IL-2R β subunit ectodomain in a prototype complex to test the feasibility of this approach (9). Displayed in a helical wheel representation (Fig.1, left),

the e and g positions of the heptad are available for favorable electrostatic interactions within a homomeric complex.

Figure 1. Helical wheel representations of the coiled-coil heptad repeat designs employed, (left) for the IL-2R β cc homotrimer, (center) for the IL-2R α cc homotrimer and IL-2R $\alpha\beta$ cc heterotrimer and (right) for the IL-2R $\alpha\beta$ cc heterotrimer. A single heptad is depicted with positions a - g viewed along the helical axis from the top. * indicates residues that may participate in interhelical electrostatic interactions.

For the preparation of heteromeric complexes, we altered the sequences at the e and g positions (LEALKEK for IL-R α and LKALEKE for IL-2R β) in order to disfavor homomeric association based on presumed electrostatic interactions (Fig. 1, center and right). These sequences were fused to their respective IL-2R ectodomains to generate a heteromeric complex (10). Using these recognition sequences, three IL-2R complexes were constructed (Fig.2)

EXPRESSION AND PURIFICATION: To express large quantities of properly folded and glycosylated fusion proteins, we utilized baculovirus mediated insect cell expression (21). This method has been employed for expression of other hematopoietin receptor ectodomains (22) and several versions of baculovirus expression vectors are commercially available. cDNA's encoding the desired fusion proteins were inserted into pBlueBac II (Invitrogen) and recombinant baculovirus were conveniently identified by color selection.

High titer virus was produced in *Sf9* insect cells, while protein expression was carried out by infection of *Trichoplusia ni* (*High-FiveTM*) cells in protein free media (10). The preparation of homomeric complexes was achieved by infection of a single virus while simultaneous co-infection by viruses harboring the IL-2R α and IL-2R β coiled-coil fusion proteins generated a heteromeric complex.

Figure 2. Schematic representations of the three coiled-coil IL-2 receptor ectodomain complexes examined in this study.

Protein purification was carried out by immunoaffinity chromatography over IL-2R subunit specific affinity columns. For homomeric complexes (IL-2R α cc and IL-2R β cc), this single step procedure yielded homogenous products. To isolate the heteromeric complex (IL-2R $\alpha\beta$ cc), the fraction bound to the IL-2R α specific immunoaffinity column was eluted and applied to the IL-2R β specific column. The fraction that bound to both columns represented the IL-2R $\alpha\beta$ cc protein complex. Reverse phase HPLC confirmed the presence of both subunit fusion proteins (Fig. 3).

Figure 3. Reverse phase HPLC analysis of the IL-2R $\alpha\beta$ cc complex demonstrating the presence of the α -subunit fusion protein (A) and the β -subunit fusion protein (B). Column: Dynamax 300A C18(4.6 x 250 mm) column (Rainin). Buffers: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile; gradient, 25-70 % solvent B over 45 min at a flow rate of 1 ml/min; absorbance was monitored at 215 nm.

PROTEIN CHARACTERIZATION: In addition to reverse phase HPLC, the complexes were analyzed for homogeneity by SDS-PAGE. The proteins migrated at the expected molecular weights corresponding to the IL-2R ectodomains fused to seven coiled-coil heptad repeats (9, 10). The IL-2R $\alpha\beta$ cc complex provided bands corresponding to both fusion proteins. Although confirming the homogeneity of the preparations, these analyses did not reveal the stoichiometry of the complexes. Non-denaturing gels as well as gel filtration experiments were also not definitive in this regard due to the expected non-globular nature of the complexes. If fully formed, the coiled-coil segment would extend as a rod-like structure approximately 75 Å in length. To confirm the size of the complexes analytical ultracentrifugation was performed. Equilibrium sedimentation analysis carried out at three to four different protein concentrations and several rotor speeds revealed molecular weights in the 105 - 120 kDa range for all of the complexes (9, 10). These values were consistent with the trimeric stoichiometry expected from the heptad designs and were further supported by sedimentation velocity experiments. Sedimentation coefficients of approximately 4.5s were obtained in addition to axial ratios (assuming a hydrated prolate ellipsoid) of 15 suggesting an elongated structure.

Since the secondary structure of the receptor ectodomains is primarily beta sheet (1, 23), circular dichroism proved to be useful in revealing the addition of the helical structure of

the coiled-coil segment. Deconvolution of far UV CD spectrum of the IL-2R β cc complex when compared to the spectrum of the IL-2R β ectodomain itself suggested that the coiled-coil segment was at least 75% formed (9). Attempts to determine the stability of the complex by monitoring the far UV CD spectrum while adding denaturants were complicated by the observation that the globular regions of the complex unfolded prior to coiled-coil segment.

Finally, automated N-terminal amino acid sequencing confirmed that the complex subunits began with the amino terminal residues predicted from the cDNA sequences and were not unexpectedly truncated. Furthermore, sequencing of the IL-2R $\alpha\beta$ cc protein predicted a subunit ratio of two IL-2R α to one IL-2R β subunits (10).

LIGAND BINDING: The ability of the complexes to interact with ligand is a critical factor for the success of this approach. To determine whether the addition of the coiled-coil complex perturbed ligand binding, we compared the ability of IL-2R β cc complex and the β -subunit ectodomain to compete the binding of 125 I-IL-2 to cell surface receptors in a solution binding assay. In this assay, the amount of labeled ligand specifically bound to cell surface receptors from a constant 125 I-IL-2 concentration (500 pM) is determined as a function of increasing concentrations of competitor (either receptor complexes, ectodomains or cold IL-2 as a control). From this data, a dissociation constant for the soluble receptor may be calculated (24). The K_d for the IL-2R β cc complex was determined to be 100 nM in this assay (9). Since this complex contains three β -subunit ectodomains, the apparent K_d for each subunit was $\approx$ 300 nM, a value that corresponds closely to that determined for the individual β -ectodomain (24). Thus, ligand recognition was not perturbed by the process of coiled-coil complexation.

A second aspect of ligand binding is the extent of cooperativity that heteromeric complexes would exhibit. The $\alpha\beta$ "pseudo high affinity" cell surface receptor displays a high degree of cooperativity in ligand capture. The affinity of this site (150 - 600 pM) is $\sim$ 100 fold and $\sim$ 1000 fold higher than the dissociation constants of the α - and β -subunits, respectively.

To examine this, we compared the ability of the IL-2R $\alpha\beta$ cc complex with a mixture of uncomplexed IL-2R α - and β -ectodomains to compete ligand binding to cell surface receptors (Fig. 4).

Figure 4. Competitive displacement of ^{125}I -IL-2 on MT-1 cells (10). % Of maximum specific radioactivity bound is plotted versus concentration (nM) of soluble receptor for (○) the $\alpha\beta$ cc complex and an equimolar mixture of α - and β -ectodomains (■). Each point represents the mean of triplicate determinations. The assay was performed at 37° C under conditions where the depletion of free ^{125}I -IL-2 is negligible.

As can be seen from this data, the heteromeric complex is ~ 100 fold more potent in competing cell surface receptor binding than the simple mixture. This observation suggests that ligand binding by the complex is highly cooperative. Quantitative determination of the effective K_d for the IL-2R $\alpha\beta$ cc complex in repeated assays provided values in the 300 - 500 pM range (10). The apparent dissociation constant for the α - and β -ectodomain mixture was ~ 40 nM. This value is similar to the K_d s obtained for the IL-2R α ectodomain and the IL-2R α cc complex (10). These data, then, not only confirm the cooperative nature of ligand binding by the heteromeric complex, but also suggest that there is no tendency of the

individual α - and β -ectodomains to associate at the concentrations examined either in the presence or absence of ligand.

SURFACE PLASMON RESONANCE: The use of biosensors employing surface plasmon resonance (SPR) detection is proving to be an extremely effective method of examining macromolecular interactions in real time and without labeling. For interacting systems whose characteristics fall within the limitations of existing instrumentation, this methodology allows the determination of both equilibrium and kinetic binding constants (25).

We employed SPR (BIAcore™, Pharmacia Biosensor) to determine the ligand binding characteristics of all three coiled-coil complexes. The biosensor surface for each complex was prepared using standard amine based coupling chemistry (BIAcore systems manual). This technique immobilizes macromolecules via surface amino groups to the carboxy-activated dextran biosensor surface. For these complexes, this coupling technique proved very efficient due to the availability of 42 Lys residues in the coiled-coil segment. Attachment to the surface in this region also oriented the complex in a manner the minimized steric interference with ligand binding.

By employing multiple surface densities of each complex, we were able to examine the binding behavior of wild type IL-2 in addition to two analogs possessing receptor subunit specific mutations (α -subunit, T41P; β -subunit, D20K). Typical SPR sensorgrams for these ligands are shown in Fig. 5.

Analysis of the binding data over an extended concentration range for these ligands on surfaces of each complex by nonlinear least squares curve fitting (BIAevaluation 2.0 software, Pharmacia) provided estimates of the kinetic and equilibrium binding parameters. Table I lists the dissociation constants obtained from these measurements.

The values obtained for the dissociation constants from Scatchard analysis of the equilibrium bound values compared favorably with the values obtained from the ratio of the

kinetic binding constants ($k_{\text{off}}/k_{\text{on}}$). These constants were also similar to those obtained in previous solution and cell surface receptor binding assays, where available (26). Of note, these data confirm that the **D20K** mutation eliminates binding to the β -subunit (Fig. 5B) but has no influence on interaction with the α -subunit, while **T41P** is primarily α -subunit directed (Fig. 5A).

Figure 5. BIAcore™ SPR sensorgrams (relative response in RU after background subtraction vs. time in sec.) of IL-2 (1) and analogs T41P (2) and D20K (3) on biosensor surfaces prepared from: **A**, the IL-2R α cc complex (ligand concs. = 200nM); **B**, the IL-2R β cc complex (ligand concs. = 1000 nM) and **C**, IL-2R $\alpha\beta$ cc complex (ligand concs. = 50nM). All reagents were dialyzed into standard HBS buffer. Biosensor surfaces were regenerated with 10 mM HCl. The flow rate was 8 μ l/min.

Values for the kinetic binding constants were obtained for most of the analogs (26). In some cases, however, the rate constants were too rapid to be measured by the instrument. Of note were the kinetic constants calculated for binding of IL-2 to the IL-2R $\alpha\beta$ cc surface ($k_{\text{on}} = 3.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{off}} = 2.0 \times 10^{-3} \text{ s}^{-1}$). These values were surprisingly similar to those

reported (27) for the cell surface "pseudo high affinity" site ($k_{on} = 1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 1.1 \times 10^{-3} \text{ s}^{-1}$).

Table I
SPR Dissociation Constants^a

	IL-2	T41P	D20K
<u>IL-2Rαcc</u>			
Kd_{kin}	33 nM	NA	34 nM
Kd_{Sca}	30 nM	650 nM	40 nM
<u>IL-2Rβcc</u>			
Kd_{kin}	NA	NA	NA
Kd_{Sca}	410 nM	625 nM	NA
<u>IL-2R$\alpha\beta$cc</u>			
Kd_{kin}	0.52 nM	6.9 nM	38 nM
Kd_{Sca}	0.56 nM (30 nM)	11 nM (787 nM)	108 nM

^a Dissociation constants as determined from the ratio of the kinetic rate constants ($Kd_{kin} = k_{off}/k_{on}$) and from Scatchard analysis (Kd_{Sca}) of the equilibrium bound values for each concentration of ligand. NA refers to values that could not be determined due to the inability to accurately measure the kinetic constants or lack of detectable binding. Values for Kd_{Sca} in parentheses are those determined for the extra low affinity α site in the $\alpha\beta$ cc complex.

CONCLUSION: This study has demonstrated that it is possible to direct the assembly of cytokine receptor subunits in solution by employing coiled-coil molecular recognition. Furthermore, heteromeric complexes may be prepared in which the subunits function cooperatively to bind ligand. The use of idealized coiled-coil designs rather than naturally occurring sequences resulted in subnanomolar complex stabilities. It is unlikely, however, that use of electrostatic interactions to direct heteromeric assembly had a dominant influence in the formation of these complexes, since stable homomeric assemblies (e.g. IL-2R α cc) were also isolated using "heteromeric" designs. It now appears that the nature of the residues at the **a** and **d** positions, not only control stoichiometry but may control

heterogeneity and helix orientation as well (28). The techniques of analytical ultracentrifugation and surface plasmon resonance proved critical in quantitatively characterizing these complexes. Using these approaches and the present knowledge of coiled-coil molecular recognition, it should be possible to prepare soluble receptor complexes of defined stoichiometries and subunit composition.

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