The Interleukin-6 (IL-6) Partial Antagonist (Q159E,T162P)IL-6 Interacts with the IL-6 Receptor and gp130 but Fails to Induce a Stable Hexameric Receptor Complex*

Annet Hammacher‡, Richard J. Simpson, and Edouard C. Nice§

From the Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research and Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia and §Ludwig Institute for Cancer Research, Parkville, Victoria 3050, Australia

The extracellular “soluble” domains of the IL-6 receptor (sIL-6R) and gp130 (sgp130) form a hexameric ternary receptor complex together with IL-6, consisting of two molecules of each component. In this report we have investigated the interactions of the partial IL-6 antagonist (Q159E,T162P)IL-6 ((QT)IL-6), with the sIL-6R and sgp130. The kinetic rate constants of the binding of sIL-6R to immobilized monomeric (QT)IL-6 or IL-6 were obtained using an optical biosensor with analysis of the primary data by linear and nonlinear regression. Both methods of analysis showed that, due to a higher off-rate, sIL-6R has lower apparent affinity for (QT)IL-6 than IL-6. The lower affinity of (QT)IL-6 was further confirmed by equilibrium binding measurements at the sensor surface and in solution. Using the biosensor it was also shown that the (QT)IL-6 complex interacts with sgp130, supporting the notion that the biological activity of (QT)IL-6 is mediated via gp130. However, the IL-6 mutant, when incubated with sIL-6R and sgp130, failed to induce a stable hexameric receptor complex, as shown by narrowbore size exclusion chromatography.

Interleukin-6 (IL-6)1 is a cytokine that exhibits pleiotropic effects on a wide range of target cells. It is involved in the growth and differentiation of B lymphocytes, differentiation and/or activation of T lymphocytes and macrophages, maturation of megakaryocytes, and enhancement of multipotential hematopoietic colony formation, expression of acute phase proteins, and bone metabolism. IL-6 has also been implicated in a variety of disease states, including lymphoid malignancies and autoimmune and inflammatory disorders such as glomerulonephritis and rheumatoid arthritis (reviewed in Ref. 1).

The biological activities of IL-6 are mediated by the IL-6 receptor (IL-6R), which binds IL-6 specifically and with low affinity (2), and gp130, which associates with the IL-6L-6R complex, resulting in high affinity binding and activation of intracellular signaling (3–5). gp130 does not associate with IL-6 in the absence of the IL-6R (3). gp130 also forms part of the receptor complexes of the cytokines IL-11, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor (reviewed in Ref. 6), and cardiotrophin-1 (7).

To obtain sufficient quantities of IL-6 for our structure-function studies we have expressed IL-6 in Escherichia coli using the pUC8 expression vector (8, 9). We have recently demonstrated that this material, purified to apparent homogeneity from bacterial inclusion bodies, can be separated into stable monomeric and dimeric IL-6 by size exclusion chromatography (SEC) (10). By SEC and analytical ultracentrifugation, using the extracellular domains of the IL-6R and gp130 (sIL-6R and sgp130, respectively), we have shown that one molecule of monomeric IL-6 binds one molecule of IL-6R in the binary complex, while the ternary receptor complex of IL-6 is a hexamer consisting of two molecules each of IL-6, IL-6R, and gp130 (10).

The present investigation compares the affinities of IL-6 and the partial IL-6 antagonist (Q159E,T162P)IL-62 ((QT)IL-6) for binding to sIL-6R and sgp130, using an optical biosensor (BIACORE2) employing surface plasmon resonance (SPR) detection for monitoring the interactions in real time (11). (QT)IL-6 has been shown to act as an IL-6 antagonist on human CESS and hepatoma HepG2 cell lines, apparently by preventing the formation of the IL-6L-6R complex (12). It was concluded that the (QT)IL-6L-6R complex was defective in its interaction with gp130. However, on HepG2 cells, at high concentrations of (QT)IL-6, an agonist activity corresponding to 10–20% of the maximal response of IL-6 was consistently observed. This activity was tentatively explained by the presence on HepG2 cells of a third receptor chain necessary for high affinity binding or by differences in relative numbers of high and low affinity receptors on CESS and HepG2 cells (12). It was later reported that the (QT)IL-6L-6R complex has residual affinity for sgp130 (13); however, it remained to be determined whether (QT)IL-6 is capable of inducing the formation of the hexameric receptor complex.

For reliable kinetic analysis using a biosensor it is important to use homogeneous binding components, as heterogeneity of the material may affect the interpretation of the results. For example, the interaction of a Fab fragment of a monoclonal antibody with its peptide antigen yielded biphasic dissociation kinetics prior to purification of the antigen but linear first-order kinetics after purification (14). Since the monomeric, but not the dimeric, form of recombinant IL-6 appears to reflect the interactions of "wild type" IL-6 with the IL-6R and gp130,3 we have used monomeric (QT)IL-6 and IL-6 for the studies de-
Detailed below.

In this report, we determine the apparent affinity of (QT)IL-6 for the sIL-6R by linear and nonlinear regression of the biosensor data (15, 16) as well as by equilibrium binding and compare the values obtained with those of IL-6. This rigorous analysis was undertaken to confirm the appropriateness of the mathematical models used, since disparity between methods has been reported recently (17, 18). Using the biosensor and narrow bore SEC we also investigated the interactions of the (QT)IL-6-sIL-6R complex with sgp130. The formation and characterization of the ternary receptor complex in the presence of (QT)IL-6 is discussed.

**EXPERIMENTAL PROCEDURES**

Construction of Human IL-6 and (QT)IL-6 Expression Vectors—Human IL-6 in pUC8 was generated as described previously (8). (Q159E,T162P)IL-6 cDNA (with an introduced Xho site) was generated by polymerase chain reaction using the internal oligonucleotides 5'-ACAGAACCAGTGCTGAGCCAACATCTCATCATTCTG and 5'-AGATGATGTCATGTCCTGGCAGCAGTCGGTCT, the external oligonucleotides “A” and “B”, and an N-terminal truncated form of IL-6 in pBluescript (8) as a template. The two polymerase chain reaction products were digested with EcoRI/XhoI and BamHI/XhoI, respectively, purified and subcloned into EcoRI/BamHI-digested pUC8. Restriction endonuclease was purchased from Boehringer Mannheim. The construct was verified by DNA sequencing (8).

Expression and Purification of Proteins—Human IL-6 and (QT)IL-6 were purified from E. coli inclusion bodies as described (8, 19). The N-terminal residues of the proteins (TM7TINSRGSSIL-6 and TM7TINS in (QT)IL-6; one-letter amino acid codes) are expression vector-derived and are followed by residues 1-184 and 20-184 of IL-6 and (QT)IL-6, respectively. The purity of IL-6 was confirmed by N-terminal sequencing and mass spectrometry (20). When analyzed by mass spectrometry, (QT)IL-6 yielded the expected molecular mass of 19,558 Da. A small amount of material lacking Thr (19,454 Da) was also present. For the kinetic studies detailed below (QT)IL-6 was considered homogeneous, such as a truncation in IL-6 does not interfere with receptor interaction (21).

Soluble human IL-6 receptor and sgp130 were expressed in Chinese hamster ovary cells transfected with pCECDhr344 and pCECdhfr620, respectively (22, 23), and purified as described (10, 24).

**Size Exclusion Chromatography**—Monomeric IL-6 and (QT)IL-6 were isolated using a Superose 12 column (300×10 mm inner diameter) (Pharmacia Biotech Inc.) operated at 0.5 ml/min in phosphate-buffered saline at 25 °C. Narrow bore SEC was performed in phosphate-buffered saline at 25 °C on a Superose 12 PC 3.2/30 SMART™ column (Pharmacia).

**Protein Estimation**—Protein concentrations were determined by amino acid analysis on a Beckman 6300 high performance amino acid analyzer equipped with a model 7000 data analyzer (Beckman Instruments Inc., Palo Alto, CA).

**Murine Hybridoma 7TD1 Assay**—The mitogenic assay on 7TD1 cells was performed as described (8).

**Analytical Ultracentrifugation**—Sedimentation equilibrium ultracentrifugation was performed as described (10) using an angular velocity of 10,000 rpm.

**Radiolabeling of Proteins**—Monomeric (QT)IL-6 and IL-6 were labeled with Bolton-Hunter reagent (Amersham Corp.) as described (8). Protein-bound 125I radioactivity was determined using a Packard multi-analyzer equipped with a model 7000 data analyzer (Beckman Instruments Inc., Palo Alto, CA).

**Analytical T-cell Proliferation**—Purified sgp130 was labeled with [3H]thymidine (NEN Research Products) as above. The specific activity of the labeled sgp130 was determined using an 4,5-diphenyloxazole luminescence counter (Beckman Instruments). Using an Optical Biosensor—The BIACore, CMS5 sensor chips, amine coupling kit, and software package for the Kinject command were supplied by Pharmacia. Soluble gp130 (10 μg/ml), (QT)IL-6 monomer and IL-6 monomer (14 μg/ml) were immobilized to the sensor chip as described for IL-6 (9), using N-ethyl-N′-(3-dimethylaminopropyl)N-hydroxy succinimidyl coupling chemistry (10). Assuming 3000 resonance units (RU) correspond to a surface concentration of 1 nM (25), sgp130, (QT)IL-6, and IL-6 were immobilized to concentrations of 4.0, 3.4, and 1.6 ng/mm², respectively. All reagents were introduced over the sensor chip in BIACore buffer (10 mM Hepes, pH 7.4, 0.15 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20) at 2 μl/min with 35-μl injection volumes. For measurements of complex formation, samples were mixed and incubated for at least 1 h prior to injection. The sensor surface was regenerated between assays by treatment for 1 min at 20 μl/min with 1.5 mM KSCN in 10 mM Tris-HCl, pH 8.0 (sgp130), or 6 mM guanidine-HCl in BIACore buffer (QTIL-6 and IL-6), followed by several injector washes to remove any residual salts. Regeneration of the (QT)IL-6- or IL-6-derivatized sensor chips with guanidine-HCl was necessary to avoid increasing background signal. To verify that regeneration had not caused inactivation of the sensor surface, a control sample was routinely assayed at both the beginning and the end of a series of sensorgrams.

To investigate whether rebinding of sIL-6R to the sensor surface derivatized with (QT)IL-6 was occurring during dissociation, excess (300 nM) soluble (QT)IL-6 was injected immediately after injection of sIL-6R (21.9 nM), from the second injector loop in the microfluidic cartridge.

Analysis of Binding Kinetics—The detection principle of the BIACore is based on SPR. One of the reactants is covalently attached to the sensor surface, whereas the other (called the analyte or ligate) is injected over the derivatized sensor chip as a soluble component. The binding of the analyte to the immobilized reactant causes a refractive index change at or near the sensor surface that is proportional to the increase in mass (25). The changes in mass are indirectly measured in RU and are plotted against time as a sensorgram.

A typical sensorgram consists of an association phase, during which the analyte passes over the derivatized sensor surface, and a dissociation phase where the analyte is replaced with buffer. The association and dissociation rate constants (k⁺ and k⁻, respectively) of the interaction can be obtained from analysis of the sensorgrams by linear regression analysis (25) or by nonlinear least squares analysis (16). Determination of the k⁺ according to the former method relies on analysis of the data from the association phases of various concentrations of analyte and assumes a pseudo-first-order (1:1) interaction between the immobilized protein and the analyte.

Linear regression of the data was performed using Equations 1-3, where R is the detector response (in RU) resulting from the interaction of analyte with immobilized component, Rmax is the maximal response at a given concentration of analyte, k⁺ and k⁻ are the association and dissociation rate constants (in M⁻¹ s⁻¹ and s⁻¹, respectively), and C is the concentration of the analyte (in M). Using this nomenclature the rate equation may be expressed in the following way.

\[ \frac{d R}{d t} = k_C (R_{max} - R) - k_R R \] (Eq. 1)

This may be rearranged as the following.

\[ \frac{d R}{d t} = k_C R_{max} - (k_C + k_R) R \] (Eq. 2)

When dR/dt is plotted as a function of R, the slope, k⁺ (in s⁻¹), is the following.

\[ k_C = k_R C + k_R \] (Eq. 3)

Thus, the slope of a plot of k⁺ as a function of C yields the k⁺, whereas the k⁻ can be obtained from the intercept on the y axis.

Using linear regression analysis the k⁺ at each individual analyte concentration can also be obtained directly from the dissociation phase of a sensorgram. After the association phase, when buffer is flowing over the sensor chip, C = 0, and Equation 1 becomes the following.

\[ \frac{d R}{d t} = - k_R R \] (Eq. 4)

This can be rewritten as the following.

\[ \ln (R/R_0) = k_C (t_1 - t) \] (Eq. 5)

where R₁ is the response at the arbitrary starting time t₁, and R₀ is the response at time t₀. Hence, the slope of a plot of ln(R/R₀) as a function of time equals k⁺.

The k⁻ for the interaction between two components can also be measured directly from analysis of the response at equilibrium (Req). Equilibrium binding is obtained at dR/dt = 0. Thus, R = R₀, and Equation 1 becomes the following.

\[ k_C (R_{max} - R_{eq}) = k_R R_{eq} \] (Eq. 6)

Since k⁺k⁻ = k₀, this may be rearranged as follows.

\[ R_{eq}/C = k_R R_{max} - k_R R_{eq} \] (Eq. 7)

Assuming a univalent interaction, a plot of R_{eq}/C as a function of R_{max} is linear, and the slope of the line equals K⁺ = 1/K₀.

Nonlinear least squares analysis of the primary data analyzes the sensorgrams using the integrated form of the rate equation (16). By contrast to linear regression analysis, it is theoretically possible to calculate both an apparent k⁺ and k⁻ using nonlinear regression anal-
ysis of a single concentration of analyte. However, in practice it is preferable to analyze the data from several concentrations to ensure that there is no variation of the kinetic rate constants with concentration (16).

The integrated form of the rate equation can be written as follows,

\[ R_s = C_{k_s} R_{max} (1 - \exp^{-C_{k_s} + k_d}) + R_0 \]  
(Eq. 8)

where \( R_s \) is the signal (in RU) at the start of the analysis. For the dissociation phase, it can be written as follows,

\[ R_s = A \exp^{k_d t} + R_\infty \]  
(Eq. 9)

where \( A \) is the amplitude of the dissociation (in RU) and \( R_\infty \) is the signal at infinite time when the dissociation is complete.

Data were analyzed using the linearized forms of the rate equation in the BIA Evaluation Kinetics package supplied by Pharmacia. Nonlinear least squares analysis was performed using Equations 8 and 9 on sensorgrams imported into Origin (Microcal Software Ltd., Northampton, MA) or analyzed using the BIA Evaluation, version 2.1, software supplied by the manufacturer and adapted with the appropriate equations. The binding kinetics in solution and at equilibrium were also plotted using Origin.

RESULTS

Preparation and Characterization of Monomeric (QT)IL-6—Purified (QT)IL-6 and IL-6 were separated into monomeric and dimeric forms by SEC as detailed under “Experimental Procedures.” The fact that (QT)IL-6, like IL-6 (10), can be separated into monomers and dimers suggests that dimerization is not unique to native pUC8-derived IL-6.

The biological activity of monomeric (QT)IL-6 was assayed on mouse hybridoma 7TD1 cells and found to be approximately 20-fold less potent than that of monomeric IL-6 (EC50 = 150 pg/ml and 7 pg/ml for (QT)IL-6 and IL-6, respectively). The activity of (QT)IL-6 on 7TD1 cells was thus in good agreement with results obtained using mouse hybridoma B9 cells, where this mutant was 7-fold less active than IL-6 (12).

We have shown previously, by SEC and analytical ultracentrifugation, that the stoichiometry of binding of sIL-6R with IL-6 fits a model whereby one molecule of IL-6 binds to one molecule of sIL-6R (10). Incubation of (QT)IL-6 with sIL-6R resulted in the formation of a binary receptor complex that had similar characteristics to the IL-6-sIL-6R complex, as determined by SEC and sedimentation equilibrium centrifugation, suggesting that (QT)IL-6 binds with a 1:1 stoichiometry to sIL-6R (data not shown).

Biosensor Analysis of Interaction with sIL-6R Using Immobilized (QT)IL-6 Monomer—An overlay of the sensorgrams for the interactions of sIL-6R (2.6–21.9 nM) with immobilized (QT)IL-6 monomer is shown in Fig. 1A. The maximal response (in resonance units, RU) increased with increasing analyte concentration and was between 159 and 557 RU. The response was measured at time = 1030 s, 15 s after cessation of injection of sIL-6R. This time point was chosen to minimize the influence of buffer-related refractive index changes and flow cell effects, which occur directly after the cessation of the injection of analyte. When buffer was injected over the sensor surface the response was negligible at 5 RU (Fig. 1A).

Linear Regression Analysis—As detailed above, the analysis of the binding affinity of sIL-6R for (QT)IL-6 was based on a 1:1 interaction of ligand with sIL-6R. A plot of \( k_s \) against C from the association phases (see Equations 1–3 under “Experimental Procedures”) of the sensorgrams in Fig. 1A yielded an apparent \( k_s = 2.4 \times 10^4 \) M\(^{-1}\) s\(^{-1}\) (slope; coefficient of correlation > 0.99) (Fig. 1B and Table I) and \( k_d = 3.5 \times 10^{-3} \) s\(^{-1}\) (ordinate intercept) (Fig. 1B). However, it is known that this method of calculating \( k_d \) may be subject to error, particularly when the \( k_d \) is low (15). Therefore, the \( k_d \) was also obtained directly by analysis of the dissociation phase (see Equations 4 and 5). Thus, for the initial 60 s of the dissociation phase observed with 21.9 nM sIL-6R, a plot of \( \ln(R/R_\infty) \) against time yielded a slope corresponding to \( k_d = 3.1 \times 10^{-3} \) s\(^{-1}\) (coefficient of correlation > 0.99) (Fig. 1C and Table I), in good agreement with the value obtained from the plot of \( k_d \) against C (Fig. 1B). The values of the individual rate constants yielded an apparent \( K_D = 13 \) nM for the interaction of sIL-6R with immobilized (QT)IL-6 monomer (Table I).

It can be seen (Equation 5) that the \( k_d \) should be independent of concentration. The dissociation rate constant for the initial 60 s was found to be constant at concentrations above 13.1 nM sIL-6R. However, at lower concentrations (10.9–2.6 nM) sIL-6R, \( k_d \) was found to be dependent on concentration, values between 2.8 and 1.7 \( \times 10^{-3} \) s\(^{-1}\) being obtained, resulting in an apparent higher affinity of interaction with immobilized (QT)IL-6. Additionally, at later time points, the plot of \( \ln(R/R_\infty) \) against time was markedly curvilinear. Analysis of the
**Table I**

| Immobilized monomer | Analyte | Analyte preincubated with | $k_a$ | $k_d$ | $K_D$ | $K_D^*$ |
|---------------------|---------|--------------------------|------|------|------|--------|
| (QT)IL-6            | sIL-6R  | (QT)IL-6                 | 2.4  | 3.1  |     |        |
| IL-6                | sIL-6R  | IL-6                     | 2.1  | 2.0  |     |        |
| (QT)IL-6            | sIL-6R  | IL-6                     | NA   | NA   |     |        |
| IL-6                | sIL-6R  | IL-6                     | NA   | NA   |     |        |

**Table II**

| Immobilized monomer | Analyte (sIL-6R) | $k_d$ | S.E. ($k_d$) | $\chi^2$ |
|---------------------|------------------|------|--------------|---------|
| (QT)IL-6            | 2.1              | 3.1  | 1.1          | 3.2     |
| IL-6                | 19.7             | 3.3  | 1.2          | 3.6     |
| (QT)IL-6            | 15.3             | 3.1  | 1.1          | 2.2     |
| IL-6                | 131.2            | 0.9  | 2.1          | 17.5    |
| (QT)IL-6            | 109.2            | 0.9  | 1.9          | 13.2    |
| IL-6                | 87.4             | 0.9  | 1.9          | 12.5    |
| (QT)IL-6            | 65.6             | 0.8  | 1.8          | 7.8     |
| IL-6                | 43.7             | 0.7  | 1.8          | 5.5     |
| (QT)IL-6            | 21.9             | 0.5  | 2.0          | 3.2     |

Later time points (300-440 s) indicated an apparent $k_d = 0.8 \times 10^{-3}$ s$^{-1}$. Such a phenomenon has been noted previously by others (26, 27) and may be due to rebinding of the analyte to the immobilized ligand during dissociation (28, 29). To investigate whether rebinding of sIL-6R to (QT)IL-6 was affecting the $K_D$, excess ligand was injected during the dissociation phase, as detailed under “Experimental Procedures.” By contrast to the system of Panayotou and co-workers (28), who found that a large excess of soluble peptide injected immediately after the analyte was able to prevent rebinding to an immobilized peptide, we observed no major differences in the dissociation kinetics in the presence or absence of over 10-fold molar excess of soluble (QT)IL-6 (data not shown). These results suggest that the $k_d$ was not influenced by rebinding of sIL-6R to immobilized (QT)IL-6.

**Nonlinear Least Squares Analysis**—To confirm the apparent rate constants and the appropriateness of the mathematical models used, the association and dissociation phases of the individual sensorgrams were also analyzed using nonlinear regression (see Equations 8 and 9). As suggested by O’Shannessy and co-workers (16), we first analyzed the dissociation phase data and then used the $k_d$ obtained to constrain the analysis of the $K_D$ from the association phase.

From the initial 50 s of the dissociation phases of 4.4-21.9 nm sIL-6R (sensorgrams shown in Fig. 1A) apparent $k_d$ = 2.3-3.3 $\times 10^{-3}$ s$^{-1}$ were obtained (Table II). The dissociation phases fitted well to a single exponential function, with S.E. between 2.7 and 1.1% ($\chi^2$ between 1.0 and 3.9) (Table I). Analysis of the association phase phases, using the above determined values for the $k_d$, yielded $K_D = 8.8-2.4 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ (Table III). Again the values for S.E. and $\chi^2$ were low. It can be seen (Table III), that at concentrations above 10.9 nm the results show little dependence on concentration, with apparent $K_D$ values ranging from 10 to 13 nm. However, as we had observed when using the linear regression analysis, at low concentrations there was a progressive reduction in the values calculated for the $K_D$ with a concomitant increase in the apparent $k_d$.

Thus, apparent $k_d = 2.4 \times 10^5$ M$^{-1}$ s$^{-1}$, $K_D = 3.2 \times 10^{-3}$ s$^{-1}$, and $K_D = 13$ nm were determined for the interaction of sIL-6R with immobilized (QT)IL-6, using nonlinear regression analysis.

**Equilibrium Binding**—The $K_D$ for interaction between two components can also be measured directly by analysis of the response at equilibrium (see Equations 6 and 7). In Fig. 2A the equilibrium response (maximal response at equilibrium corrected for bulk refractive index changes, measured in RU at time 1030 s) from the sensorgrams in Fig. 1A is plotted as a function of sIL-6R concentration. From the plot of $R_{eq/C}$ against $R_{eq/C}$ an apparent $K_D$ of 14 nm was calculated for the equilibrium binding of sIL-6R with immobilized (QT)IL-6 (Fig. 2B and Table I). However, as can be seen in Fig. 2B, the datapoints corresponding to the lowest concentrations of sIL-6R deviate from the line of best fit. This may be explained by the apparent lower $K_D$ (with corresponding higher affinity) as observed by both linear and nonlinear regression analysis and discussed above. Using concentrations up to 131.2 nm sIL-6R an apparent $K_D$ of 14 nm was also obtained (data not shown).

**Solution Binding**—To investigate whether immobilization of (QT)IL-6 had affected its ability to interact with sIL-6R, we also measured the affinity of binding of soluble (QT)IL-6 monomer to sIL-6R in solution. The rationale behind this is that surface heterogeneity can be introduced during immobilization (15). Immobilization through lysines located in IL-6R binding sites could potentially directly interfere with IL-6R binding. Furthermore, immobilization through lysines located elsewhere in the ligands could also influence IL-6R binding due to steric hindrance.

In these experiments the biosensor was used to measure the concentration of free sIL-6R following preincubation with various concentrations of ligand. As shown by the sensorgrams in Fig. 2C, sIL-6R (21.9 nm) preincubated with 20, 40, 80, or 160 nm (QT)IL-6 yielded a lower response with immobilized (QT)IL-6 than 21.9 nm sIL-6R alone, indicative of the formation of binary complex in solution. As these values (in RU) all fell within the range of the standard curve obtained between 2.6 and 21.9 nm sIL-6R (Fig. 2A), the concentration of uncomplexed sIL-6R could be determined accurately. Based on a 1:1 inter-
action of ligand with sIL-6R, from the total sIL-6R concentration (21.9 nM) and the concentration of uncomplexed sIL-6R, the concentration of sIL-6R in the binary complex could be calculated. The latter equals the concentration of complexed ligand, B. Ligand not bound to sIL-6R, F, equals total ligand in solution minus complexed ligand. According to the Scatchard relationship (30), when B/F is plotted as a function of B, the slope of the line yields $K_a = \frac{1}{K_D}$. Such a plot yielded a $K_D$ of 20 nM for the interaction of (QT)IL-6 monomer with sIL-6R in solution (Fig. 2D and Table I).

The sIL-6R standard curve in Fig. 2A could also be used to determine the concentration of free sIL-6R and hence calculate the $K_D$ for the interaction of IL-6 monomer with sIL-6R in solution. From results obtained by preincubation of 21.9 nM sIL-6R with 20, 40, 60, and 80 nM IL-6 (sensorgrams not shown), a $K_D$ of 9 nM was calculated (Fig. 2D and Table I).

Biosensor Analysis of Interaction with sIL-6R Using Immobilized IL-6 Monomer—For comparison with the data obtained using immobilized (QT)IL-6, we investigated the interaction of sIL-6R with 1L6 monomer. An overlay of the sensorgrams of
phases (see Equations 1–3) yielded an apparent $k_a$ concentration (13.1–2.6 nM) sIL-6R the dissociation over the equilibrium.

The interaction of sIL-6R (21.9–131.2 nM) with immobilized IL-6 is shown in Fig. 3A.

A plot of $k_a$ against $C$ from the corresponding association phase (see Equations 1–3) yielded an apparent $k_a = 2.2 \times 10^5$ M$^{-1}$ s$^{-1}$ (slope coefficient of correlation $> 0.99$). Direct analysis of the dissociation phases (see Equations 4 and 5) gave an apparent $k_d = 1.0 \times 10^{-3}$ s$^{-1}$. The resultant $K_{D}$ was 5 nM. In a separate experiment, analysis of lower concentrations of sIL-6R (2.6–21.9 nM) yielded an apparent $k_a = 2.1 \times 10^5$ M$^{-1}$ s$^{-1}$ (slope coefficient of correlation $> 0.98$) and $k_d = 1.2 \times 10^{-3}$ s$^{-1}$ (ordinate intercept data not shown). The $k_d = 1.2 \times 10^{-3}$ s$^{-1}$ obtained from the intercept of a plot of $k_a$ against $C$ compared well with the $k_d = 2.0 \times 10^{-3}$ s$^{-1}$ (slope of a plot of $\ln(R_U/R_s)$ against time; coefficient of correlation $> 0.99$) calculated from analysis of the initial 60 s of the dissociation phase of 21.9 nM sIL-6R. As was observed with (QT)IL-6, at lower concentrations (13.1–2.6 nM) sIL-6R, the dissociation over the initial 60 s was found to be concentration-dependent, ranging between $k_d = 1.8–1.0 \times 10^{-3}$ s$^{-1}$ (data not shown). Taken together, using linear regression analysis, apparent $k_a = 2.1 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_d = 2.0 \times 10^{-3}$ s$^{-1}$ and $K_D = 10$ nM were determined for the interaction of sIL-6R with immobilized IL-6 monomer (Table I).

Nonlinear least squares analysis of the initial 50 s of the dissociation phases of 21.9–131.2 nM sIL-6R, using a single exponential function (see Equation 9), yielded apparent $k_d = 0.5–0.9 \times 10^{-3}$ s$^{-1}$, with standard errors between 1.8 and 2.1% ($\chi^2$ values between 3.2 and 17.5) (Table II). These values were used to constrain the calculations of the $k_a$, yielding apparent $k_a = 1.9–2.1 \times 10^5$ M$^{-1}$ s$^{-1}$, and hence $K_D = 3–5$ nM (Table I).

It should be noted that for the IL-6 data an improved fit ($\chi^2 = 0.5–0.9$) could be obtained using a second exponential function (data not shown). This analysis suggested that a small component of the dissociation (~3%) had a rapid off-rate ($k_d = 5 \times 10^{-2}$ s$^{-1}$) but that the remainder was described by an apparent $k_d = 0.5 \times 10^{-3}$ s$^{-1}$, i.e., similar to that calculated using the single exponential decay. In a separate experiment, using 4.4–21.9 nM sIL-6R, apparent $k_a = 1.4–2.1 \times 10^{-3}$ s$^{-1}$, $k_d = 1.6–1.8 \times 10^5$ M$^{-1}$ s$^{-1}$, and $K_D = 8–13$ nM were determined using a single exponential function (data not shown).

As for (QT)IL-6, the apparent $K_D$ was calculated independently by analysis of the equilibrium data (see Equations 6 and 7). From the maximal responses in the concentration range of sIL-6R shown in Fig. 3A, the apparent $K_D$ was calculated to be 10 nM (Fig. 3B).

The interaction in solution of sIL-6R with (QT)IL-6 and IL-6 was also measured on a sensor chip derivatized with IL-6 (sensorgrams not shown). From results obtained by preincubation of 21.9 nM sIL-6R with various concentrations of ligand (40, 60, 80, 120, and 160 nM (QT)IL-6 or 20, 40, 60, 80, and 100 nM IL-6) a $K_D$ of 16 nM for (QT)IL-6 and 6 nM for IL-6 were calculated (Table I).

Binding of (QT)IL-6-sIL-6R Complex to sgp130, as Detected by a Biosensor—Having shown that (QT)IL-6 has a similar, albeit slightly lower, affinity than IL-6 for binding to the sIL-6R, the interaction of the (QT)IL-6-sIL-6R complex with sgp130 was investigated (Fig. 4).

On a sensor chip derivatized with sgp130, a response of 697 RU was obtained upon injection of 40 nM IL-6 preincubated with 40 nM sIL-6R (curve A). By comparison, injection of 40 nM (QT)IL-6 preincubated with 40 nM sIL-6R yielded a response of only 117 RU (curve E). When a large molar excess of (QT)IL-6 (1000 nM) was preincubated with 40 nM sIL-6R, the response increased slightly to 210 RU (curve D) but was still substantially reduced compared with that of the binary receptor complex of IL-6 (curve A). No significant binding was obtained with 1000 nM (QT)IL-6 (40 RU; curve F), IL-6, or sIL-6R alone (not shown). These data thus suggest that the (QT)IL-6-sIL-6R complex can interact with sgp130, albeit at a much lower level than the IL-6-sIL-6R complex.

Upon injection of 1000 nM (QT)IL-6 preincubated with 40 nM IL-6 and 40 nM sIL-6R a response of 278 RU was obtained (curve C). This response was lower than that obtained by the IL-6-sIL-6R complex alone (697 RU; curve A), showing that (QT)IL-6 can compete with IL-6 for binding to the sIL-6R, thereby reducing the interaction of the IL-6-sIL-6R complex with sgp130. Similarly, when 40 nM (QT)IL-6 was preincubated with 40 nM IL-6 and 40 nM sIL-6R, the response obtained (645 RU; curve B) was lower than that of the IL-6-sIL-6R complex alone.

Narrowbore Size Exclusion Chromatography of Receptor Complexes of (QT)IL-6—To determine whether (QT)IL-6 can induce the formation of the hexameric ternary receptor complex, the interaction of the (QT)IL-6-sIL-6R complex with sgp130 was further investigated using narrowbore SEC (Fig. 5).

(QT)IL-6 monomer (panel A), sIL-6R (panel B), sgp130 (panel D), and IL-6 monomer (not shown) eluted essentially as single peaks, confirming the homogeneity of the purified proteins. As shown with IL-6 (10), incubation of ligand with sIL-6R prior to chromatography results in both an increased peak height and a slight shift in elution position compared with the sIL-6R, indicative of the formation of the binary IL-6 receptor complex. Similarly, preincubation of (QT)IL-6 with sIL-6R yielded a
decreased peak height of the ligand as well as an increased peak height and a minimal shift in elution position of the sIL-6R, compared with (QT)IL-6 and sIL-6R alone, indicating the formation of a binary complex of (QT)IL-6 with sIL-6R (compare panel E with panels A and B).

Whereas incubation of IL-6 with sIL-6R and sgp130 resulted in the formation of the hexameric receptor complex (see peak indicated by arrow in panel C), no significant formation of a
The hexameric IL-6 receptor complex was noticed when (QT)IL-6 was used as a ligand (panel F). In a separate experiment, when IL-6, sIL-6R, and sgp130 were incubated together with (QT)IL-6 (6-fold molar excess over IL-6), no significant decrease in the peak height of the hexameric IL-6 receptor complex was noticed (data not shown).

Narrowbore Size Exclusion Chromatography of Receptor Complexes of 125I-labeled (QT)IL-6 and 125I-IL-6—Since it was possible that the levels of receptor complexes formed with (QT)IL-6 were too low for detection by UV absorption, (QT)IL-6 and IL-6 were mixed with a trace amount of the corresponding 125I-labeled ligand, were incubated with 0.4 µg sIL-6R alone (A) or sIL-6R (0.4 µg) and sgp130 (0.74 µg) (B). Fractions were collected every 30 s, and the associated counts were determined using a γ counter (125I-(QT)IL-6, solid line; 125I-IL-6, dashed line). The elution positions of the binary and hexameric receptor complexes obtained from the corresponding UV traces (data not shown) are indicated.

The hexameric complex was detected when (QT)IL-6 was used as a ligand (panel F). In a separate experiment, when IL-6, sIL-6R, and sgp130 were incubated together with (QT)IL-6 (6-fold molar excess of (QT)IL-6 over IL-6), no significant decrease in the peak height of the hexameric IL-6 receptor complex was noticed (data not shown).

Using a biosensor employing surface plasmon resonance detection and narrowbore SEC we have investigated the interactions of an IL-6 antagonist, (QT)IL-6, with the sIL-6R and sgp130, the extracellular domains of the subunits of the IL-6 receptor complex.

Affinity data for the interaction of monomeric (QT)IL-6 with sIL-6R were obtained from the association and dissociation rate constants as well as from equilibrium binding measurements at the sensor surface and in solution. With (QT)IL-6 immobilized to the sensor chip these methods of calculation yielded Ka values that were in good agreement with each other and with the binding kinetics in solution as well as with the corresponding values for immobilized monomeric IL-6. The small, but consistent, difference between the apparent Ka values of (QT)IL-6 and IL-6 was due to the difference in the apparent dissociation rate constants. Linear and nonlinear regression analysis yielded Ka = 3.1 and 3.2 s⁻¹ × 10⁻³ for (QT)IL-6 and Ka = 2.0 and 0.9 s⁻¹ × 10⁻³ for IL-6, respectively. Our data using soluble IL-6R are in good agreement with the relative affinity of (QT)IL-6 to that of IL-6 for the IL-6R on human CESS cells (500 and 360 pm, respectively) (12).

Similar to (QT)IL-6, in the case of the interaction of sIL-6R with immobilized IL-6 monomer, the equilibrium binding data yielded an apparent KD = 10 nm, which is in excellent agreement with the apparent KD = 10 nm calculated from linear regression analysis. The apparent association rate constants for the interaction of sIL-6R with IL-6 obtained by linear and nonlinear regression analysis were indistinguishable (ka = 2.1 × 10⁵ M⁻¹ s⁻¹). However, the apparent kd = 9.9 × 10⁻³ s⁻¹ obtained by nonlinear regression analysis was slightly lower than the apparent kd = 2.0 × 10⁻³ s⁻¹ calculated using linear regression, resulting in an apparent KD = 5 nm. A similar phenomenon was also observed in a study on the interaction of soluble CD4 with an immobilized antibody, where the nonlinear regression yielded a similar apparent kd, but a 6-fold lower apparent KD than the linear regression analysis (16).

To measure the interaction between two proteins using SPR, it is preferable to immobilize the smaller component, as the detector response will be proportional to the mass of bound analyte (25). In the case of the interaction between IL-6 (∼21 kDa) and sIL-6R (∼53 kDa) it is possible to immobilize either protein. In the present investigation, using linear regression analysis, we have determined apparent kₐ = 2.1 × 10⁵ M⁻¹ s⁻¹ and kₐ = 0.002 s⁻¹ (apparent KD = 10 nm) for the interaction of sIL-6R with immobilized IL-6 monomer. Using the same method of analysis, but with the assay performed in the opposite orientation, the interaction of IL-6 monomer with immobilized sIL-6R yielded an apparent kₐ = 3.8 × 10⁵ M⁻¹ s⁻¹ and kₐ = 0.018 s⁻¹ (apparent KD = 47 nm) (31). As similar discrepancy was found in a biosensor study on the interaction of human IL-5 with the human soluble IL-5 receptor (31). In this study, the KD was calculated to be 1.7 nm using immobilized receptor with IL-5 as the analyte; however, when the assay was performed in the opposite orientation, the apparent KD was extrapolated to be 5.5 nm (31).

The difference in the apparent KD (10 nm and 47 nm for immobilized IL-6 monomer and immobilized sIL-6R, respectively) is mainly due to the difference in the calculated off-rates for the analytes. One possible explanation for the difference could be the immobilization procedure used. The N-ethyl-N-(3-diethylaminopropyl)/N-hydroxysuccinimide coupling chemistry, which, as evidenced by the good correlation between solution phase and solid phase binding, appeared not to interfere with the ability of (immobilized) IL-6 to interact with sIL-6R, may have affected the interaction of (immobilized)
sIL-6R with IL-6. Additionally, the conditions used to regenerate the sensor chip between measurements (4 mM MgCl2 in 10 mM Tris-HCl, pH 7.4) may have altered the properties of the immobilized sIL-6R. In this respect it is worth mentioning that the sIL-6R is considerably less stable than IL-6 when immobilized on the sensor surface, with an average life-span of days as compared to weeks for IL-6.

In this report the comparative studies of the solution binding of sIL-6R with (QT)IL-6 and IL-6 have been performed with analysis of uncomplexed sIL-6R concentration, using sensor chips derivatized with both monomeric (QT)IL-6 and monomeric IL-6. The Kd values observed (20 and 16 nM for (QT)IL-6, 9 and 6 nM for IL-6; Table 1) were essentially independent of whether (QT)IL-6 or IL-6 was used for analysis, implying that the immobilization had not drastically altered the ability of (immobilized) (QT)IL-6 to interact with sIL-6R. Our data on the solution binding of monomeric IL-6 with sIL-6R compare well with previous results, where the concentration of uncomplexed sIL-6R was measured using a sensor chip derivatized with an essentially dimeric preparation of IL-6. In the latter study, the solution binding constant (Kd) of sIL-6R with monomeric and essentially dimeric preparations of IL-6 was 5 × 10^7 M^-1 (Kd = 20 nM) for both (24).

Interaction of the IL-6IL-6R complex with gp130 induces high affinity binding (4). On the basis of detecting low but not high affinity binding of (QT)IL-6 to CESS cells, Brakenhoff and co-workers (12) concluded that the (QT)IL-6IL-6R complex did not interact with gp130 and that the antagonistic activity of (QT)IL-6 on human HepG2 and CESS cells was caused by interference with the formation of the IL-6IL-6R complex. However, because of the apparent inability to associate with gp130, it was difficult to explain the small agonist activity of this mutant on HepG2 cells. Furthermore, (QT)IL-6 also had residual bioactivity on the human myeloma cell line XG-1 (13). Using a sandwich ELISA, de Hon et al. (13) showed that (QT)IL-6, in the presence of sIL-6R, has residual affinity for sgp130. Here, using biosensor technology, we have shown that (QT)IL-6 interferes with the association of the IL-6sIL-6R complex with sgp130 (Fig. 4). We also found that the (QT)IL-6sIL-6R complex is capable of interacting with sgp130, further suggesting that the biological activity of (QT)IL-6 is mediated by gp130.

We (10) and others (32) have recently reported that the ternary receptor complex of IL-6 is a hexamer consisting of two molecules each of IL-6, IL-6R, and gp130 (2:2:2 complex). Our recent data suggest that the formation of the 2:2:2 complex may involve the dimerization of an intermediate complex consisting of one molecule each of IL-6, IL-6R, and gp130 (30). The existence of such a trimer in the signaling pathway of IL-6 was previously suggested by Davis et al. by analogy with the sequential formation of the receptor complexes of LIF and CNTF (33). (QT)IL-6 apparently does not induce the formation of significant amounts of a stable hexameric complex using the extracellular domains of the IL-6R and gp130 (Figs. 5 and 6). However, the bioactivity of (QT)IL-6 may be explained by residual ability of this molecule to induce a hexameric complex on the cell surface. The reason why CESS cells are unresponsive to (QT)IL-6 (12) remains to be determined.

Simultaneous incubation of (QT)IL-6 with IL-6, sIL-6R, and sgp130 did not reduce the formation of the hexameric complex, as determined by SEC (data not shown). Since the affinity of (QT)IL-6 for the sIL-6R is only approximately 2-fold lower than that of IL-6, the 6-fold molar excess of (QT)IL-6 used in this experiment should be sufficient to compete with IL-6 for binding to the sIL-6R. The apparent inability of (QT)IL-6 to interfere with the formation of the 2:2:2 complex suggests that the hexameric complex is of higher affinity than the binary complex of IL-6 and sIL-6R. This conclusion using the extracellular domains of IL-6R and gp130 is in agreement with previous results on cells showing that association of gp130 with the IL-6IL-6R complex confers high affinity binding (4).

The three regions of IL-6 apparently involved in the association of the IL-6IL-6R complex with gp130 are residues Gln152–His164 (denoted the (p1) site) (12), residues Lys41, Ala56 (p2 site) (34) and residues Tyr51, Gly35, Ser118, and Val121 (p3 site) (35). Replacement of the (p2) region with the corresponding mouse IL-6 residues resulted in drastically reduced bioactivity of the mutant protein without disrupting its affinity for the IL-6R (34). An IL-6 mutant with substitutions of residues Gln159 for Glu, Thr162 for Pro, and the (p2) region for the corresponding mouse IL-6 residues was inactive on XG-1 cells and weakly antagonized IL-6 bioactivity on these cells (13). By introducing two additional substitutions the affinity of this mutant for the IL-6R was enhanced 5-fold, yielding an antagonist that completely inhibited the activity of IL-6 on XG-1 cells (13).

In homologous models of human and mouse IL-6 based on the structure of G-CSF (8, 34), the affinity of the IL-6R complex confers high affinity binding (4). We have shown that the (QT)IL-6IL-6R complex is of higher affinity than the binary receptor complex. Our data suggest that the soluble hexameric IL-6 receptor complex is of higher affinity than the binary complex of IL-6 and sIL-6R.

Acknowledgments—Chinese hamster ovary cells expressing sIL-6R and sgp130 were a kind gift of Dr. K. Yasukawa, Tosoh Corp., Japan. We thank Dr. G. J. Howlett for analytical ultracentrifugation, N. Koutouzi for tissue culture, J. Eddes for amino acid analyses, T. Guo-fen for automated DNA sequencing, R. M. Moritz for purification and N-terminal sequence analysis of the proteins, G. E. Reid for mass spectrometry analyses and digonucleotide synthesis, and Dr. A. W. Burgess for critical review of the manuscript.

REFERENCES

1. Akira, S., Taga, T., and Kishimoto, T. (1993) Adv. Immunol. 54, 1–78
2. Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., and Kishimoto, T. (1988) Science 241, 825–828
3. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989) Cell 58, 573–581
4. Hibi, M., Murakami, M., Saito, M., Hirano, T., Tago, T., and Kishimoto, T. (1990) Cell 63, 1149–1157
5. Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, A., Yasukawa, K., Yamanishi, K., Taga, T., and Kishimoto, T. (1993) Science 259, 1808–1810
6. Kishimoto, T., Tago, T., and Akira, S. (1994) Cell 76, 253–262
7. Pennica, D., King, K. L., Shaw, K. J., Lu, E., Rullman, J., Luh, S-M., Darbonne, W. C., Knutzon, D. S., Yen, R., Chien, K. R., Baker, J. B., and Wood, W. I. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 1142–1146
8. Hammacher, M., Ward, L. D., Weinstock, J., Treutlein, H., Yasukawa, K., and Simpson, R. J. (1994) Protein Sci. 3, 2389–2393
9. Ward, L. D., Hammacher, A., Chang, J., Zhang, J.-G., Discolo, G., Moritz, R. L., Yasukawa, K., and Simpson, R. J. (1994) in Techniques in Protein Chemistry (Crabill, W. J. W., ed) pp. 333–338, Academic Press, Inc., San Diego
10. Ward, L. D., Howlett, G. J., Discolo, G., Yasukawa, K., Hammacher, A., Moritz, A. W.,Flushner, G. R., Discolo, G., Yasukawa, K., Hammacher, A., Moritz,
11. Jo¨nsson, U., Fa¨gerstam, L., Ivarsson, B., J ohnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönberg, I., Sjölander, S., Stenberg, E., Ståhlberg, R., Urbaniczky, C., Ostlin, H., and Malmqvist, M. (1991) Bio-Techniques 11, 620–627

12. Brakenhoff, J. P. J., de Hon, F. D., Fontaine, V., ten Boekel, E., Schoottink, H., Rose-John, S., Heinrich, P. C., Content, J., and Aarden, L. A. (1994) J. Biol. Chem. 269, 23286–23289

13. De Hon, F. D., Ehlers, M., Rose-John, S., Ebeling, S. B., Klaasse Bos, H., Aarden, L. A., and Brakenhoff, J. P. J. (1994) J. Exp. Med. 180, 2395–2400

14. Nöe, E. C., Lackmann, M., Smyth, F., Fabri, L., and Burgess, A. W. (1994) Anal. Biochem. 217, 319–325

15. Chaiken, I., Rose´, S., and Karlsson, R. (1992) Anal. Biochem. 201, 197–210

16. O'Shannessy, D. J., Brigham-Burke, M., Soneson, K. K., Hensley, P., and Brooks, I. (1993) Anal. Biochem. 212, 457–468

17. Fisher, R. J., and Fivash, M. (1994) Curr. Opin. Biotechnol. 5, 389–395

18. Morton, T. A., Myszka, D. G., and Chaiken, I. M. (1995) Anal. Biochem. 227, 176–185

19. Zhang, J., Moritz, R. L., Reid, G. E., Ward, L. D., and Simpson, R. J. (1992) Eur. J. Biochem. 207, 903–913

20. Ward, L. D., Hammacher, A., Zhang, J.-G., Weinstock, J., Yasukawa, K., Morton, C. J., Norton, R. S., and Simpson, R. J. (1993) Protein Sci. 2, 1472–1481

21. Brakenhoff, J. P. J., Hart, M., and Aarden, L. A. (1989) J. Immunol. 143, 1175–1182

22. Yasukawa, K., Saito, T., Fukunaga, T., Sekimori, Y., Koshihara, Y., Fukui, H., Ohsugi, Y., Matsuda, T., Yawata, H., Hirano, T., Taga, T., and Kishimoto, T. (1990) J. Biochem. 108, 673–676

23. Yasukawa, K., Futatsugi, K., Saito, T., Yawata, H., Narazaki, M., Suzuki, H., Taga, T., and Kishimoto, T. (1992) Immunol. Lett. 31, 123–130

24. Ward, L. D., Howlett, G. J., Hammacher, A., Weinstock, J., Yasukawa, K., Simpson, R. J., and Winzor, D. J. (1995) Biochemistry 34, 2901–2907

25. Stenberg, E., Persson, B., Roos, H., and Urbaniczky, C. (1991) J. Colloid. Interface Sci. 143, 513–526

26. Fagerstam, L. G., Frostell-Karlsson, Å., Karlsson, R., Persson, B., and Rönberg, I. (1992) J. Chromatogr. 597, 397–410

27. Gruen, L. C., Kortt, A. A., and Nice, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4902–4906

28. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672

29. Payne, G., Shoelson, S. E., Gish, G. D., Pawson, T., and Walsh, C. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4902–4906

30. Panayotou, G., Gish, G., End, P., Truong, O., Ghout, I., Himes, I., Pawson, T., and Waterfield, M. D. (1993) Mol. Cell. Biol. 13, 3567–3576

31. Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1993) Science 260, 1805–1808

32. Ehlers, M., Gro¨tzinger, J., de Hon, F. D., Müllberg, J., Brakenhoff, J. P. J., Liu, J., Wollmer, A., and Rose-John, S. (1994) J. Immunol. 153, 1744–1753

33. Savino, R., Ciapponi, L., Lahm, A., Demartis, A., Cabibbo, A., Toniatti, C., Delmastro, P., Altamura, S., and Ciliberto, G. (1994) EMBO J. 13, 5863–5870